Bioconversion of Agricultural Residues into Value-added Products by Pectinase-producing Bacteria and Expression of Pectinase Gene in *E. coli* for Biomass Valorization

A dissertation submitted to the Department of Biotechnology, Lakehead University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Pectinase is a group of enzymes that degrade pectin and is one of the most influential industrial enzymes, which helps produce varieties of good-quality products. These enzymes are eco-friendly, highly specific, sustainable, and non-toxic. The importance and implications of pectinases are rising in diverse areas, including bioethanol production, extraction of DNA, protoplast isolation from a plant, fruit juice industries, wine industries, paper and pulp industries, and wastewater treatment. Furthermore, pectinases are employed in retting and degumming plant fiber, preparing animal feed, saccharification and liquefaction of biomass, bio-scouring of cotton fiber, coffee and tea fermentation, and oil extraction. Therefore, the market demand and application of pectinases in new sectors are continuously increasing. However, due to the high substrate cost of growing microorganisms, pectinase production using microorganisms is limited.

At the same time, population growth and waste production are continuously increasing. Waste production is estimated to rise by 73% by 2050. The massive amount of waste needs to be appropriately disposed of; otherwise, this may pollute the air and water and contaminate the soil, creating adverse effects on human health, including gastrointestinal tract infections, respiratory tract infections, eye infections, and skin diseases. Landfilling or dumping is an easy and common way to dispose of waste; however, it is not safe. The air near or surrounding area of the dumping site may have air pollution due to the gases produced and the suspended particles in the air. The leachate from landfills may contain toxic byproducts and contaminate water bodies or soil, affecting living things, including human and aquatic life. In addition, lignocellulosic biomasses (agricultural biomasses) are abundant in nature and can be considered major biological feedstocks for enzyme and energy production. Lignocellulosic material comprises cellulose,

hemicellulose, lignin, and pectin as biopolymeric constituents, broken down by various enzymes into reactive biomolecules and transformed into valuable products and fuels. Thus, this study emphasized and exploited various agricultural biomasses in their biodegradation and producing pectinases and other value-added products.

In chapter 3, the study isolated and identified the pectinase-producing bacteria from local forest soil and illustrated that 17 of 29 bacteria (58.62%) were pectinolytic. Four bacteria (S-5, S-10, S-14, and S-17) showing high pectin hydrolysis zones were designated as *Streptomyces* sp. (S-5, S-14), *Cellulomonas* sp. (S-10), and *Bacillus* sp. (S-17). They were Gram-positive, non-hemolytic, not proteolytic (based on gelatin hydrolysis), non-capsulated, and lipase producers. Interestingly, bacteria showed multi-enzyme (pectinase, cellulase, and xylanase) activities. The soil isolates exhibited auto-aggregation capacity in an order S-5 (66.67%) > S-14 (56.86%) > S-17 (49.77%) > S-10 (27.06%). Further, the bacteria were found to be weak biofilm producers and non-hydrophobic. S-5 illustrated the highest macerating capacity for both potato and cabbage (18.49 and 42.59%, respectively) among the isolates, and UV radiation increased pectinase activity. The isolated soil bacteria are not pathogenic and have the potential to be used as probiotics. The crude enzyme extracts of those bacteria were used in oil and juice extraction from sesame seeds and apples, respectively.

In chapter 4, the Box-Behnken design was used to optimize the cultural parameters of *Streptomyces* sp. for maximum pectinases production. The bacterium produced maximum pectinases at 35℃, pH 7, 58 hours upon submerged fermentation in yeast extract-containing media. Also, the pectinase activity of *Streptomyces* sp. was enhanced in the media containing 1.5% pectin, 1% casein as a nitrogen source, 0.5 mM MgSO4, and 5 mM NaCl.

On SDS-PAGE and zymogram, the molecular mass of the pectinase protein was observed as 25 and 75 kDa. The partially purified pectinase protein illustrated the maximum pectinase activity at 70℃ and two different pHs (5 and 9). The residual activity of the protein was about 30 to 40% at different temperatures, even after 120 mins. Further, the bacterium illustrated the ability to decolorize crystal violet dye efficiently. While optimizing cultural conditions for *Streptomyces* sp., the bacterium C-19 significantly increased pectinase activity from the contaminated broth and was identified as *Bacillus* sp. that produced different polysaccharides degrading enzymes, such as pectinase, polygalacturonase, xylanase, and cellulase. Furthermore, *Bacillus* sp. produced more than 20% lipid content indicating the bacterium has the potential to produce lipids and can be the potential feedstock in producing renewable biofuels and environmental resilience.

Chapter 5 supports different agro-wastes as potential low-cost resources for innovative and competitive production of phytochemicals, including total flavonoid, phenolic content, and pectin. The study revealed higher flavonoid, total phenolic content, and antioxidant capacity from pomegranate peel and maple leaf. However, different solvents used in extraction showed different potentials for evaluating total phenolic content, total flavonoid, and antioxidant capacity. In addition, agro-wastes were explored for immobilization of whole cells, adsorption of dye, saccharification, and ethanol production. *S. thermocarboxydus* is a potential candidate for producing multi-enzymes and degrading agro-wastes. Scanning electron microscopy images of biomasses before and after bacterial treatment and weight loss of agro-wastes revealed the bacterium degraded the biomasses. Extreme vertices mixture design was used in formulating the agro-waste mixture for multi-enzyme production and illustrated the bacterium produced the highest enzymes by formulating the agro-waste mix of orange peel, pomegranate peel, and

pumpkin pulp+seeds. The aqueous extract of pomegranate peel exhibited the highest inhibition zone against *Cellulomonas* sp. (S-10) and *Bacillus* sp. (S-17), while pumpkin pulp+seeds extract did not show any inhibition. The traditional and microwave-assisted methods showed no significant difference in pectin yield from most agro-wastes. However, the study obtained a higher pectin yield from pumpkin pulp+seeds followed by orange peel, banana peel, pomegranate peel, and others.

In addition, the study revealed the adsorption of crystal violet was higher in orange peel. However, the adsorption might depend on the type of agro-wastes, the concentration of crystal violet, and the time of exposure. Further, the study illustrated that laboratory-scale composts were formed within 90 days with changes in the visual appearances of agro-wastes mixtures, temperature, and pH of composts. The temperature increased in the initial first week of composting and later declined, while the pH of the compost was acidic in the beginning and alkaline (around pH 9) at the end. 100% moong seeds germinated on those composts; however, the weights of the seedlings were different with the different composts. The number of bacteria in all composts was 10^7 colony-forming units per gram of compost with seven different colony morphologies.

Furthermore, the hydrolysate produced from a mixture of 43.33% orange peel, 33.33 % pumpkin pulp+seeds, and 23.33% pomegranate peel exhibited significantly high saccharification (22.36±0.54 mg/g dry weight). The hydrolysate when supplemented with 2% w/v fructose produced a maximum of 7.86±0.08% v/v ethanol by the yeast isolated from the brewer's spent grains. Thus, readily available waste could be a promising source for yeast isolation and feedstock for ethanol production.

In chapter 6, the pectinase genes of about 750 bp from *Streptomyces* sp. (S-5 and S-14) were expressed in *E. coli* and encoded a product of approximately 25 kDa molecular weight. Both expressed pectinase proteins showed optimal activity at 2 different pHs (5 and 9) and 50℃. The recombinant pectinases retained their activity for 120 mins indicating both have important thermostable properties with great potential in industrial biotechnological processes. Therefore, the agricultural residues can be employed for enzyme production by pectinase-producing bacteria and valorized to other value-added products such as pectin, polyphenols, and compost with potential industrial applications. This would represent a remarkable alternative to add value to economic development and waste management. In addition, the cloning and expression of the pectinase gene from local forest soil bacterium in *E. coli* add value to industrial biotechnological processes.

Acknowledgments

I would like to express my sincere thanks to my PhD supervisor Dr. Wensheng Qin, who has provided me with an opportunity to undertake my PhD studies under his supervision. I acknowledge the motivation, encouragement, continuous support, and insightful guidance Dr. Qin has provided throughout my study. Besides, I will always be thankful to him for sharing his experiences, playing the role of guardian, and giving us the space to freely express ourselves. Words cannot describe my sincere gratitude to him. In addition to my supervisor, I would like to thank Dr. Jinqiang Hou, Dr. Jinwen Chen, and Dr. Nur Alam for being part of my PhD research committee and providing me with their insightful comments and encouragement throughout the study. Dr. Chengbo Yang from the University of Manitoba for agreeing to serve as the external examiner. I highly appreciate their time and insightful suggestions and comments. My special thanks to Dr. Brenda Magajna, Science and Environmental Studies PhD programs facilitator, for her helpful comments, official reminders, and advice. In addition, I further extend thanks to Dr. Kam Leung, Dr. Heidi Schraft, Dr. Guosheng Wu, Dr. Susanne Walford, Michael Moore, and Grzegorz Kepka, for their help during my research. Without their help, my research would not have been completed on time.

I would also like to thank Algae Corp., USA for providing the algal biomass (Spirillum). I would like to acknowledge the financial support provided by the Graduate Assistantship at Lakehead University.

I thank everyone in my lab family for making the lab a better place to work and making a PhD life quite joyful and conducting research smoothly. Each one of you has made a significant impact during this period in a unique way. I owe a great debt to visiting scholars Dr. Xiaodong Zhang, Dr. Yuen Zhu, Dr. Feifei Chen, and all my friends (Chonlong Chio, Janak Raj

Khatiwada, Aristide Laurel Mokale Kognou, Nadia Ali, Xuantong Chen, Hem Kanta Sharma, and Rabindra Chaulagain) both on-campus and off-campus for their constant support and understanding me through this journey.

I express my gratitude to all my community members for supporting me and encouraging me. Without their support and encouragement, it would have been difficult to adapt to this new environment and I never would have been able to accomplish my goals.

Last but certainly not least, I would like to thank all the members of my family, particularly my life partner (husband), Prakash Shrestha, who is more confident in my capability, build my confidence, and has been always there to help me reach this milestone. He encouraged me to come out of my comfort zone and participate in various activities, and a balanced life. I should not forget to thank my two beautiful angels for their multifaceted support, understanding, and compromising of many things. They are the brightness of my life.

Finally, my respect and gratitude to everyone whom I have not been able to mention here but have directly or indirectly contributed their effort and helped me accomplish my PhD study (journey) on time.

Thank you.

I dedicate this thesis

to

my late father '**Duttaram Shrestha'**

and

all family members who have been my constant source of inspiration.

List of Published Works During PhD

Publication related to this thesis

- **Shrestha, S.**, Khatiwada, J.R., Kognou, A.L.M., Chio, C., Qin, W. Biomass-degrading enzyme(s) production and biomass degradation by a novel *Streptomyces thermocarboxydus*. *Current Microbiology,* 80, 71 (2023).
- **Shrestha, S.**, Chio, C., Khatiwada, J.R., Kognou, A.L.M., Chen, X., Qin, W. Optimization of Cultural Conditions for Pectinase Production by *Streptomyces* sp. and Characterization of Partially Purified Enzymes. *Microbial Physiology,* 33, 12-26 (2022).
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Work related to this thesis submitted for publication (Under review)

- **Shrestha, S.,** Kognou, A.L.M., Chio, C., Khatiwada, J.R., Qin, W. A Sustainable Source for Phytochemicals and Potential Antibacterial Applications. (International Journal of Environmental Science and Technology)
- **Shrestha, S.,** Chio, C., Khatiwada, J.R., Li, O., Qin, W. Saccharification of Agricultural Residues by *Streptomyces* sp. and Ethanol Production From Agro-waste Mixture Hydrolysate. (Waste Management Bulletin)

Other contributions during PhD

- **Shrestha, S.**, Khatiwada, J.R., Kognou, A.L.M., Chio, C., Qin, W. A comparative study of *Cellulomonas* sp. and *Bacillus* sp. in utilizing lignocellulosic biomass as feedstocks for enzyme production. *Archives of Microbiology*, *205*(4), 130 (2023).
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Abbreviations

LB	Lignocellulosic biomass
DM	Degree of methylation
HG	Homogalacturonan
RG	Rhamnogalacturonan
GalA	Galacturonic acid
DA	Degree of acetylation
PL	Pectin lyases
PE	Pectin esterase
SSF	Solid state fermentation
SmF	Submerged state fermentation
BDO	1,4-butanediol
CNC	Cellulose nanocrystals
CNF	Cellulose nanofibrils
DALA	5-aminolevulinic acid
FDCA	2,5-furandicarboxylic acid
GBL	γ-butyrolactone
HMF	Hydroxymethylfurfural
MFC	Microbial fuel cell
SCP	Single-cell protein
LA	Levulinic acid
3-HPA	Hydroxypropionic acid
LiP	Lignin peroxidase
MFCs	Microbial fuel cells
MnP	Manganese peroxidase
PG	Polygalacturonase
PMG	Polymethylgalacturonase
PGL	Polygalacturonate lyase
PMGL	Polymethylgalacturonate lyase
THF	Tetrahydrofuran

DNS	3,5-dinitrosalicylic acid
UV	Ultraviolet
Et-Br	Ethidium bromide
YEP	Yeast extract pectinase production media
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MR	Methyl red
VP	Voges-Proskauer
H_2S	Hydrogen sulfide
TSI	Triple sugar iron
VRB	Violet red bile
PBS	Phosphate buffered saline
TSB	Tryptic soy broth
BATH	Bacterial adherence to hydrocarbon
OD	Optical density
EPS	Exopolysaccharides
ANOVA	Analysis of variance
RSM	Response surface methodology
BBD	Box-Behnken design
CTAB	Cetyltrimethylammonium bromide
YEP’	Yeast extract peptone media
MSM	Minimal salt media
EVMD	Extreme vertices mixture design
TPC	Total phenolic content
YPD	Yeast peptone dextrose media
TBP	Tri-n-butyl phosphate

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Chapter 1

General Overview Rationale and Objectives

1. Introduction

Population growth and waste production are directly proportional (Supangkat and Herdiansyah, 2020), and it is estimated that waste production to rise as much as 73% by 2050 (https://datatopics.worldbank.org/what-a-waste/trends_in_solid_waste_management.html). The fruit processing industries alone produce >0.5 billion tons of waste worldwide (Banerjee et al., 2017). Similarly, agricultural waste of about 180 billion tons is estimated to be produced globally (Dahmen et al., 2019). The massive amount of waste needs to be appropriately disposed of; otherwise, this may pollute the air, water and contaminate the soil, creating adverse effects on human health like gastrointestinal tract infection, respiratory tract infection, eye infection, and skin diseases (Parvin and Tareq, 2021; Siddiqua et al., 2022). Although landfilling or dumping is an easy and common way to dispose of waste, it is not safe. The air near or surrounding the area of the dumping site may have air pollution due to the gases produced and the suspended particles in the air (Siddiqua et al., 2022). The leachate from landfill may contain toxic byproducts and may contaminate water bodies or soil which finally may affect living things including human and aquatic life (Parvin and Tareq, 2021; Siddiqua et al., 2022). As per the Eurostat report on 2020, waste is reported as the fourth largest source of greenhouse gas emissions, accounting for 3% of global greenhouse gas emissions. However, greenhouse gas emission has decreased by 42% between 1995 and 2017 due to the recovery of landfill gas and reduction in landfilling, and an increase in waste reuse and recycle practices (https://ec.europa.eu/eurostat/web/products-eurostat-news/-/DDN-20200123-1). Furthermore, there is an increase in demand for food and energy for

increasing population. Therefore, alternative sustainable production of food and energy is needed. Organic wastes, including all agricultural waste, kitchen waste, and various industrial organic wastes are commonly known as lignocellulosic biomass. Lignocellulosic biomass, being more abundant in nature, is considered the major biological feedstock for enzymes, and energy production. Globally, it is estimated that nearly 180 billion tons of lignocellulosic biomass are produced annually, having the potential to be converted into various high value-added goods, including biofuels, biochemicals, and biomaterials via the biorefinery process (Bharathiraja et al., 2017; Sadh et al., 2018). Lignocellulosic material comprises cellulose, hemicellulose, pectin and lignin as biopolymeric constituents that can be broken down into reactive biomolecules, which are transformed into valuable products and fuels (Baruah et al., 2018; Shrestha et al., 2020).

Pectin is the complex heteropolysaccharides present in cell wall and middle lamella of plant cell. Pectinase, a group of pectin degrading enzymes, is one of the most influential industrial enzymes, helpful in producing a wide variety of products with good qualities. These enzymes are biocatalysts and are highly specific, non-toxic, sustainable, and eco-friendly. The market demand and application of pectinases in new sectors are continuously increasing. However, due to the high cost of the substrate used for the growth of microbes, the production of pectinase using microorganisms is limited. Therefore, in this study, low-cost, or no-cost substrates, such as various agricultural biomasses, are emphasized and exploited in producing pectinases. The importance and implications of pectinases are rising in diverse areas, including bioethanol production, extraction of DNA, protoplast isolation from a plant, in fruit juice industries, wine industries, paper and pulp industries, wastewater treatment. Furthermore, pectinases are employed in retting and degumming of plant fiber, preparing animal feed, saccharification and liquefaction of biomass, bio-scouring of

cotton fiber, coffee and tea fermentation, and oil extraction (Kubra et al., 2017; Oumer, 2017; Shrestha et al., 2021b).

The aim of this research is to isolate the local pectinase producing bacteria having capacity to degrade agro-wastes and further contribute to developing greener industrial materials and applications. In the first chapter of this thesis (**Chapter 1**), a summary of subsequent chapters, as well as the research motivation and objectives are presented.

Chapter 2 involves concepts and literature relevant to lignocellulosic biomass, pectin and pectinases, as well as a more detailed discussion of their types, sources, classification, and applications.

Chapter 3 describes the isolation and identification of pectinase-producing bacteria from the forest soil. The primary screening (qualitative) and quantitative analysis were discussed in this chapter. Besides, the characterization of pectinase-producing bacteria, their maceration properties and the strain development by mutagenesis are also described in this chapter.

Chapter 4 includes the optimization of fermentation conditions for pectinase production and characterization of enzymes. The optimization of cultural condition was performed using Box-Behnken design response surface methodology too.

Chapter 5 details the agricultural waste utilization for pectinase and other enzyme production and their degradation by pectinase-producing bacteria. Different agro-waste mixtures were formulated for maximum pectinase production. In addition, pectin extraction, flavonoid and antioxidant of agro-wastes were also included to valorize agro-waste. Furthermore, agro-waste was used as an adsorbent for crystal violet dye and immobilizing agent for the *Streptomyces* sp. to produce pectinase.

Chapter 6 describes the cloning and expression of the pectinase gene from *Streptomyces* sp. to *E. coli* BL21 and the characterization of expressed pectinase. The effects of temperature, pH, and the stability of pectinases in different temperatures were also performed.

Chapter 7 concludes major contributions of this thesis and reveals future work recommendations.

2. Objectives

Objectives of this thesis are to:

- Isolate, screen, and identify pectinase-producing bacteria from the local forest soil and characterize them
- Optimize cultural conditions for the maximum pectinase production and characterization of produced enzymes
- Valorize agricultural wastes into value added products
- Express pectinase gene in *E. coli* and characterize the expressed pectinase

3. Significance of the research

Isolating the pectinase-producing bacteria from the forest soil is the first important work in this research which further is exploited to produce pectinase enzyme. The population growth and generation of waste is continuously increasing and demand for pectinase too. Thus, to mitigate the pectinase demand and reduce the production cost, different low-cost agricultural waste is used. The effective utilization of agricultural residues obtained from various activities is globally important for reducing waste disposal problem, air and water pollution, soil contamination, and finally reducing the greenhouse gas emission and waste management problems. The renewable value-added products derived from waste will not only minimize our dependence on non-renewable products but will also help the economic development. Thus, bioconversion processes

of agricultural residues into different value-added products using microorganisms are advantageous and promising. This project is mainly focused on valorization and bioconversion of low value agricultural waste to value-added bioproducts, including enzymes, bioactive compounds and bioethanol. The successful completion of this research may result in reducing pollution, greenhouse gas emission, waste disposal problem, and finally sustainable development of economical and efficient technology.

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Chapter 2 Literature Review

A part of this chapter has been published in '**Waste and Biomass Valorization**' (IF: 3.703) and another part is published in '**Applied Microbiology and Biotechnology**' (IF: 4.813).

Different Facets of Lignocellulosic Biomass Including Pectin and Insight in Pectinase Production Development and Industrial Applications

Abstract

The plant matter, lignocellulosic biomass, is a renewable and inexpensive abundant natural resource in the world. The development of inexhaustible energy rehabilitated from agricultural waste is an alternative to fossil fuels to reduce CO_2 emissions and prevent global warming. The amount of waste generated has a direct correlation with the human population. Thus, the waste generated by the population is being added to the environment as municipal, agricultural waste, and waste produced from forest-based industries. Moreover, there are high possibilities of producing environment-friendly valuable bio-based products, including biofuels, biogas, enzymes, and biochar from biomass without competing with the food supply chain.

Pectinase, a group of pectin degrading enzymes, is one of the most influential industrial enzymes, helpful in producing a wide variety of products with good qualities. These enzymes are biocatalysts and are highly specific, non-toxic, sustainable, and eco-friendly. Consequently, both pectin and pectinase are crucially essential biomolecules with extensive applicatory perception in the biotechnological sector. The market demand and application of pectinases in new sectors are continuously increasing. However, due to the high cost of the substrate used for the growth of microbes, the production of pectinase using microorganisms is limited. Therefore, low-cost, or no-cost substrates, such as various agricultural biomasses, are emphasized in producing pectinases. The importance and implications of pectinases are rising in diverse areas, including bioethanol production, extraction of DNA, and protoplast isolation from a plant.

Therefore, this review highlights the significance of lignocellulosic biomass, pretreatment of biomass focusing on biological pretreatment. It briefly describes the different valuable products

like biochemicals, biochar, enzymes, single-cell protein, dye dispersant, and bioplastic from lignocellulosic biomass emphasizing their applications briefly. Natural biomass utilization would lead to solving the energy shortage, food security issues, and obstacles for developing technological solutions in agriculture, agro-processing, and other related manufacturing sectors. Further, this review describes the structure of pectin, types, and source of pectinases, substrates and strategies used for pectinases production, and emphasizes diverse potential applications of pectinases. The review also has included a list of pectinases producing microbes and alternative substrates for commercial production of pectinase applicable in pectinase-based industrial technology.

Keywords: Lignocellulosic biomass; bioproducts; syngas; single-cell protein, enzyme, bioenergy

1. Introduction

The organic matter which is easily and abundantly found in the earth like wood, grasses, crop residues, animal manures, kitchen, forestry, industrial, and municipal wastes are lignocellulosic biomass (LB) (Dashtban et al. 2009; Saini et al. 2015). Agricultural and fruit waste are used as feed for animals or used to make organic fertilizer (Sabiiti 2011) or are burned out (Ezcurra et al. 2001). LB has the potential to be used as resources in paper industries, animal feed, biomass fuel production (Sanchez 2009), and can be converted into different value-added products (Iqbal et al. 2013; Kumar et al. 2016). The burning of LB increases air and environmental pollution which in turn indirectly affects the health of humans and living things (Ezcurra et al. 2001). LB is easily obtainable, renewable, and recyclable, and is an excellent economical and eco-friendly alternative to fossil carbon source for biofuel and other bio-based products like biochemicals production (Gupta et al. 2015; Soccol et al. 2019). Additionally, a carbon-neutral renewable source can reduce greenhouse gases emissions and environmental pollution (Isikgor and Becer 2015; Soccol et al.

2019). The report of Bioproducts production and development survey of 2015 mentioned that about 21 million metric tons of agricultural and forestry biomass in Canada per year is used for bioproducts production (Rancourt et al. 2017). Similarly, the report has stated that bio-based products are expected to make 11% of global chemical sales and bio-based sales of $375-$441 billion by 2020 (Biotechnology Innovation Organization 2016). When biofuel is produced from starch and sugar crops, there may arise the question of sustainability, and may lead to a struggle with food production (Cherubini 2010). However, lignocellulosic feedstocks have crucial advantages over other biomass supplies because they are the non-edible portion of plants and do not interfere with food supplies (Soccol et al. 2019). Moreover, the utilization of biomass prevents different serious environmental problems for example a large amount of forestry, agricultural and agro-industrial wastes accumulated can be used resulting in fewer disposals of these wastes to the soil or landfill (Gupta et al. 2015; Soccol et al. 2019). Agriculturally important biofuel feedstocks such as corn starch, soybeans, and sugar cane can be cultivated for energy purposes and can be produced in a short time at a lower cost than others. Huber has described that these biofuel feedstocks are significantly cheaper than crude oil (Huber 2008). When these feedstocks are cultivated in the land, the production per unit land area increases and land-use efficiency also increases (Larson 2008). In Bio-based Chemicals- IEA Bioenergy, it is mentioned that Carrez et al. have made the statement on biomass use as “Indeed, its use in green chemistry and green materials is saving more CO_2, is more resource-efficient and leads to more employment than using the equivalent land area for the production of bioenergy” (De Jong et al. 2013).

Thus, LB has a hopeful future as a predictable, feasible and maintainable resource for biofuels and other value-added products (Larson 2008; Saritha and Arora 2012; Soccol et al. 2019). In general, the bioconversion process includes different steps such as pretreatment and hydrolysis of biomass

that help biomass to break down into simple sugars, which further undergo fermentation and other methods to produce additional value-adding products. Although LB is abundant and usually low-priced, there is a big challenge to convert LB into fine chemicals and polymers at an economical cost (Zhou et al. 2011; Alonso et al. 2013). This challenge is due to the resistance of LB to enzymatic and chemical degradation (Cherubini 2010). Another challenge is to decrease high oxygen content from biomass and produce low-value high-volume biofuel with high energy density having physical and chemical properties similar to fossil fuel and the need to develop the integrated biocatalyst technology (Melero et al. 2012). Pretreatment can enhance low-value high-volume biofuels and other high-value low-volume chemical production from LB on an industrial scale. However, their pretreatment is not easy and there is a debate about the pretreatment of those materials (Melero et al. 2012). Pretreatment of LB is expensive, relating to both cost and energy but is essential for changing the physical and chemical properties of the lignocellulosic matrix (Saha 2005, Rajendran et al., 2017). More about pretreatment is explained later in the section "Pretreatment of LB". Biorefinery and biofuel technologies are developed for renewable oil and green monomers production from LB compatible with petrochemistry (Stocker 2008).

Pectinase is a complex heterogeneous group of enzymes that acts on pectic substances. The pectic content is the generic name for a natural heteropolymer compound, acidic in nature, present commonly in plants and fruits (Picot-Allain et al., 2020; Satapathy et al., 2020). The homemade winemaker used pectinases in producing wine two centuries ago. However, the first commercial use of pectinases started in 1930 to clarify fruit juice, and it was later used in making wine and fruit juice (Tapre and Jain, 2014; Bhardwaj et al., 2017). Only after 1960 other enzymes were recorded, the chemical nature of plant tissues becomes apparent, and researchers started to use more enzymes proficiently. Of all these enzymes, pectinases are essential catalysts of the

commercial sector for the maximum yield of stable and clarified fruit juices (Tapre and Jain, 2014). Applications of pectinases are increasing continuously, and the worldwide enzyme market accounts for 25% of pectinases (Amin et al., 2017). The different natural sources for pectinase production are yeast, bacteria, fungi, and plants. Microbial enzymes are replacing the chemical catalysts because enzymatic catalysts are more specific, less aggressive, ecofriendly, and saves energy (Garg et al., 2016; Amin et al., 2019).

This review article highlights the significance of LB, the structure and sources of LB, different enzymes of each component, different valuable products from the degradation of LB and their applications. As the demand for pectin and pectinase is upgoing, this review additionally aims to briefly explain the different avenues of pectinase applications so that the review encourages the researcher to develop different strategies for maximum pectinase production and to study applications in different areas. Further, the review will help to know the ideas regarding sources, production strategies, and alternative sources of substrate for pectinase production and are described in following paragraphs.

2. Structure and sources of lignocellulosic biomass

LB is mainly composed of cellulose, hemicelluloses, and lignin as the main component of plant cell walls (Dashtban et al. 2009; Saini et al. 2015). In some plant biomass, pectin like carbohydrate polymer is present (Ezcurra et al. 2001; Doran-Peterson et al. 2008; Mohen 2008; Edwards et al. 2011). In addition, other components like acetyl groups, minerals, phenolic substituents (Scheller and Ulvskov 2010) and ash are present in a very small amount (Bajpai 2016). The composition of LB differs between the species and their sources such as hardwoods, softwoods, and grasses (Table 1). Moreover, the composition also differs with age, stage, and conditions of plant growth even in a single species. Depending on the type of LB, these carbohydrate polymers are organized into

complex non-uniform three-dimensional structures to different degrees and varying relative composition (Scheller and Ulvskov 2010; Vassilev et al. 2012). The resistance in degradation and toughness or recalcitrance of LB is due to the crystallinity of cellulose, hydrophobicity of lignin, encapsulation of cellulose (Agbor et al 2011; Morais et al. 2012; Saini et al. 2015), and complex structure of pectin (Mohen 2008).

Table 1 The contents of cellulose, hemicellulose, and lignin in various lignocellulosic biomass (modified from Kumar et al. 2016; Isikgor and Becer 2015; and Banerjee et al. 2017)

Lignocellulosic biomass [a]	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Pectin (%)**
Corn cobs (Isikgor and Becer 2015)	33.7 - 41.2	31.9 - 36.0	6.1 - 15.9	-
Rice straw (Isikgor and Becer 2015)	29.2 - 34.7	23.0 - 25.9	17.0 - 19.0	-
Barley straw (Isikgor and Becer 2015)	36.0 - 43.0	24.0 - 33.0	6.3 - 9.8	-
Ray straw (Isikgor and Becer 2015)	36.2 - 47.0	19.0 - 24.5	9.9 - 24.0	-
Oat straw (Isikgor and Becer 2015)	31.0 - 35.0	20.0 - 26.0	10.0 - 15.0	-
Soyabean straws (Kim 2018)	44.2	5.9	19.2	-
Corn stover (Kumar et al. 2016)	41.7	20.5	18.0	-
Sugarcane bagasse (Sanchez 2009; Isikgor and Becer 2015)	25.0 - 44.0	27.0 - 32.0	15.0 - 25.0	-
Sugarcane (de-Souza et al. 2014)	28.0	58.0	6.0	8.0
Sweet sorghum bagasse (Sanchez 2009)	45.0	25.0	18.0	-
Rice husks (Sanchez 2009)	31.0	24.0	14.0	-
Banana peels (Emaga et al. 2008)	6.4 - 9.6	2.0 - 8.4	6.0 - 16.8	8.9 - 21.7
Bamboo (Rabemanolontsoa and Saka 2013)	39.8	19.4	20.8	-
Miscanthus (Rabemanolontsoa and Saka 2013)	38.0 - 40.0	18.0 - 24.0	24.0 - 25.0	-

Apple pomace (Bhushan et al. 2008; Banerjee et al. 2017)	7.2 - 43.6	4.26 - 24.4	15.3 - 23.5	3.50 -14.2
Watermelon waste (Banerjee et al. 2017)	20.0	23.0	8.6	16.0
Sweet potato peel (Mei et al. 2010)	27.6 - 36.5	8.7 - 15.9	8.9 - 22.6	9.0 - 22.9
Mandarin peel (Boluda-Aguilar et al. 2010)	22.5	6.0	10.0	3.0 - 4.0
Orange peel (Boluda-Aguilar et al. 2010)	37.1	11.0	7.5	23.0
Tomato pomace (Banerjee et al. 2017)	39.1	11.0	5.3	-
Carrot waste (Banerjee et al. 2017)	30.0	12.3	32.2	3.8
Pomegranate peel (Banerjee et al. 2017)	26.2	10.8	5.6	27.9
Switchgrass (Sanchez 2009; Isikgor and Becer 2015)	5.0 - 45.0	25.0 - 31.0	12.0 - 20.0	2.0
Wheat straw (Sanchez 2009)	29 -35	26 - 32	16 -21	-
Tobacco stem (Zheng et al. 2017)	63.2	9.1	-	0.67
Agave (Wang et al. 2019)	-	25.8	14.08 - 16.46	5.87 - 6.17
Hardwood biomass				
Beech (Di-Balsi et al. 2010)	45.0	33.0	20.0	-
Poplar (Di-Balsi et al. 2010)	49.0	24.0	20.0	-
Cherry wood (Di-Balsi et al. 2010)	46.0	29.0	18.0	-
Eucalyptus (Isikgor and Becer 2015; Coetzee et al. 2011)	54.1	18.4	21.5	1.52 - 2.58
Softwood biomass				
Pine (Isikgor and Becer 2015)	42 - 50	24.0 - 27.0	20.0	-
Japanese cedar (Rabemanolontsoa and Saka 2013)	38.6	23.1	33.8	-
Fir (Isikgor and Becer 2015)	44.0	11.0	27.0	-

‘[a]’ References, ‘-‘ not mentioned

2.1. Cellulose

Cellulose is the major component of LB, which is fibrous, insoluble and has high molecular weight constituting 35 to 50% of LB. The homopolymer of anhydrous glucose units are linked by β-1,4-glycosidic linkages in cellulose that is the primary structural component of plants (Morais et al. 2012). The crystalline region of cellulose is formed by unbranched long polysaccharide chains that are arranged parallelly whereas in the amorphous region of cellulose, the polysaccharide chain is less orderly arranged (Gardner and Blackwell 1974; Li et al. 2009). The cross-polarization/magic angle spinning study reveals the crystalline structure of cellulose has two forms called Iα and Iβ (VanderHart and Atalla 1984). The cellulose chain length or degree of polymerization varies from 250 to 10000 sugar units per molecule depending on the source of material and treatment methods. The chain length also impacts the physiological, mechanical and biological properties of the cellulose (Kuhad et al. 1997; Klemm et al. 2005). Cellulose is regarded as the best saccharide for fuel production due to its environment-friendly characteristics, such as renewability, biocompatibility, and biodegradability (Klemm et al. 2005).

2.2. Hemicellulose

Hemicellulose is a heterogeneous polymer of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose, and galactose), and sugar acids (Saha 2003; Scheller and Ulvskov 2010) resulting in a complex, randomly branched and amorphous structure. Typically, hemicellulose is composed of five different sugars; L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose along with other components, such as acetic, glucuronic and ferulic acids. These sugar units are linked together by β-1,4-glycosidic and sometimes by β-1,3-glycosidic bonds (Scheller and Ulvskar 2010). The configuration of different sugars varies with different plant source, wood and cultivation conditions (Sorieul et al. 2016). Hardwood hemicelluloses mostly

consist of xylans, whereas softwood hemicelluloses mostly consist of glucomannans (Saha 2003; Sorieul et al. 2016). Usually, hemicelluloses comprise 15 to 35% of LB and the degree of polymerization ranges from 100-200 sugar units per molecule (Kuhad et al. 1997; Sorieul et al. 2016). Hemicelluloses are entrenched in the plant cell walls to form a complex network (cross-linked network) of bonds providing structural strength by linking cellulose fibers into microfibrils (Scheller and Ulvskor 2010; Agbor et al. 2011).

2.3. Lignin

Lignin is an abundant heterogeneous polymer of LB constituting up to 30% of LB and is a complex amorphous hetero biopolymer. Lignin has a three-dimensional polymer of phenylpropanoid units joined together by carbon-carbon and aryl-ether linkages. The complex heteropolymer provides rigidity and comprehensive strength to the plant tissue and the individual fibers, stiffness to the cell wall, resistant to water, insects, pathogens, and chemicals (Sorieul et al. 2016; Soccol et al. 2019). Lignin along with cellulose is considered the most abundant biopolymer in nature (Perez et al. 2002) and formed by three phenylpropanoid units; p-coumaryl, sinapyl and coniferyl alcohol (Perez et al. 2002; Soccol et al. 2019). The various aromatic chemicals are produced due to the different structural and chemical properties of lignin which varies with the plant source, type of plants and wood. Softwood is constituted of more than 90% of coniferyl alcohol, while hardwood is composed of varying degrees of coniferyl and sinapyl alcohols (Sorieul et al. 2016).

2.4. Pectin

Pectin is the most complex heteropolysaccharides and contains galacturonic acids (70%), rhamnose, xylose, arabinose, and galactose. It is acidic and negatively charged polysaccharides, naturally occurring biopolymer and provides rigidity and structure to the cell. The type of pectin plays an essential role in the texture of vegetables and fruits during growth, ripening, and storage.

Enzymatic and chemical modification affect the pectin that is present in fruits. During the ripening of fruits, solubilization of pectin and softening of fruits take place due to the pectinase (Paniagua et al., 2014). The molecular weight of pectin ranges from 60,000–318,000 g/mol and varies with the plant source, stage of fruit/plant, extraction condition, and methods (Sayah et al. 2016; Yang et al. 2019). Pectin has an important role in the growth of the plant, development of plant morphology, the defense system of the plant, and has the gelling and stabilizing properties (Sorieul et al. 2016). Due to this gelling and stabilizing properties, pectin is used in diverse food and special product production that significantly affects human health and has biomedical uses (Mohen 2008). Although the amount of pectin is low in many biomasses, it plays an essential role in secondary wall development in addition to primary wall synthesis and modification (Xiao and Anderson 2013). It is highly accountable in fruits and vegetable wastes. Dragon fruit (Hylocereus sp.) peel and pulp contain 38 to 47% water soluble pectic substance (Liaotrakoon et al. 2013) murta fruit (*Ugni molinae* Turcz) 30% by dry weight (Taboada et al. 2010), pomegranate peels between 6.8 - 10.1% (Saulnier and Thibault 1987), grape berries pulp represents 20·8% (Saulnier and Thibault 1987), and passion fruit peel 14.8 g/100 g of dried peel (Kulkarni and Vijayanand 2010). Similarly, pectic polysaccharides in the edible flesh of the loquat fruit contribute up to 70% of total cell wall polysaccharides (Femenia et al. 1998), orange peel 23% pectin, and mandarin peel 16% (Boluda-Aguilar et al. 2010). Hilz et al., 2005 mentioned the cell wall pectin contents in black currants and bilberries vary between 0.20-1.79 g/100 g, and 0.10-0.78 g/100 g respectively (Hilz et al. 2005). The yield of pectin extracted from creeping fig seed (*Ficus pumila* Linn.) ranged from 5.25 - 6.07% (w/w) dry weight (Liang et al. 2012).

It is reported that sugar beet pulp, apple pomace, and citrus waste, like high pectin biomasses, possess about 12% to 35% pectin by dry weight and grass and woody biomass, which are not

regarded as high pectin biomass contain 2-10% and 5% of pectin respectively (Ridley et al. 2001). The pectin content in wood ranges from 10 mg/g to 40 mg/g of wood and eucalyptus wood, about 15.2 to 25.8 mg/g (Coetzee et al. 2011). Likewise, when there is less pectin in biomass, lipid concentration is high and vice versa, showing the non-reciprocal relation between pectin and lignin (Ridley et al. 2001, Edward and Doran-Peterson 2012). Biosynthesis of pectin takes place in the Golgi apparatus and secreted in apoplast. It is suggested the pectin might block other enzymes to degrade cellulose and/or hemicellulose present in different biomasses (Marcus et al. 2008). Pectin-rich biomasses are used as bioenergy, biofuel feedstocks (Edwards et al. 2011). It can act as a determinant of cell wall porosity because it has cross-linking and water complexation properties (Willats et al. 2008). Pectinase, a group of enzymes, degrades pectin through depolymerization and de-esterification reactions (Pedrolli et al. 2009) and leads for other enzymes for the degradation of other polysaccharides present in biomasses (Marcus et al. 2008).

Besides, pectin has some drawbacks like the gelation of highly methylated pectin in high sucrose concentration; therefore, it is not appropriate for diabetes patients. The reduction in the ability to effectively regulate the release of drugs is due to the hydration, swelling, and water-solubility properties of pectin. However, these kinds of drawbacks can be overcome by pectin modifications and derivatives formation using different techniques such as amidation, sulfation, grafting, cross-linking, β-elimination, hydrolysis, degradation, etc. The pectin amendment has increased its application more widely (Chen et al. 2015).

2.4.1. Structure of pectin

The structure of pectin is complex, not known entirely, and is still in debate. However, two pectic models are prevalent; the “smooth and hairy region” model and the “RG I backbone” model.

The main constituent of pectin polysaccharides is D-galacturonic acid, joined by α (1→4) glycosidic bonds. Other sugar units, including ribose, galactose, arabinose, and sucrose, are also found inserted into the polymers, and about 70% galacturonic acid is present in the cell wall (Harholt et al., 2010; Picot-Allain et al., 2020; Shrestha et al., 2020). The structural components and molecular mass of the pectin differ with their origin, cell types, different stages of cellular development, extraction condition and methods, and the thickness of a given cell wall (Harholt et al., 2010; Pancerz et al., 2021). However, the pectin molecule of the same molar mass may have different hydrodynamic properties due to the difference in the degree of methylation (DM), branching, and neutral sugar content. The most common polysaccharide structural components of pectin are; homogalacturonan (HG), rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) (Lara-Espinoza et al., 2018; Picot-Allain et al., 2020; Ropartz and Ralet, 2020).

Homogalacturonan (HG): This is the simplest and most abundantly found in pectin, accounting for about 75 – 100% pectin. HG represents the backbone chain of the pectin molecule and contains linear polymers of α-D galacturonic acid (GalA) residue linked with α (1→4) glycosidic bonds. The minimum length of HG is estimated to be 100 GalA residues. GalA residues can be acetylated or/and methyl esterified. The number of acetyl esterified GalA and methyl esterified GalA in 100 GalA residues is known as the degree of acetylation (DA) and the degree of methylation (DM), respectively. Both DA and DM have a massive effect on the functional properties of pectin. This HG region is considered as smooth zone of pectin (Lara-Espinoza et al., 2018; Ropartz and Ralet, 2020).

Rhamnogalacturonan I (RG I): RG I is present in the highly branched area containing many neutral sugars such as arabinose, galactose, and mannose as the side chains of a-1, two-linked resides of L-rhamnopyranose. Moreover, the backbone of RG I is formed by the repeated disaccharide

structure of rhamnose and galacturonic acid. The galacturonic residue can undergo acetylation and bind with other neutral sugars (galactose, arabinose, and xylose). Therefore, RG I accounts for about 10 - 25% of pectin (Lara-Espinoza et al., 2018).

Rhamnogalacturonan II (RG II): RG II is the most complex branch of a pectic domain, containing an HG backbone, and is the most conserved and wide spread domain of the plant. RG II accounts for 0 - 10% of pectin. A short HM of RG II is substituted by four side chains of several unusual sugar residues. RG I and RG II region of pectin are known as hairy regions of pectin (Lara-Espinoza et al., 2018; Mellinas et al., 2020).

2.4.2. Application of Pectin

Pectin has a wide range of applications such as in plant growth, development, morphogenesis, defense, cell-cell adhesion, wall structure, signaling, cell expansion, wall porosity, binding of ions, growth factors and enzymes, pollen tube growth, seed hydration, leaf abscission, and fruit development (Khan et al., 2013; Chen et al., 2015; Yamada et al., 2015). Pectin has remarkable properties; therefore, pectin is utilized as a gelling, stabilizing, and emulsifying agent in the food and cosmetic industries. Also, pectin has multiple positive effects on human health, including lowering blood cholesterol and serum glucose levels, inhibiting the growth of cancer cells, and stimulating the immune response (Jackson et al., 2007; Mohnen, 2008; Lara-Espinoza et al., 2018). Moreover, pectin is used to produce various exclusive products, including edible and biodegradable films, adhesives, paper substitutes, foams and plasticizers, surface modifiers for medical devices, materials for biomedical implantation. Besides, pectin is used as a carrier material in colon-specific drug delivery systems (Chambin et al., 2006; Vityazev et al., 2017; Lara-Espinoza et al., 2018), effectively removing toxic chemicals like lead, cadmium, arsenic, and mercury from the gastrointestinal tract and respiratory organs (Kohn, 1982; Lara-Espinoza et al.,

2018). Moreover, pectin has been used as a pectin hydrogel in controlled-release matrix tablet formulations and water purification (Aydìn & Akbuğa, 1996; Thakur et al., 2019).

3. Pretreatment of LB

Different components present in LB are complex and impart their role differently in different types of biomasses in converting LB to other value-adding products. The major constituents of biomass are cellulose, hemicellulose, lignin, and pectin. Among these various constituents, lignin acts as the protective covering, and it is responsible for the delay in the degradation of hemicellulose and cellulose (Sharma et al. 2019; Chaturvedi 2013). Also, pectin prevents the degradation of other polysaccharides present in biomass (Marcus et al. 2008). This kind of action makes pretreatment more important so that lignin, pectin, and other polysaccharides disintegrate efficiently into smaller fragments and simple sugars (Marcus et al. 2008; Sharma et al. 2019; Chaturvedi 2013).

The pretreatment of LB is the fundamental step that affects the efficiency of conversion and downstream processes to produce different products. The goal of pretreatment is i) to disrupt the structure matrix of LB and make smaller fragments ii) to increase the surface area and pore volume iii) to reduce crystallinity and recalcitrant nature of LB iv) to increase the yield of simple fermentable sugars, improving hydrolysis (Rajendran et al. 2017; Chaturvedi 2013; Puligundla et al. 2016). There are different types of pretreatment methods developed, and the techniques are expensive. For considering the effective pretreatment method, the method needs to have different advantages like the method should use low energy and cost effective, should be applicable for different kinds of biomass, should depolymerize hemicellulose, reduce crystallinity and recalcitrant properties of LB, should not produce the inhibitors resisting the hydrolysis and growth of microorganisms, and should be able to recover all the lignocellulosic components (Agbor et al. 2011; Chaturvedi 2013). In addition, the pretreatment process should have low sugar

disintegration, less chemical consumption, should be safe to operate, and less risky (Puligundla et al. 2016). Factors like cost, disposal, and toxicity need to be focused before planning or choosing the pretreatment method (Tu and Hallett 2019).

3.1. Classification of pretreatments

The pretreatment methods are broadly classified into physical, chemical, biological, and combination or multiple or hybrid methods (Fig. 1). Nowadays, the combination pretreatment method is commonly employed because it is more effective in breaking down biomass (Rajendran et al. 2017; Sharma et al. 2019; Puligundla et al. 2016).

3.1.1. Physical pretreatment

This method includes grinding, chipping, milling, etc. like processes that generally reduce the size of biomass, increasing surface area, reduce the crystallinity of cellulose, and reduce the degree of polymerization (Puligundla et al. 2016). Physical treatment is also known as mechanical treatment. Radiation, ultrasonication, heat treatment, microwave, and sonication are included in physical pretreatment methods. Physical pretreatment enhances hydrolysis by increasing surface area and transferring heat and mass (Rajendran et al. 2017). In physical treatment, high temperatures with high mechanical shearing are applied. However, when treated biomass is taken out from the chamber, explosion or damage in lignin may occur due to a rapid decline in temperature and pressure. The disadvantages of physical pretreatment are high energy requirements and operational cost, not suitable for lignin removal, and a high chance of equipment devaluation (Sharma et al. 2019).

3.1.2. Chemical pretreatment

In this process, varieties of chemicals such as alkali, acids, organic solvents, ozone, ionic liquids are used for treatment. The chemical pretreatment process has been useful for various biomasses,

although there is a chance of less production of sugars from softwoods (Tu and Hallett 2019). Sodium, calcium, potassium, and ammonium hydroxides are commonly used chemicals in alkaline pretreatment. In such a pretreatment process, swelling of biomass occurs, surface area increases, polysaccharides are exposed by breaking down intramolecular linkages, and sometimes the crystalline structure of cellulose as well (Rajendran et al. 2017). Alkali pretreatment is suitable for those biomasses containing less lignin (Agbor et al. 2011). In acidic pretreatment, commonly hydrochloric acid, phosphoric acid, sulphuric acid, etc. are used. Dilute acids are more preferred because strong acids are corrosive and non-environment friendly. However, dilute acid may degrade carbohydrates and form humins, which further impact sugar yield and produces unwanted byproducts (Tu and Hallett 2019). Organic solvents such as ethanol, methanol, acetone, oxalic acid, glycerol, ethylene, and salicylic acid are also applied in the pretreatment process. These organic solvents enhance retention and enzymatic digestibility of cellulose and help to remove lignin and hemicellulose. However, some organic solvents are inflammable and may cause fire and explosion. Moreover, organic solvent increases the cost and impacts the environment (Sharma et al. 2019). Ionic solvents treatment is a new and expensive method. However, these solvents are nonvolatile, environmentally friendly, thermostable, non-toxic, non-explosive, and can be recovered and reused in various ways. In the ionic solvent treatment process, cation interacts with lignin, whereas anions interact with cellulose. Thus, the combined action of cations and anions effectively degrades LB. Ionic solvents such as 1-butyl-3-methylimidazonium chloride, triethylammonium hydrogen sulfate dissolve biomass (Rajendran et al. 2017; Tu and Hallett 2019).

3.1.3. Biological pretreatment

This method uses different microorganisms, mostly fungi like brown-rot fungi, white-rot fungi, soft-rot fungi, and some actinomycetes and bacteria are used for hydrolysis of LB. Those

microorganisms produce different enzymes so that degradation and hydrolysis of different cell wall components occur (Rajendran et al. 2017; Sharma et al. 2019; Tu and Hallett 2019). Other enzymes useful in the degradation of biomasses are described in section "Lignocellulolytic enzymes". Since biological pretreatment uses different microorganisms, the conditions applied should be favorable for microorganisms. The size of particles, temperature, aeration, pH, organic compounds, or inorganic compound, a ratio of carbon and nitrogen source, and strain of bacteria or fungi, etc. should be optimized for efficient and effective biological treatment (Tu and Hallett 2019). Here, degradation and fermentation co-occur, resulting in hydrolysis of cellulose, hemicellulose, lignin, and pectin producing organic acids, biofuels, enzymes, etc. (Sharma et al. 2019). This method requires less energy, environmentally friendly, sustainable, cost-effective, no production of inhibitors, and no chemical is required. However, this method takes a long time to take a few days to months, large space, and careful growth conditions (Agbor et al. 2011; Tu and Wallett 2019).

In biological pretreatment, bacteria such as *Clostridium* sp., *Cellulomonas* sp., *Streptomyces* sp., *Bacillus* sp., *Azospirillum lipoferum*, *Mucilaginibacter* sp., etc. and fungi like *Trichoderma reesei, Aspergillus niger, Aspergillus nidulans, Fusarium gramineum, Neurospora crassa,* etc. are used. Many studies have also shown that microbial consortia are more effective. The consortium may be of more than two bacteria or more than two fungi or a combination of bacteria and fungus. Some insects, ruminant animals, gastropods, and worms also can degrade lignocellulose in their specific way (Sharma et al. 2019)

3.1.4. Hybrid pretreatment

This pretreatment class includes the combination of other pretreatment methods, for example, steam explosion, ammonia fiber explosion, CO_2 explosion, etc. which enhance the degradation and

hydrolysis of LB (Sharma et al. 2019). This class of pretreatment exploits the chemicals and the use of conditions that affect the physical and chemical properties of biomass (Agbor et al. 2011). Such type of pretreatment method consumes less chemical and energy, degrades hemicellulose and cellulose, addition of chemicals enhances hydrolysis and is applicable in industries. However, this method needs high pressure and produces inhibitory products, there is a chance of low yield, high energy consumption, and chemical threat (Sharma et al. 2019).

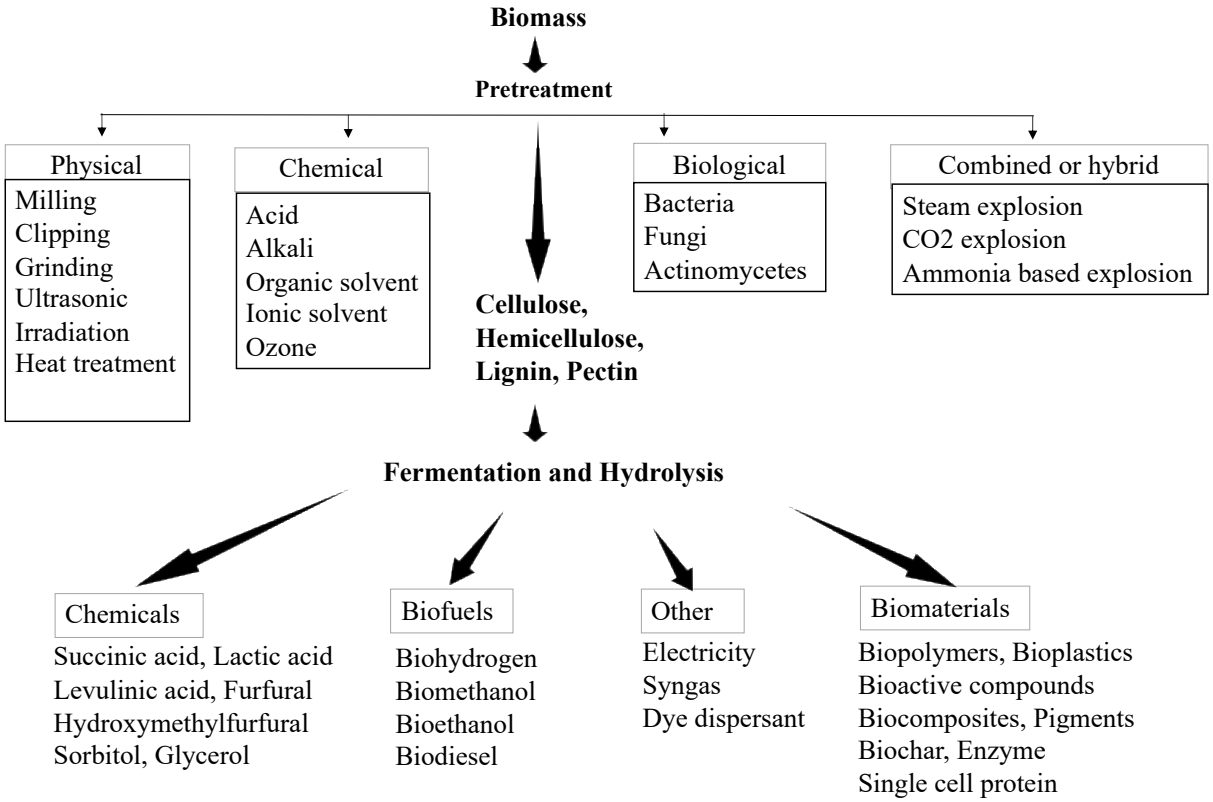

Fig. 1 Overview of value-added products from LB

4. Lignocellulolytic enzymes

Lignocellulolytic enzymes are a complex array of microbial enzymes that are required for degradation of a complex structure of LB. Those lignocellulolytic enzymes are found in bacteria, fungi, yeast, plants, and actinomycetes (Saini et al. 2015). These enzymes are categorized into ligninolytic, cellulolytic, hemicellulolytic, and pectinolytic enzymes. Those enzymes are used in

converting LB into value-added commodities, bio-scorning and bio-polishing of jeans, improving efficacy of detergents, maceration and color extraction from juices. In addition, those enzymes are employed for enzymatic deinking, pulping, wastewater treatment, improving the nutritional properties of animal feed, retting of flax, producing oligosaccharides, clarifying juices, treating dyes and other organic pollutants, bioethanol production, and developing biosensors, etc. (Kuhad and Singh 2007; Kuhad et al. 2011; Mtui 2012; Saini et al. 2015). The different lignocellulolytic enzymes when applied together, are more effective and work better. Agrawal et al. (2018) analyzed synergism between cellulase and accessory enzymes (xylanase, pectinase, glucosidase etc.) for hydrolysis of wheat straw. The study revealed the enzyme cocktail or multiple enzymes were more effective and is sustainable approach for efficient hydrolysis of LB (Agrawal et al. 2018).

4.1. Cellulolytic enzymes

Cellulolytic enzymes are the third most important industrial enzymes and they hydrolyze cellulose into fermentable sugars which can be used for further applications. These cellulolytic enzymes are used in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture (Kuhad et al. 2011).

Cellulase consists of endo-glucanase, exo-glucanases or cellobiohydrolase, and β-glucosidase which are hydrolytic enzymes, act synergistically and belong to glycosyl hydrolase family (Henrissat and Davies 1997). The endo-glucanase hydrolyzes the glycosidic bonds on amorphous sites in between the chain randomly and produces small fibers with free reducing and non-reducing ends whereas exo-glucanase hydrolyzes on chain ends of cellulose to release cellobiose and some glucose (Beguin and Aubert 1994; Lynd et al. 2002). So far, cellobiose has inhibitory activities during cellulose hydrolysis, the β-glucosidase is essential to break the final glycosidic bonds of cellobiose and produce sufficient glucose molecules. In some cellulase complexes, β-glucosidase

is not present or is present in the small amount resulting in insufficient hydrolysis (Beguin and Aubert 1994; Bhat and Bhat 1997; Maki et al. 2009). Some cellulase producing microbes are; *Aspergillus niger, Aspergillus nidulans, Aspergillus oryzae, Penicillium brasilianum, Penicillium occitanis, Penicillium brasilianum, Penicillium fumigosum, Neurospora crassa, Trichoderma atroviride, Sporotrichum thermophile, Trametes versicolor, Agaricus arvensis, Pleurotus ostreatus, Phlebia gigantea, Acinetobacter junii, Acinetobacter amitratus, Bacillus subtilis, Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus circulan, Bacillus flexus, Bacteriodes* sp., *Cellulomonas biazotea, Paenibacillus curdlanolyticus, Pseudomonas cellulose, Streptomyces drozdowiczii,* and *Streptomyces lividans* (Kuhad et al. 2011).

4.2. Hemicellulolytic enzymes

Hemicellulases include a group of enzymes; xylanases, β-mannanases, β-D galactanases, β-xylosidases, and arabinofuranosidases involved in the breakdown and hydrolysis of xylans, mannans, galactans, xylobiose, and arabans (Sajith et al. 2016). Xylan and mannan are the most copious components of the hemicelluloses. In hardwood hemicellulose, xylan is the major component whereas in softwood mannan is the major component. These different hemicellulases are interdependent (Sajith et al. 2016), act synergistically in the hydrolysis of hemicellulose to form several monomeric sugars and expose the surface for cellulase activities (Perez et al. 2002; Sweeney and Xu 2012). *Thermobifida halotolerans, Actinomadura* sp., *Cellulomonas flavigena, Streptomyces cyaneus, Cellulosimicrobium cellulans, Enterobacter s*p., *Penicillium* sp., *Bacillus pumilus* like microbes produce hemicellulases (Walia et al. 2017).

Xylanases catalyze the hydrolysis of internal β-1,4-xylosidic linkages in xylan to oligomers. The enzymes like endo- and exo-xylanases hydrolyze the cross-linked of hemicelluloses that cleave the xylene to generate oligosaccharides (Perez et al. 2002).

Mannanases are the second important hemicellulases after xylanases. Mannanases hydrolyze randomly β-D-1,4-mannopyranosyl linkages in mannose containing polysaccharides such as glucomannans, and galactomannans to produce short β-1,4-manno-oligomers. These short oligomers are finally hydrolyzed into mannose by β-mannosidase. The side group connected to xylan and glucomannan chains can be cleaved by α-glucuronidase, α-arabinosidase, and α-D-galactosidase (Dekker 1985; Chauhan et al. 2012).

Galactanases hydrolyze the D-galactans and L-arabino D-galactans. Endo-galactanases degrade D-galactans randomly at the β-1,4-D-galactosyl linkage and produce D-galactose and galactose oligosaccharides. Another type of endo-galactanases degrade β-1,3-D-linked galactosyl bonds of arabinogalactans and produce D-galactose, L-arabinose and other oligosaccharides (Dekker 1985). β-xylosidase hydrolyzes xylobiose and small xylooligosaccharides to xylose and facilitates the hydrolysis of xylan. Arabinofuranosidase catalyzes the removal of arabinose substituents and facilitates an increase in the access points of xylanase to the xylan (Dekker 1985).

4.3. Ligninolytic enzymes

The ligninolytic enzymes are concerned in the degradation of lignin into low molecular weight compounds which generally comprise a group of three principal enzymes; lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Niladevi 2009; Saini et al. 2015). Other enzymes like aryl alcohol dehydrogenase, vanillate hydroxylate, dioxygenase, catalase, aromatic aldehyde oxidase, etc. act as mediators of lignin degradation. Those mediator enzymes either help by producing H_2O_2 required for the activity of peroxidases or catalyze the breakdown products of lignin degradation (Niladevi 2009). Ligninolytic enzymes are produced by several bacteria, fungi, and actinomycetes particularly white-rot fungi having ligninolytic function (Hofrichter 2002; Sokan-Adeaga et al. 2016). Ligninolytic microbes are *Pseudomonas fluorescens*, *Pseudomonas*

Putida, *Enterobacter lignolyticus*, *Escherichia coli*, *Streptomyces viridosporus*, *Streptomyces paucinobilis*, *Rhodococcus jostii* (Tian et al. 2014), *Panaeolus papilionaceus*, and *Coprinopsis friesii* (Heinzkill et al. 1998). The white rot fungus species like *Bjerkandera adusta*, *Cyathus stercoreus*, *Dichmitus squalens*, *Phanerochaete chrysosporium* produce multiple isoenzymes of lignin and manganese peroxidases but do not produce laccase (Huang et al. 2013).

Laccase is a polyphenol and multicopper oxidase having four copper atoms per molecules at their active site. It acts as an oxidizing agent and cofactor thus it is involved in efficient oxidation, cleavage, and polymerization of several biological as well as synthetic phenolic and non-phenolic compounds (Hofrichter 2002; Fisher and Fong 2014; Saini et al. 2015). Laccases do not require H_2O_2 to oxidize different substrates because it has four copper ions (Niladevi 2009). Laccase converts polyphenol of the biomass to yield phenoxy radicals and quinines (Singh et al. 2016).

LiP, also known as ligninase, is a member of the oxidoreductases family. LiP oxidizes the compounds having high redox potential in the presence of hydrogen peroxide and can oxidize both phenolic and non-phenolic compounds. LiP also catalyzes the oxidation of a wide range of aromatic substrates. Thus, LiP having a wide range of substrate specificity and high redox potential they are utilized in various industrial applications (Maciel et al. 2010).

MnP, another important enzyme for lignin degradation, is also a member of the oxidoreductases family. MnP has heme peroxidases with low redox potential and requires hydrogen peroxide for the activity. This enzyme is not able to oxidize nonphenolic compounds and can be manganese dependent or versatile peroxidases (Niladevi 2009; Fisher and Fong 2014; Placido and Capareda (2015). MnP oxidizes Mn^{2+} to highly reactive Mn^{3+} that catalyzes the oxidation of phenolic structure to phenoxy radicals. MnP also depolymerizes natural and synthetic lignin as well as lignocelluloses in free form. The molecular weight of MnP ranges from 38 to 62.5 kDa and 11

different isoforms in *Ceriporiopsis subvermispora* were observed (Lobos et al. 1994; Hotrichter 2002; Hammel and Cullen 2008; Niladevi 2009).

4.4. Pectinolytic enzymes

Pectinases are biocatalyst comprising complex enzymes that degrade pectic substances (Khan et al. 2013; Kavuthodi et al. 2015; Oumer 2017). In the past, pectinases were only known as the virulence factor in the decomposition of a plant cell wall. But, today, pectinases are becoming necessary enzymes due to its multiple applications in a wide range of industrial sectors such as food, textile, and biofuel industries and accounts for about 25% of total enzyme market sale (Oumer, 2017; Shrestha et al., 2020). Nevertheless, pectinases are the upcoming important biocatalyst because of its versatility, broad-substrate specificity, inducibility, stability, and ability to act on a large variety of pectic substances (Pedrolli et al., 2009; Martín et al., 2019).

Esterase enzyme commonly known as pectin methylesterase catalyzes the de-esterification of methyl ester linkage of pectin and produces methanol and acidic pectin. Depolymerizing enzyme like polygalacturonase (PG), and pectin lyases (PL) break the main pectin chain. PG catalyzes the breakdown of α-1,4-glycosidic bonds between galacturonic acid chain while PL transeliminates pectin molecule, producing an unsaturated product (Jayani et al. 2005). Pectinases are produced from *Bacillus* sp., *Pseudomonas* sp., *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus flavus, Penicillium chrysogenum*, *Penicillium expansum*, *Trichoderma viride*, *Mucor piriformis*, *Yarrowia lipolytica*, *Saccharomyces sp.*, *Candida sp.*, *Actinomycetes sp.*, *Lactobacillus sp.*, *Aeromonas caviae* (Pandey et al. 2009; Geetha et al. 2012).

4.4.1. Sources of pectinase

There are many sources for pectinase that are widely distributed in nature. The different sources of pectinases are plants, bacteria, fungi, yeasts, insects, nematodes, and protozoa. However, the

microbial source is significant due to the fast growth, diversified widespread, shorter fermentation, and more accessible genetic modifications. Microbial pectinase plays a vital role in plant pathogenesis, symbiosis, and decomposition of plant deposits (Polizeli et al., 2016; Amin et al., 2019). Also, the microbial pectinase production process has no political or social issues as in plant and animal sources. It is reported that 35% of pectinase is from a bacterial source, 55% from fungi and yeast, while only 15% from plant or animal sources (Kavuthodi and Sebastian, 2018). Many studies are undergoing related to a high quality of pectinase like the high stability to different physicochemical conditions from microbes of different sources. Some of the microbes capable of producing pectinase at their optimum condition are listed in Table 2. The enzyme extraction from a diseased animal may have a substantial risk of contamination from diseases and difficulties in isolation and purification, thereby discourage the production of pectinase from animal sources (Bhardwaj et al., 2017).

Table 2 Optimal conditions of different microbes for pectinase production and pectinase activity (-: not mentioned)

Source			**Optimal conditions**			
			Time	**Temperature**		
	Microorganisms	**Pectinase activity**	**(hours)**	**(°C)**	**pH**	**References**
Bacterial	*Bacillus* sp.	27.0 U/ml (polygalacturonate lyase)	60	34	8.5	(Guo et al., 2019)
	Bacillus sp.	40.0 U/ml (polygalacturonase)	60	34	8.5	(Guo et al., 2019)
	Bacillus sp.	110.47 U/mg	48	34	5	(Yu and Xu, 2018)
	Enterobacter	14.16 U/mg	72	30	9	(Abdollahzadeh et al., 2020)

	Bacillus sp.	1431 U/ml (wheat bran)	24	37	7.2	(Karthik et al., 2011)
	Streptomyces fumigatiscleroticus	45.93 U/ml to 98.65 U/ml	48	35	6	(Govindaraji and Vuppu, 2020)
	B. licheniformis	39 U/ml to 219 U/ml	120	37	9.5	(Bibi et al., 2016)
	Bacillus subtilis SAV	3315 U/gds	144	35	4	(Kaur and Gupta, 2017)
	Bacillus subtilis SAV	10.5 U/gds (pectin lyase)				(Kaur and Gupta, 2017)
	Bacillus subtilis starin Btk 27	66.3±1.2 U/ml (SmF)	144 48	35 37	4 6.5	(Oumer and Abate, 2018a)
	Bacillus subtilis starin Btk 27	1272.4±25.5 U/g (SSF)	48	37	6.5	(Oumer and Abate, 2018a)
	Bacillus tequilensis CAS-MEI-2-33	1370 U/ml	40	40	7	(Zhang et al., 2019)
	Bacillus tequilensis SV11-UV37	1371.15 U/gds (pectate lyase)	72	37	6	(Chiliveri et al., 2016)
	Bacillus tequilensis SV11-UV37	85.45 U/gds (polygalacturonase)	72	37	6	(Chiliveri et al., 2016) (Takcı and Turkmen, 2016)
	Bacillus subtilis	62.18 U/mg	72	37	7	
	Geotricum candidum	0.554 IU/ml (immobilization)	48	25	7	(Ejaz et al., 2018)
Fungal	*Aspergillus awamori*	-	72	30	5	(Dasari, 2020)
	Aspergillus sp.	106.7 U/ml	48	30	5.8	(KC et al., 2020)

	Candida sp.	19.5 U/ml	120 - 144	55	5.5	(Aggarwal et al., 2020)
	Penicillum notatum	82.92 U/gds (wheat bran)	72	35	3	(Amin et al., 2017)
	Coriolus versocolar	73.21 U/gds (wheat bran)	96	30	5	(Amin et al., 2017)
	Rhizopus sp.	11.63 IU/ml	168	30	-	(Handa et al., 2016)
	S. fibuligera	117.55 U/ml (pectin lyase)	24	28	-	(Haile and Kang, 2019)
Yeast		8.28 U/ml (polygalacturonase)	48			
	S. fibuligera *W. anomalus*	16.96 U/ml (pectin lyase)	24			
		8.01 U/ml (polygalacturonase)	24			
	W. anomalus			28	-	(Haile and Kang, 2019)

4.4.1.1. Bacterial source

Different bacteria are the ideal source for pectinase production. Bacteria are easy to grow in different laboratory environmental conditions like temperatures, and pH, have a shorter lifetime, easy to manipulate genetically, and are environmentally friendly (Amin et al., 2019; John et al., 2020). *Bacillus* sp. is an essential and highly efficient bacterial source for industrial applications (Kavuthodi and Sebastian, 2018) and be perceived from Table 2.

4.4.1.2. Fungal source

The most crucial fungal strain for pectinase production is *Aspergillus* sp. This strain has outstanding importance because the strain produces non-toxic and economically important

metabolites. Also, *Aspergillus* sp. is assigned as Generally Regarded as Safe microbe (John et al., 2020). Table 1 also illustrates that the fungi such as *Aspergillus, Candida, Geotricum,* and *Rhizopus* have a longer optimum time on pectinase production than bacteria. In the study, *Penicillium notatum, Coriolus versicolor*, *Ganoderma lucidum*, and *Trametes hirsuta* produced 83.46 U/gds, 73.21 U/gds, 62.29 U/gds, and 47.15 U/gds pectinases respectively when incubated at 30℃ (Amin et al., 2017).

4.4.1.3. Yeast pectinase

Yeast also acts as the source for pectinase. For example, *Wickerhamomyces anomalus, Saccharomycopsis fibuligera, Papiliotrema flavescens, Pichia kudriavzevii,* and *Saccharomyces cerevisiae* have the potential to be used as the starter for coffee fermentation (Haile and Kang, 2019). Moreover, *Rhodotorula glutinis* isolated from fruit has pectinase and tannase activity. It was stated that this yeast could be higher (503.1 U/ml) polygalacturonase producer using immobilized cells (Taskin, 2013). The polygalacturonase activity was also observed in *Aureobasidium pullulans*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola*. However, Belda et al. selected *M. pilcherrima* as a potential candidate to use on a semi-industrial scale for red wine upgrading (Belda et al., 2016).

4.4.1.4. Insect pectinase

Commercial pectinases are generally produced from a microbial source. However, insects can also be the source of pectinase. The sugarcane weevil, *Sphenophorus levis*, has pectin methylesterase and endo-polygalacturonase. The pectinase from sugarcane weevil has been expressed in *Pichia pastoris* and *P. pastoris* could be used as an alternative resource for industrial pectinase (Habrylo et al., 2018). Some of the forest pests and wood-boring cerambycid beetle, *Apriona japonica*, have cell wall degrading enzymes, including pectinase (Pauchet et al., 2014).

4.4.1.5. Plant and animal pectinase

The plants and animal sources are less popular because many social and political issues need to be considered, less yield, time-consuming, not appropriate to produce pectinase over a wide range of environmental conditions. In addition, plant and animal tissues may contain some harmful materials. Furthermore, sometimes pectinase extracted from diseased plants and animals may potentially contaminate with diseased cells. Besides, plant tissue contains phenolic compounds and animal tissue, some inhibitors, and proteases (Bhardwaj et al., 2017; Kavuthodi and Sebastian, 2018). Thus, the microbial source is the most valuable source for the pectinase enzyme. However, there should be more exploration for plant and animal pectinases.

4.4.2. Alternative substrates for pectinase production

The extracted pectin from different plant sources has humongous applications. Thus, it is wise to use agricultural wastes as alternative sources for pectinase production to benefit economically and reduce agricultural waste disposal. The agro-wastes are renewable, inexpensive, and natural resource, acting as a cost-effective and eco-friendly source for pectinase production. Natural resource utilization will help solve the energy shortage problem, pollution concerns, waste disposal issues and will not compete with the food supply chain (Govindaraji and Vuppu, 2020; Shrestha et al., 2020). Various agricultural wastes are incredibly nourishing, which enable and promote the growth of different microbes. Apple pomace, sunflower heads, orange peel, barley grain, and grape pomace have extensively been used as the carbon source for pectinase production. However, there are lots of agro-waste that have the potential to be used as the source of pectin for pectinase production.

4.4.2.1. Crop waste

Wheat bran is one of the common agro-industrial waste accounts 15 to 20% and gets discarded during the wheat flour production process. Wheat bran has the potential to be used for industrially important enzymes. It is reported that wheat bran and tea extract together produce a 15.28-fold increase in polygalacturonase with a specific activity of 33.47 U/ml under solid-state fermentation (SSF), at 50℃ and pH 4 (Anand et al., 2017). In another study, when *Aspergillus ojae* was cultured on media with wheat bran at 37℃ for 4 days under SSF, polygalacturonase of 535.4 U/g substrates was produced (Demir et al., 2014). Similarly, Oumer and Abate observed maximum pectinase production (1272.4±25.5 U/g) by *Bacillus subtilis* using wheat bran under SSF having initial pH 6.5 and at 37℃ (Oumer and Abate, 2018a). Kaur and Gupta screened 25 different agro waste such as orange peel, coconut fiber, paddy straw, mustard straw, mustard oil cake, rice bran, lemon peel, etc., for pectinase and pectin lyase production. The maximum pectinase (450.50±12.8 U/gds) was observed in orange peel-containing media at pH 7 and 35℃. However, the mixture of orange peel and coconut fiber at a ratio of 4:1 revealed as the prominent substrate producing 3315 U/gds of pectinase with moisture content 60% at 35℃ and pH 4 after 4 days of incubation (Kaur and Gupta, 2017).

Tobacco stalks are also considered waste, have a high amount of pectin, and *Bacillus tequilensis* CAS-MEI-2-33 showed pectinase activity of 1370 U/ml in optimal fermentation conditions; 40 hours, pH 7, and inoculum amount 3% (Zhang et al., 2019). *Aspergillus awamori* was exploited to use leaves of *Ficus religiosa* in solid-state fermentation for producing pectinase. The maximum pectinase was observed in 72 hours, at 30℃, 60% v/w moisture content, and pH 5 (Dasari, 2020).

Onion waste has the potential to be used as a substrate in pectinase production. Pereira et al. produced 4.82 U/ml of thermostable pectinase under SSF by using onion waste (Pereira et al., 2017).

4.4.2.2. Fruit and vegetable waste

When peels from fruits like orange, mango, pomegranate, mosambi were studied for pectinase activity, orange peel gave maximum pectinase of 98.65 U/ml at 35℃, pH 6, and 48 hours of incubation (Govindaraji and Vuppu, 2020). Mango peel utilization for pectinase production using *Aspergillus foetidus* gave the highest polygalacturonase and pectin lyase under SSF. The pectinase produced by this way gave the maximum mango juice clarification (92.5±0.26%) at 40℃ and 150 min of incubation (Kumar and Sharma, 2012). Similarly, Reddy and Saritha used mango fruit processing waste as a carbon source for pectinase production by using *Enterobacter* sp. under submerged fermentation (SmF) (Reddy and Saritha, 2016). Grape skin and olive pomace were exploited to produce lignocellulosic enzymes, including pectinase. Two local species of *Aspergillus*, *A. niger* and *A. fumigatus,* were grown in solid media containing grape skin and olive pomace, and in some cases, media was supplemented with wheat (Sánchez et al., 2015). Orange peel was added in Czapeck media as a carbon source under SmF to produce pectinase from *Aspergillus*. They also showed the maximum pectinase yield (117.1± 3.4 μM/mL/min) at 30℃ and pH 5.5 after four days of incubation (Ahmed et al., 2016b). In another study, *Bacillus licheniformis* produced 219 U/ml of pectinase under SmF and media supplemented with orange peels (Bibi et al., 2016). Similarly, orange peel and coconut fiber in 4:1 ratio were also used under SSF for pectinase production. In this condition, maximum pectinase (3315 U/gds) and pectin lyase (10.5 U/gds) were recorded at pH 4, moisture content of 60%, and in 4 days and 8 days of incubation, respectively (Kaur and Gupta, 2017). Papaya is a tropical worldwide popular fruit

having medicinal and nutritional values and contain pectin. Therefore, papaya peel has been used for pectin methylesterase production (246.83 U/gds) by exploiting *Aspergillus tubingensis.* The methylesterase thus produced was applied for pineapple juice clarification (Patidar et al., 2016).

4.4.2.3. Algal biomass

Algal biomass has also been used as a natural resource to produce pectinase. The presence of a significant amount of pectin in the middle lamella of the algal cell wall makes it applicable for this application. For example biomass of charophyte green algae, *Penium margaritaceum,* has been studied in pectinase production (Domozych et al., 2014). In addition, brown or green algae such as *Dictyopteris polypodioides, Sargassum wightii, Dictyopeteris divaricate, Ulva lactuca* and *Codium tomentosum,* were used for pectinase production. Besides, the algal biomass can be used as the feedstock for other hydrolytic enzymes production. The green algal biomass from *U. lactuca* was observed to be the most suitable substrate than the rice husk and sugarcane bagasse for *Bacillus licheniformis* in producing pectinase under SmF (2457±3.31 U/mg) compared to SSF (1432±1.46 U/mg) (Pervez et al., 2017).

4.4.3. Pectinase production strategies

4.4.3.1. Fermentation strategies

In general, two fermentation techniques solid-state fermentation (SSF) and submerged state fermentation (SmF), are traditional methods of pectinase production. During fermentation, different parameters like growth medium, cultivation conditions, pH, temperature, aeration, moisture, salts, carbon source, nitrogen source, inoculum volume, inoculum age, type of strain, and inducers are considered for boosting pectinase production using microorganisms (Amin et al., 2017). However, the processes are tedious and time consuming. The microbial recombinant DNA

technology tools such as gene cloning and metabolic engineering are applied to increase pectinase production.

Most of the enzyme manufacturing industries produce enzymes by using submerged state fermentation because this process can easily be accessed and the production is topped up (Oumer and Abate, 2018a). For the commercial production of pectinase, SmF is generally used but due to the high cost of medium ingredients, low yield, high energy consumption, and high effluent production, SSF is replacing SmF. To find out the economical and appropriate source of carbon for pectinase production, different agricultural waste, including wheat bran, orange bagasse, sugarcane bagasse, banana peel, etc., are studied using SSF (Sharma et al., 2013). The main differences between SmF and SSF with advantages and limitations are listed in Table 2. A little or no water is added to a solid substrate in SSF so that microbes, mostly fungi, grow as in their natural habitat and produce maximum pectinase. Also, microbes get sufficient nutrients from the substrate added, and no need for extra nutrients in SSF (John et al., 2020). Some of the pectinase produced following SmF and SSF with the optimum fermentation conditions in Table 2.

4.4.3.2. Immobilization strategies

Immobilization of enzyme or microbes onto the solid carrier is also another effective technique to enhance enzyme production. Enzymes in the form of whole cells are immobilized using various methods such as adsorption, covalent bonding, and entrapment. Several nanocarriers, such as magnetic nanoparticles, are used for enzymes. However, different types of carriers need to have some unique properties like biocompatibility, low toxicity, biodegradability, tailored surface chemistry, etc. The immobilization is advantageous to recover enzyme easily when it is in free form and has short time stability. Therefore, this immobilization process minimizes enzyme loss, contamination and separates products easily (Hosseini et al., 2020; John et al., 2020). The pectinase

enzyme was immobilized onto the surface of magnetic nanoparticles by dextran polyaldehyde as a cross-linking agent and applied in apple juice clarification. The immobilized pectinase showed superior thermal stability than the free enzyme, 87% residual activity after seven cycles of recyclability, and rapid reduction of turbidity up to 74% (Sojitra et al., 2017). The polyaldehyde pullulan can be a potential alternative for the immobilization of pectinase on the glass beads (Hosseini et al., 2020). Similarly, pectinase was immobilized in magnetic chitosan particles and exploited in the clarification of juices (Magro et al., 2019).

4.4.3.3. Genetic modification strategies/ strain development

Microbial strain development or improvement is another technique/method for improving bacterial strain applied in the commercial development of fermentation processes to enhance bioproducts productions. The method implies the blending of classical techniques, biochemical engineering, and molecular genetics. The strains of bacteria are developed or improved by different processes like mutagenesis and molecular process, which impart mainly in changing the microbial DNA (Heerd et al., 2014). Strain development also benefits from an economic point of view by decreasing the production cost by not increasing the capital expenditure (Parekh et al., 2000; Ademakinwa et al., 2017). Many factors influence bacterial strain development, such as the type of processes, methods, treatment duration, and microorganisms type, etc. (Munir et al., 2020). Chemicals such as N-methyl-N-nitro-N-nitrosoguanidine (NTG), hydroxylamine, nitrous acid are commonly used chemical mutants. Ultraviolet (UV), X-ray, and gamma-ray radiation are physical mutants. UV radiation induces cross-links and pyrimidine dimerization in DNA. The intercalating agents, for example, ethidium bromide, acridine dyes, intercalate the base pairs between nucleotides resulting in frameshifts or loss of plasmids (Parekh et al., 2000; Heerd et al., 2014).

Heerd et al. 2014 applied UV radiation and/or NTG for *Aspergillus sojae* strain development and studied their effects on pectinolytic activities. The study demonstrated the PG production was enhanced by strain development and could be applied industrially (Heerd et al., 2014). In another study, *A. tamatii* was treated with UV radiation, sodium azide, nitrous acid, and ethyl methane sulphonate for strains development. The study illustrated sodium azide treated strain produced high polygalacturonase activity (Munir et al., 2020). Similarly, the strain enhanced the production of fructosyltransferase when chemicals such as ethidium bromide and ethyl methane sulfonate were used as mutagens (Ademakinwa et al., 2017). Thus, the strain development of microorganisms plays an important role in meeting the increasing demand for enzymes in different avenues of applications.

4.4.4. Classification of pectinase

Based on the action of pectinase on a substrate; the pectinase enzymes can be classified into three groups; protopectinase, pectinesterase, and depolymerase (hydrolase and transeliminase) (Garg et al., 2016; Oumer, 2017; Patidar et al., 2018) which is shown in Fig 2.

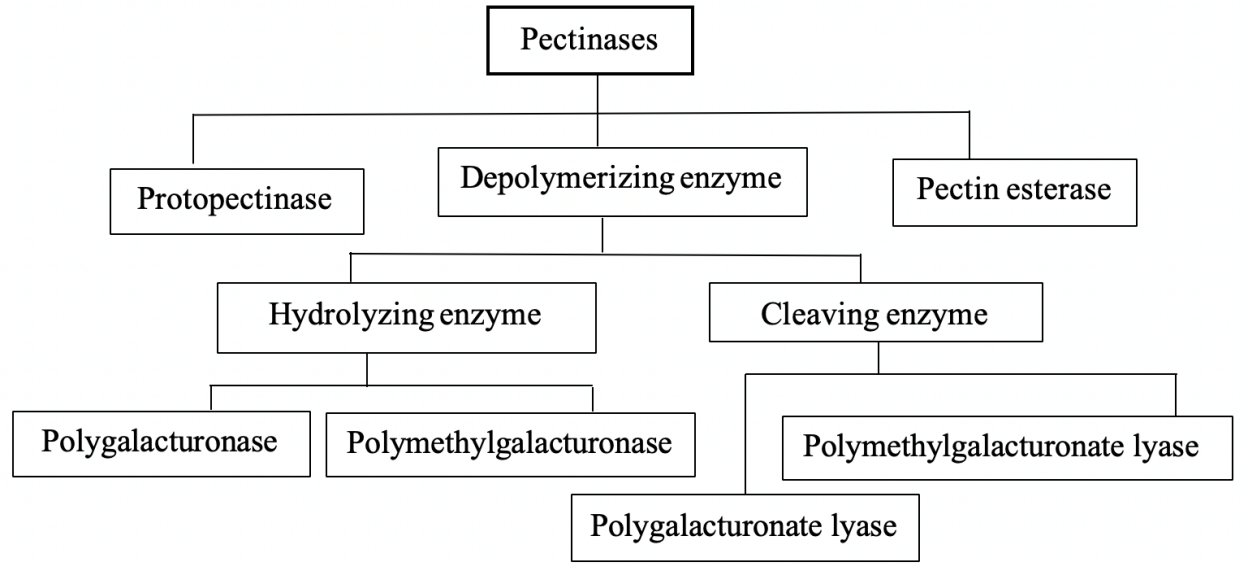

Fig. 2 Classification of pectinase enzymes based on their action

Protopectinase: This type of pectinase is generally present in unripe fruits, converts insoluble protopectin into a soluble form of pectin. Thus, protopectinase is supposed to be the first enzyme necessary for pectin degradation. Pectinosinase is alternately termed protopectinase (Tapre and Jain, 2014; Patidar et al., 2018).

Pectin esterase (PE, de-esterase): This pectinase is also known as pectin methyl hydrolase, pectin methylesterase, and pectase. PE is a carboxylic acid esterase that catalyzes the de-esterification of methyl ester linkage of galacturonan backbone of pectin to yield pectic acid and methanol (Jayani et al., 2005; Garg et al., 2016). It is reported that after the action of PE, the resulting pectin is then acted upon by PG and lyases. PEs are found in higher plant's fruits, leaves, flowers, stems, and roots. They are believed to shift pH from acidic to alkaline conditions (Kavuthodi and Sebastian, 2018; Nighojkar et al., 2019).

Depolymerizing enzyme: This class constitutes hydrolyzing enzymes and cleaving enzymes.

a. Hydrolyzing enzymes (hydrolases) include polygalacturonase (PG) and polymethylgalacturonase (PMG). Both PG and PMG hydrolyze the pectin chain by acting on α

1→4 glycosidic bond in the presence of water molecule and produce polygalacturonate and polymethylgalacturonate, respectively(Jayani et al., 2005; Garg et al., 2016).

b. Pectin transaminase (Transeliminase) includes polygalacturonate lyase (PGL) and polymethyl galacturonate lyase (PMGL), which cleave α (1→4) glycosidic linkage by transelimination reaction producing unsaturated galacturonates. The breakage of the pectin chain is non-hydrolytic; the action is by eliminating H from C-5 and producing D4:5 unsaturated products (Tapre and Jain, 2014).

The depolymerizing enzymes, both hydrolases and transeliminases, can be exo and endo based on the site of pectinase action on the pectin chain. If pectinase acts randomly in a long chain of pectin, it is known as endo-pectinase, and if pectinase works at the reducing end of the chain terminally, it is exo-pectinase. The summary of different types of pectinases with their mode of action and final products is shown in Fig. 3. Pectinases are also classified as alkaline or acidic pectinase depending on the pH range in which pectinases operate. Most fungi produce acidic pectinase as they work at acidic pH, while most bacteria produce alkaline pectinase able to function at alkaline pH (Kavuthodi and Sebastian, 2018; Nighojkar et al., 2019). Based on the application of temperature, pectinases are classified into psychrophilic, thermophilic, and mesophilic pectinase. Generally, most of the pectinase exploited in industries are mesophilic, which works in a wide temperature range between 20℃ to 50℃. However, pectinases which show activity at 37℃ are not applicable in such industries that use low or high temperature processes. Besides, extremophiles can be the best alternatives (John et al., 2020). Due to the increasing demand for pectinase in various industries, there is a need to produce hyperactive, resisting a wide range of pH, and thermostable characteristics pectinase.

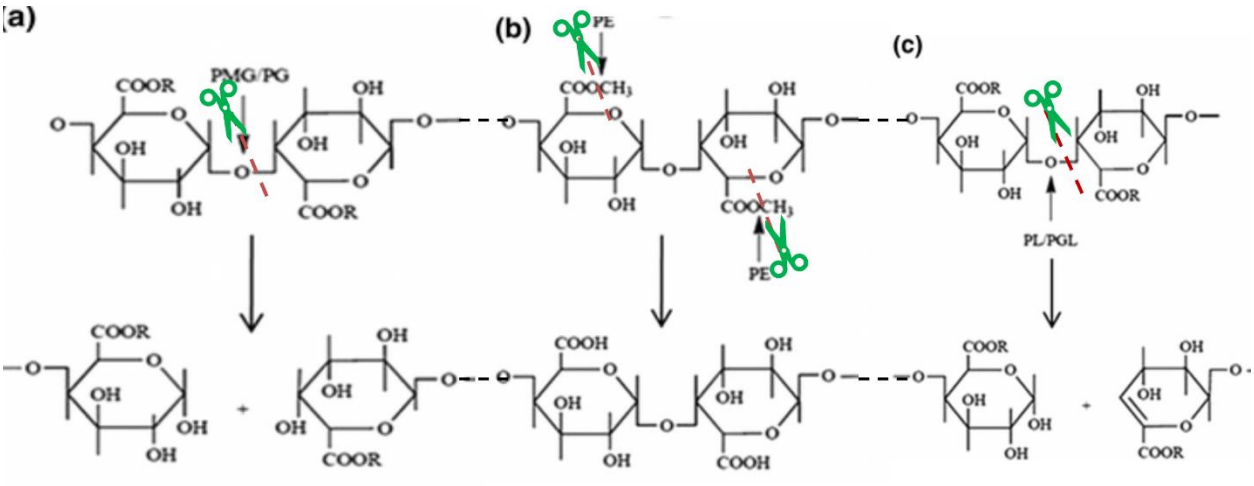

Fig. 3 Mode of action of pectinases a) polymethylgalacturonase and polygalacturonase b) pectin esterase c) pectin lyase and polygalacturonate lyase [Adopted and modified from (Garg et al., 2016)]

Table 3 Summary of pectinases classification and their mode of action [adopted and modified from (Jayani et al., 2005; Garg et al., 2016)]

Pectinase type	Pectinase name	Common name	E.C.No.	Substrate	Mode of action and cleavage site	Product
De-esterifying enzyme						
Esterase	Polymethylgalacturonate esterase (PMGE)	Pectin esterase	3.1.1.11	Pectin	Random cleavage of the methyl ester group of galacturonate unit Pectic acid	Methanol
De-polymerising enzyme						
a) Hydrolases	i. Polygalacturonase (PG)			Cleavage of α -1,4-glycosidic linkage in pectic acid		
	Endo-polygalacturonases (endo-PG)	Polygalacturonase	3.2.1.15	Pectate	Random cleavage of pectic acid	Oligosaccharides

	Exo-polygalacturonase 1 (exo-PG1)	Polygalacturonase	3.2.1.67	Pectate	Terminal cleavage from the nonreducing end of polygalacturonic acid	Monosaccharides
	Exo-polygalacturonase 2 (exo-PG2)	Polygalacturonase	3.2.1.82	Pectate	Penultimate Cleavage	Di-galacturonates
	ii. Polymethylgalaturonases (PMG)			Hydrolytic cleavage of α -1,4glycosidic linkage in pectin		
	Endo-PMG	Pectin hydrolase		Pectin	Random cleavage	Oligo methylgalacturonates
	Exo-PMG	Pectin hydrolase		Pectin	Terminal cleavage from the nonreducing end of pectin	Methyl monogalacturonate
b) Lyases	i. Polygalacturonate lyase (PGL)			α -1,4-glycosidic linkage in pectic acid by trans-elimination forming unsaturated galacturonates		

Endo-PGL	Pectate lyase	4.2.2.2	Pectate	Random cleavage	Unsaturated oligogalacturonates
Exo-PGL	Pectate lyase	4.2.2.9	Pectate	Cleavage of penultimate bonds from the nonreducing end	Unsaturated digalacturonates
Oligogalacturonate lyase	Pectate lyase	4.2.2.6	Oligo-galacturonate	Terminal cleavage	Unsaturated monogalacturonates
ii. Polymethylgalacturonate lyase (PMGL)			Cleavage of α -1,4-glycosidic linkage in pectin by trans-elimination forming unsaturated methyl galacturonates at the nonreducing end		
Endo-PMGL	Pectin lyase	4.2.2.10	Pectin	Random cleavage	Unsaturated methyl oligogalacturonates
Exo-PMGL	Pectin lyase		pectin	Terminal cleavage	Unsaturated methyl monogalacturonase

4.4.5. Applications of pectinase

Pectinases are economically very important due to their vast implications. The discovery of the pectinase enzyme brought a revolution in the economic and commercial sectors. Although it was used in making fruit juice and wine in the past, recently its application is increasing continuously. The applications of pectinases in fruit juice industries, wine industries, paper and pulp industries, wastewater treatment, bioethanol production, extraction of DNA from a plant, and protoplast isolation from a plant are also significant. Furthermore, pectinases are employed in preparing animal feed, saccharification and liquefaction of biomass, retting and degumming of plant fiber, bio-scouring of cotton fiber, coffee and tea fermentation, and oil extraction (Kubra et al., 2017; Oumer, 2017; Shrestha et al., 2021b).

4.4.5.1. Pharmaceutical industries

The most substantial industrial enzymes, pectinases, have remarkable applications in the pharmaceutical industry. Humans are consuming a variety of dietary fiber foods of plant origin, which need to be properly digested in our digestive system. However, pectinase plays an important role in digesting the fermentable dietary fibers as well as improving the immune system. Many pharmaceutical products are produced from the fermentation of pectic substances present in fruits and vegetable peels using pectinase. The products thus produced are used as a fiber supplement for the treatment of diabetes and obesity (Satapathy et al., 2020). Moreover, plant essential oils are used for the treatment of various microbial infections, including wound healing and cancer. The organic solvent in oil extraction from different medicinal plants may damage some essential properties and lose the concentration of important phytochemicals. Thus, pectinase is applied in pharmaceutical industries to minimize such possible losses. The pectinase stimulates the liquefaction of cell wall components of the plant and destroys emulsifying properties of pectin to

increase the product yields (Satapathy et al., 2020). Also, pectinase is used to produce enzyme-sensitive colon-specific tablets in the pharmaceutical industries for oral colon-specific drug delivery application. Generally, oral drugs have a short half-life and an absolute bioavailability and are destroyed by acidic pH in the stomach. This may need high doses or a large amount of drug administration, resulting in many side effects such as increasing toxic levels and gastrointestinal problems. Therefore, a controlled delivery system such as an oral colon-specific drug delivery system is appropriate where the drugs are not damaged by acidic gastric juice in the stomach and are shipped to the colon. Furthermore, the tablets are degraded by pectinase and attain an accurate controlled release of drug in the colon (Zhu et al., 2019).

4.4.5.2. Fruit juice industries

The fruit juice industries use pectinases, and other cell wall degrading enzymes such as cellulases and proteases to soften fruits, facilitate the extraction, increase the juice yield, and clarify the juice (Patidar et al., 2018; Verma et al., 2018; Nighojkar et al., 2019). The fruits, mainly tropical fruits, have more pectin and are pulpy (Tapre and Jain, 2014). The mechanical grinding of fruits in juice extraction may contribute to jelly, viscous, and cloud-like appearance products due to the positively charged protein that gets coated around by negatively charged pectin. The addition of pectinase in fruit juice would result in degrading pectin and exposing the positively charged protein. The exposition of positively charged protein reduces the electrostatic repulsion between cloud particles and the formation of a more massive particle, which further settles down and gives clear juice (Tapre and Jain, 2014). Also, the starch complex is responsible for the characteristic viscosity and turbidity of the juice. However, pectinase supplement breaks the glycosidic bonds present between the galacturonic acid monomers to increase the juice yield. In addition, pectinase decreases the water holding capacity of pectin, and reduces the viscosity and turbidity (Tapre and

Jain, 2014; Nighojkar et al., 2019; Shrestha et al., 2021b). Furthermore, the enzymatic treatment clears the juice by breaking down the pectin, helps to settle down the suspended particles, and eliminates undesirable changes in color, smell, and stability. The pectinases are applicable in manufacturing fruit purees, peeling of fruits segments, and wine clarification. The implication of biocatalyst reduces the cost and increases the yield of products and is highly competitive than the other different established processes. It represents a simple alternative method for diversifying production, mainly in tropical fruit juice, and increasing market share (Tapre and Jain, 2014; Verma et al., 2018).

4.4.5.3. Paper and pulp industry

In paper industries, the sheet formation step is the most crucial, and the presence of pectin in pulp reduces dewatering. In the past, the filter fiber used to have bigger holes so that water gets easily removed from paper, and peroxide bleaching reduces cationic demand (Reid and Ricard, 2000; Samanta, 2019). The use of chemical and bleaching treatment aids in retaining properties of paper, but these are not environmentally friendly; therefore, enzymatic treatment is becoming more popular. In paper manufacturing, pectinases are used to lower the cationic demand of pectin polymers and bleaching treatment of pulp. Pectinase depolymerizes the long pectin chain and reduces the cationic need because pectin, more than hexamers, has cationic demand, whereas shorter than hexamers do not have cationic demand (Reid and Ricard, 2000; Samanta, 2019). The alkaline pectinases produced from *Streptomyces* sp., *Bacillus* sp., *Erwinia carotovora*, and some fungi are applied in making the paper more uniform, softer to touch, brighter, and to increase the pulp strength compared to the conventional soda-ash cooking method (Kubra et al., 2017; Samanta, 2019).

4.4.5.4. Oil extraction

An organic solvent like hexane, which has the potential to cause cancer, was used in the past for the extraction of edible oil from oil-producing crops like rapeseed, coconut germ, sunflower seed, palm, and olives (Ortiz et al., 2017). Nowadays, pectinase and other cell wall degrading enzymes, cellulases and hemicellulases, are used to extract oil from plants, olive, sunflower, coconut, canola, and other oil seeds. During the grinding process, enzymes are added to degrade pectin in the middle lamellae of plant cells and promote the extraction of oils from the inner part of the seeds or germ layer, thus, enhancing oil release from different sources. The pectinase addition also increases the stability, yield, rheological properties of oil and improves the level of vitamin E contents, polyphenols, and organoleptic quality of oil (Hoondal et al., 2002; Iconomou et al., 2010; Ortiz et al., 2017). Another study illustrated that pectinase from bacteria facilitated the oil extraction from sesame seeds increasing the yield, and clarity and decreasing the relative viscosity (Shrestha et al., 2021b).

4.4.5.5. Textile industries

In textile industries, pectinase is used with other enzymes like amylase, lipase, cellulase, and hemicellulase to remove sizing agents from cotton safely and eco-friendly (Hoondal et al., 2002; Bristi et al., 2019). The enzymes are specific; the action is quick and saves energy, cost, raw material, water, and energy; thus, the enzymes are replacing the harsh, costly, and environment polluting chemical method for scouring of cotton fiber in textile industries (Bristi et al., 2019). Pectinase treatment helps to decrease the fabric weight and wet. The addition of enzymes is an eco-friendly process that maintains the quality, whiteness, and brightness of the textile, like the conventional method, and lowers the release of eco-toxic agents (Vigneswaran et al., 2012; Singh et al., 2020).

4.4.5.5.1. Bio-scouring of cotton

Bio-scouring means removing undesirable non-cellulosic contaminants like minerals, pectin, natural colorants, fats, waxes, protein, and water-soluble compounds. These impurities can be removed using enzymes but not with chemicals. For example, pectinase is used for bio-scouring of cotton without any harmful side effects (Kubra et al., 2017) but improves the dyeing properties, water absorption capacity, texture of cotton (Vigneswaran et al., 2012), and lowers the weight. This biological scouring method decreases total dissolved solids (TDS) value but increases biological oxygen demand (BOD) amounts of bio-scouring effluent (Aggarwal et al., 2020).

4.4.5.5.2. Degumming and retting fiber

Degumming is the procedure of removing heavily coated gum from outside the xylem, phloem, or pericarp of the plant fiber like ramie and sun hemp before its use for textile making. The enzymatic processing of fiber is energy conservative, eco-friendly, non-toxic, non-biodegradable, and non-polluting. The combination of chemical and enzymatic treatment in fiber reduces chemical and energy consumption (Iconomou et al., 2010; Chamani et al., 2012). Ramie fiber is the best natural fiber but contains pectic substances and hemicellulosic material; thus, pectinase and xylanase are applied for effective degumming in textile. This green enzymatic process prevents the use of the hot alkaline solution, high energy consumption, and environmental pollution (Singh et al., 2020). In addition to this, pectinase improves its malleability and good separation of blast fiber from decorticated ramie fiber, producing better quality yarn (Banik and Ghosh, 2008).

4.4.5.6. Coffee, cocoa, and tea fermentation

Pectinases are used to remove the mucilaginous coat from coffee beans and accelerate coffee's fermentation process, cocoa, and tea. The characteristics of tea depend on oxidation products like theaflavins, thearubigins, and other inherent components. Generally, non-volatile compounds are

present in tea leaves, such as polyphenols, flavonols and flavonol glycosides, flavones, phenolic acids, etc. Also, amino acids, chlorophyll, carbohydrates, other pigments, organic acids, caffeine, enzymes, vitamins, and minerals, etc. are found in tea leaves (Chaturvedula and Prakash, 2011). Pectinases decrease foam formation in the instant coffee/tea powders, remove the thick layer consisting of pectic substances from coffee beans, accelerate the fermentation of coffee/tea, and develop chocolate flavor in cocoa fermentation (Samanta, 2019). The concentration of pectinase should be maintained to get maximum efficiency. Also, the enzyme must be added in a proper level because an excess of enzyme lowers the glaze and quality of tea leaves, decreases the color, flavor, aroma, and increases the rate of spoilage of tea leaves (Chaturvedula and Prakash, 2011; Sharma et al., 2013; Samanta, 2019). A study illustrated that pectinase from *Bacillus tequilensis* effectively removed mucilage from the coffee beans (Koshy and De, 2019).

4.4.5.7. Wine industries

Pectinases containing low pectin methylesterase are used not only in the extraction of wine. Still, they are also used to increase juice yield and accelerate filtration. The main problem in winemaking is cloudiness that makes filtration of wine difficult due to the presence of pectin. The addition of pectinase hydrolyzes pectin and removes the cloudiness present in wine. Also, pectinase enhances the aroma and taste of the wine (Garg et al., 2016; Rollero et al., 2018). However, the phenolic compound is a secondary metabolite; the enzymatic action intensifies and stabilizes the color of wine by increasing the phenolic compound of wine (Busse-Valverde et al., 2011). The immobilized pectinase has been used recently for decreasing the viscosity of grape must in winemaking and facilitating in clearing and filtering wine (Martín et al., 2019). Pectinase is applied as a biocatalyst in winemaking because it enhances the extraction of compounds and improves the properties, volatile flavor, and aroma of wine (Rollero et al., 2018; Jiang et al., 2020).

4.4.5.8. Protoplast preparation

Protoplast preparation is the initial and important step in producing a new plant with specific and interesting traits/characteristics. Protoplast from different plant leaves such as maize, Arabidopsis, tobacco, wheat, barley, rice, etc., has shown a significant effect in genetic modification, plant physiology, and development. Protoplasts can be isolated from different sources, either mechanically or enzymatically. The mechanical process may cause more breakage and result in more osmotic shrinkage. Thus, enzymatic protoplast preparation is beneficial compared to the mechanical process (Chamani et al., 2012). The protoplast isolated from plant tissues has revealed high transformation efficacy with low maintenance and holds their cell identity. The protoplast transient expression systems have contributed to elucidating intracellular signaling mechanisms in a plant. The studies in plant protoplast systems provide a framework for fundamental plant analysis. Enzymes like pectinase are useful to extract protoplast for plant tissue culture by which new genetic traits are induced in plant cells. Pectinase, in combination with cellulase, is used for the efficient isolation of protoplast (Solís et al., 1996). An enzyme mixture of cellulase, crude pectinase, and chitinase is also used for maximizing protoplast yield. A high output of protoplast can be obtained by adding pectinase, cellulase, and macerozyme (Shen et al., 2017). In another study, 4% cellulase and 1% pectinase treatment produced the highest number of viable protoplast and illustrated the best combination of enzymes for protoplast isolation from Lilium (Chamani et al., 2012).

4.4.5.9. Purification and detection of viruses

According to the types of viruses, several viral purification systems can be selected for use. In certain conditions, a mixture of enzymes such as alkaline pectinase and cellulase is employed for the viral purification process (Sharma et al., 2013). The cell wall degrading enzymes can be

exploited to liberate virus from plant tissues generally when the virus is constrained to the phloem (Hoondal et al., 2002). In a study, tobacco necrotic dwarf virus and potato leafroll virus transmitted by aphids were purified using enzymes. The plant tissue macerating commercial enzyme named Driselase mainly contains pectinase and cellulase. The study revealed the enzymatic treatment increased the purification yield and is limited to a phloem-related virus (Takanami and Kubo, 1979). The pectinase facilitates detecting hepatitis A virus and norovirus from different fresh and frozen berries and vegetables (Butot et al., 2007).

4.4.5.10. Wastewater treatment

Wastewater produced from vegetables and fruit processing industries, especially fruit juice industries or citrus processing industries, contains pectinaceous material that needs treatment before they are integrated into the environment. The physical and chemical treatments like physical dewatering, chemical coagulation, chemical hydrolysis, and methanogenesis are time-consuming, expensive, and hazardous to the environment and health (Hoondal et al., 2002). Therefore, biological enzymatic treatment has become the best alternative for sewage water treatment. Pectinases can quickly degrade pectic substances present in wastewater, which, in turn, helps in the decomposition of pectin residue by activated sludge treatment. The microbes may not have decomposed this wastewater during the activated-sludge treatment. Thus, nowadays, alkalophilic microorganisms producing pectinase are used to treat wastewater and increase biological oxygen demand (Mahesh et al., 2016). The pectinase enzyme can remove oil and grease from kitchen wastewater too, and the process is both cost-effective and eco-friendly. Besides, pectinase helps to remove biological oxygen demand and grease and oil from wastewater (Kamaruddin et al., 2019). Tobacco wastewater can be the source for culturing pectinase-producing microbes like *Rhizopus oryzae*. The pectinase made from *R. oryzae*, in turn, hydrolyze pectin-containing biomass

to acquire fermentable sugars, which has possibilities for profitable biofuels production (Zheng et al., 2017). The wastewater produced from domestic, industrial, and agricultural water activities contains different organic and inorganic substances, thus polluting the environment. However, thus produced wastewater can be used as a carbon or nitrogen source for the growth of microbes for reutilization purposes (Zheng et al., 2017). Immobilized pectinase on nanoporous activated carbon has been studied for treating pectin-containing wastewater and the study revealed 94% of pectin treated from wastewater (Mahesh et al., 2016).

4.4.5.11. Animal and poultry feed

An animal rumen contains some bacteria, fungi, and protozoa, which produces pectinolytic enzymes and plays an essential role in digestion. *Lachnospira multiparus* is a common bacterium found in rumen. This ruminant bacterium produces pectin lyase and pectin methylesterase, which is helpful in the breakdown of pectin present in food. The supplement of enzymes helps complete organic matter breakdowns in the rumen (Murad and Azzaz, 2011). Pectinases when supplemented in animal feed production, improve the feed quality by lowering the viscosity and boosting the nutrients absorptivity. Similarly, enzymes reduce the fiber content of the feed and enhance the liberation of nutrients trapped in fibers (Jayani et al., 2005). Some animals cannot lyse pectin present in plants, so the addition of pectinases in the feed helps the captivation of nutrients by liberating the nutrients after the breakdown of non-degradable fibers or releasing nutrients blocked by these fibers and decreases the fecal amount. Pectinases reduce feed viscosity, which directly intensifies the absorption of the nutrients and reduces animal defecation, and finally increases the weight of poultry (Garg et al., 2016; Abdulla et al., 2017; Bhardwaj et al., 2017).

4.4.5.12. Recycling of wastepaper

The recycling of paper products is increasing recently, and deinking of the used paper is a necessary step. Enzymatic deinking improves brightness, reduces chemical consumption, improves drainage, lowers residual ink of paper. However, excess use of the enzyme during deinking may result in fiber loss by more depolymerization and high BOD in effluents (Pathak et al., 2010). The enzymatic deinking process is less polluting, energy-saving, friendly, gives better performance to achieve the desired deinked pulp properties, and results in lowering disposal problems. Contrary to the enzymatic process, the chemical deinking process uses many chemicals that are harmful to the environment. Pectinase alters bonds near the ink particle and removes the ink from the surface washed out by washing or floatation (Pathak et al., 2010). Pectinase, in combination with xylanase, has been used for deinking of wastepaper to reduce the effects of harmful chemicals to 40% and improve the level of physical and optical properties, enhancing the quality of paper sheets (Singh et al., 2019a). The xylano-pectinolytic enzyme, when applied in the deinking of wastepaper from school to reuse, decreased the use of chemicals by 50%, biological oxygen demand 20.15%, and chemical oxygen demand 22.64% compared to the conventional chemical method. Also, this enzymatic deinking method was successful in attaining the same properties of paper like whiteness, brightness, etc. Thus, this approach was noticed to be cost-effective, environmentally friendly, and safe for recycling wastepaper from school (Singh et al., 2012).

4.4.5.13. Hydrolysis of biomass and bioethanol production

Different polysaccharide degrading enzymes like pectinases, hemicellulases, and cellulases are used in the saccharification of plant cell wall polysaccharides, including pectic substances, into simple sugars, which are used in bioethanol production. These enzymes are used in the

pretreatment of various feedstock for bioethanol production enhancement. The improvement in glucose yield, ethanol concentration, and productivity was attained by treating the feedstock with pectinase (Chen et al., 2012). The enzyme cocktail containing high pectinase activity saccharifies various biomasses effectively and aid in bioethanol production (Wang et al., 2019; Mihajlovski et al., 2020). The rye bran as a substrate achieved the maximal enzymatic activities for cellulase, amylase, xylanase, and pectinase by *Streptomyces fulvissimus*. Thus, *S. fulvissimus* was exploited for enzymatic degradation and saccharification of different agricultural biomass, including corn stover, cotton material, horsetail waste, yellow gentian waste. The bioethanol was successfully produced by employing *Saccharomyces cerevisiae*, horsetail waste, and treated cotton waste after enzymatic hydrolysis of wastes (Mihajlovski et al., 2020). Similarly, Sethi et al. (2016) obtained maximum pectinase activity by *Aspergillus terreus* by hydrolyzing banana peel under both SSF and SmF. Bioethanol was also produced by utilizing *S. cerevisiae* and different agro-waste biomass (Sethi et al., 2016b).

4.4.5.14. Enhance antimicrobial activity

The antibacterial activity of *Emblica officinalis* was enhanced when treated with pectinase. The study demonstrated the thermostable pectinase-producing bacteria isolated from hot water springs by using inexpensive agricultural residues as macronutrients promoted the antibacterial activity of *Emblica officinalis* (Sarsar and Pathak, 2019). The isozyme of pectinase associated with polygalacturonase activity from *Aspergillus niger* exhibited hydrolysis of chitosan into chitooligosaccharides and monomers. Thus, formed chitooligosaccharides and monomers mixture revealed antibacterial effects towards *Bacillus cereus* and *E. coli* (Kittur et al., 2005). Pectic heteropolysaccharides produced from the action of pectinase demonstrated antibacterial activity. The antibacterial activity was enhanced significantly when combined with lactic acid, tea

polyphenols, nisin, and sodium acetate. Due to this property, the pectic oligosaccharides are used as a food preservative or food additive to increase the self-life of food or inhibit the growth of the undesirable microorganisms in food (Li et al., 2013). The pectic oligosaccharides produced from an orange can inhibit the adherence and invasion of *Campylobacter jejuni* to Caco-2 cells to some extent. Thus, pectinase-induced pectic oligosaccharides might be useful as alternatives in foodborne pathogen control (Ganan et al., 2010).

4.4.5.15. Concrete crack healing

Recently, the study has found the pectinase application in the construction area and in the production of reinforced natural fiber composites. The natural fibers retted with the pectinase, either pectate lyases or polygalacturonases, retain the strength of fibers with variable fineness. This important eco-friendly property is useful in the development of natural polymer composites and is used in packing, construction, and automobile applications (Sisti et al., 2018). The incorporation of natural fibers in composites enhances the thermal stability, tensile strength, elastic characteristics, flexural strength, and compressive strength of the composites. The addition of oil palm fibers in cement composites proved to increase setting time and reduced the workability of fresh cement, decreasing the cost (Osofero et al., 2018). In addition, the pectinase produced from spore-forming microbes that are resistant to high pH can heal the concrete crack. Those microbes in cracks form calcite complex and precipitate calcium carbonate in the presence of water providing compatibility and compressive strength to concrete. When pectinases get incorporated as a catalyst or mineral precursor, the biological healing process prevents various environmental and health problems. The method is very cost-effective and environmentally friendly. Inorganic materials like silica fume, epoxy resin, and fly ash, when used for crack healing, may cause concrete thermal expansion (Govindaraji and Vuppu, 2020).

5. Valuable products from LB

For the production of useful, high value-added products from LB multi-steps as mentioned are normally require: (i) pretreatment (mechanical, chemical or biological) (Grethlein and Converse 1991), (ii) hydrolysis of the polymers to produce readily metabolizable molecules (e.g. hexose or sugars), (iii) bio-utilization of these molecules to support microbial growth or to produce chemical products and (iv) the separation and purification (Howard et al. 2003). Various sources of LB need to be considered separately since the different constituents like cellulose, hemicelluloses, and lignin vary in different resources. The depolymerization process plays an important role in all different feedstocks to produce different types of products like chemicals, enzymes, single-cell protein, microbial polysaccharides nanocellulose, bioactive compounds, bioenergy etc. (Garcia et al. 2016; Cho et al. 2019; Liu et al. 2019). Some of the value adding products produced from different biomass are listed in Table 4 with their references.

5.1. Platform chemicals

From biomass, the first possible chemicals in the biorefinery can be sugar compounds (C5 and C6 sugars) and further these C5 and C6 sugar compounds can subsequently be converted to any chemicals or materials. The degradation of these C5 and C6 compounds also depends on the depolymerization step (Kobayashi and Fukuoka 2013). The report of the US Department of Energy (DOE) describes sugar derivable building block chemicals that can be transformed into new families of useful molecules. These C5 and C6 sugar derived platform chemicals include 1,4-diacids (succinic acid, fumaric acid, and malic acid), 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol (Werpy and Petersen 2004). Bozell and Petersen (2010) explained that all these platform chemicals can be produced from biomass-

derived carbohydrate sources except glycerol. Bio-based products sales are expected to increase by 4 % annually and reach 11 % making bio-based products of $3,401 billion in the global chemical market by 2020 (Biotechnology Innovation Organization 2016).

5.1.1. Furfural

Furfural can be produced from various renewable agricultural resources like corn stalk, sugarcane bagasse, and eucalyptus wood (Dutta et al. 2012). Conventionally xylose is used to produce furfural and in turn, furfural is used as a chemical feedstock to produce furfuryl alcohol (Yan et al. 2014), furoic acid, maleic acid, 5-membered oxygen-heterocycles, succinic acid, and levulinic acid (Mariscal et al. 2016). Reduction of furfural produces tetrahydrofurfuryl alcohol which can be further converted into 1,5-pentanediol, a precursor to polyesters and polyurethanes (Kobayashi and Fukuoka 2013). Furfural has been extensively used in plastics, pharmaceutical and agrochemical industries, adhesives, and flavor enhancers (Takkellapati et al. 2018).

5.1.2. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) is a C6 sugars aromatic compound, are produced by dehydration of hexoses in acidic media. Various furan derivatives like 2,5-diformylfuran, 2,5-dimethylfuran, 2,5-bis(hydroxymethyl)furan, 2,5-bis(aminomethyl)furan, 2,5-dihydroxymethyltetrahydrofuran, 1,2,6-hexanetriol, 1,6-hexanediol, 2,5-bis(aminomethyl) tetrahydrofuran, caprolactone, caprolactam are formed after hydrogenation, condensation, reduction, and oxidation of 5-HMF (Melero et al. 2012). Some of the derivatives of HMF like 2,5-furandicarboxylic acid (FDCA), and 2,5-bis(hydroxymethyl)furan can be used as polyester (Kobayashi and Fukuoka 2013; Isikgor et al. 2015). Other derivatives like 2,5-dimethylfuran, 5-ethoxymethylfurfural, ethyl levulinate, and γ-valerolactone are upcoming biofuels (Van-Putten et al. 2013). 1,6-hexanediol is used in the preparation of polycarbonatediols for the production of polyurethanes that are used in coatings,

Table 4 Different biomass used and value-added products with the references

Biomass used	Organism used	Pretreatment method	Products produced	References
Agrave, miscanthus, ginkgo leaf, corn stover, wheat straw, barley straw, wheat bran, wood dust	*Aspergillus niger*	Physical treatment (drying and milling)	enzyme cocktail (cellulase, xylanase, pectinase) with high pectinase activity	Wang et al. 2019
Citrus peel, wheat husk, sugarcane bagasse, barley husk	*Bacillus* sp.	-	Pectinase, xylanase	Thite and Nerurkar 2018
Wheat straw	-	Steam explosion	Hydrolysis of biomass	Agrawal et al. 2018
Tobacco stem	*Rhizopus oryzae*	Cutting, soaking, Steam explosion	Pectinase	Zheng et al. 2017
Silybum marianum hairy root	*Agrobacterium rhizogenes*	Cutting	Silymarin (flavonolignan) and lipoxygenase	Rahimi and Hasanloo 2016
Lettuce plant	-	Far-red LEDs equipped with combined blue and red LEDs	Phenolic compounds	Lee et al. 2016
Olive tree pruning, grapevine pruning, almond shells free	*Saccharomyces cerevisiae*	Hydrothermal, dilute acid, and steam explosion	Bioethanol	Nitsos et al. 2018
Sugarcane bagasse, wheat straw	-	Thermal-enzymatic treatment	Levulinic acid	Schmidt et al. 2017

Bagasse pulp	-	Cold alkali and cellulase pretreatment	Cellulose nanofibers	Tao et al. 2019
Soybean straw	*Saccharomyces cerevisiae*	Alkali pretreatment followed by enzymatic treatment	Bioethanol	Kim 2018
Coconut waste, used oil	-	Microwave assistant treatment	Biodiesel	Thushari and Babel 2020
Banana peels, plantain peels	-	Drying, grounding	Dietary fiber and pectin	Emaga et al. 2008
Mandarin citrus peel	*Saccharomyces cerevisiae*	Steam explosion followed by enzymatic pretreatment	Bioethanol, limonene, galacturonic acid	Boluda-Aguilar et al. 2010
Corn cob	*Candida tropicalis, Aspergillus terreus*	Steam explosion	Xylitol	Guo et al. 2013

'-' refers not mentioned

elastomers, and adhesives. 1,6-hexanediamine and ε-caprolactone are used in the synthesis of various polymers. FDCA has many potential applications in polyesters, polyamides, and plasticizers (Isikgor et al. 2015).

5.1.3. Glycerol

Glycerol is a simple polyol (the simplest trihydric alcohol) compound and not a carbohydrate, but it has a mini sugar like structure. Glycerol is regarded as an important material because many lipids contain glycerol backbone (Bozell and Petersen 2010). There are many derivatives formed from

Glycerol after undergoing fermentation, reduction, and rehydration. Some of them are diglycerol, mannitol, 1,3-propanediol, propylene glycol, glycerol carbonate, ethylene glycol, glycidol, dihydroxyacetone, propane, acrolein, acrylic acid, glyceraldehyde, allyl alcohol, 3-methoxy-1-propene, glyceric acid, acetol (Bozell and Petersen 2010; Isikgor et al. 2015). Glycidol like a derivative of glycerol has prospective for production of other industrially valuable chemicals like epoxy resins, polyurethanes, and polyglycerol esters (Bai et al. 2013). Some microorganisms produce 1-butanol, 2,3-butanediol, 1,3-propanediol, ethanol, lactic acid, succinic acid, propionic acid, and dihydroxyacetone by utilizing glycerol (Almeida et al. 2012). Another derivative like glycerol carbonate has been used in the synthesis of industrially important chemicals such as glycidol, and in polymers, polyurethane foams, coatings, adhesives, and lubricants. Glycerol has also been utilized in the commercial production of epichlorohydrin (used as polymer and resins) (Bozell and Petersen 2010; Santacesaria et al. 2010).

5.1.4. Succinic acid

Succinic acid, C4 dicarboxylic acid, is a useful chemical in pharmaceutical, agricultural and food industries. From succinic acid, different high-value derivatives like adipic acid, 1,4-butanediol, methyl ethyl ketone, 1,3-butadiene, ethylene diamine disuccinate can be produced. Polyethylene succinate, polypropylene succinate and polybutylene succinate are the most studied polyesters of succinic acid. Through hydrogenation of succinic acid, 1,4-butanediol (BDO), γ-butyrolactone (GBL) and tetrahydrofuran (THF) are obtained (Luque et al. 2009; Isikgor et al. 2015). Thus, Succinic acid can be used for the production of succinate esters which are precursors for BDO, THF, and GBL. Succinic acid produces succinic anhydride under dehydrogenative cyclization and acts as a precursor of fumaric acid and maleic acid synthesis (Delhomme et al. 2009). In the food market, they are used as pH modifiers, flavoring agents, anti-microbial agents, health-related

agents, and biodegradable plastic (Kobayashi and Fukuoka 2013; Isikgor et al. 2015; Nhuan et al. 2017).

5.1.5. Lactic acid

Lactic acid is one of the most versatile organic acids produced from the fermentation of carbohydrates. It is transformed into other valuable chemicals like lactate ester, lactide, acetaldehyde, 2,3-pentanedione, pyruvic acid, lactate, oxalic acid, propylene oxide, propanoic acid, acrylates via esterification, hydrogenolysis, dehydration, oxidation, and reduction processes (Maki-Arvela et al. 2014; Isikgor et al. 2015). Lactic acid and its derivatives are applicable for the production of biodegradable polymers in food and beverage sectors, pharmaceutical and personal care products. It is also applicable in the production of cellulose-acetate-propionate polymers used as composite, adsorbent, membrane and coating material for furniture and automobile seating, bedding and carpet underlay thermal insulation (Kahlich et al. 2011; Alsaheb et al. 2015). Lactic acid has moisturizing, pH regulating and skin lightening properties. Thus, lactic acid is a common ingredient in personal care products. Lactic acid can also be used as herbicides and pesticides, in textile and tanning industries, fermentation of food in food industries, production of dairy products like yogurt, buttermilk, acidophilus milk, cottage cheese, etc. Polylactic acid which is biodegradable and produced from the polymerization of lactic acid is used in food packaging (Nee'Nigam 2009).

5.1.6. Levulinic acid

Levulinic acid (LA) is a linear C5 keto acid produced from the hydration of 5-HMF (Kobayashi and Fukuoka 2013). Traditionally, LA was produced from maleic acid in large cost and low volume but now LA is produced at a lower cost and large volume from lignocellulose (Isikgor et al. 2015). LA can replace bisphenol A as a plasticizer (Biotechnology Innovative Organization 2016) and is the foundation of the levulinic family. The presence of two reactive functional groups

in LA helps to produce many valuable new compounds with novel applications. The most important compounds obtained from LA are levulinic esters, 5-aminolevulinic acid (DALA), angelica lactones, 2-butanone, 4-hydroxypentanoic acid or its esters, γ-valerolactone and 1,4-pentanediol (Fernandes et al. 2012; Isikgor et al. 2015). LA and its derivatives have a range of applications in the preparation of pharmaceuticals and textile products, plasticizers, animal feed, coating material, and antifreeze. They also serve as a valuable chemical constituent from almost all sugars manufactured in the biorefinery (Bozell et al. 2000). DALA has agricultural value as it can be used as herbicide and to increase photosynthesis resulting in the growth of plants (Kobayashi and Fukuoka 2013).

5.1.7. Sorbitol

Sorbitol is sugar alcohol produced by hydrogenation of glucose. There are many compounds formed from sorbitol like isosorbide, sorbitan, sorbose, 2-ketogulonic acid, and vitamin C (Kobayashi and Fukuoka 2013; Isikgor et al. 2015). Sorbitol and its derivatives are extensively used as sweeteners, thickener, dispersant in food, cosmetics, toothpaste, polyurethane coatings, biocomposites, and hydrophilic interaction chromatography. Sorbitol protects food by preventing denaturation of protein, oxidation of fat, and retrogradation of starch. By fermentation, sorbitol is converted to L-ascorbic acid (vitamin C) which can be used as an antioxidant for foods (Pappenberger and Hohmann 2014), also has nutritional value to prevent scurvy and other pharmacological value (Kobayashi and Fukuoka 2013). Dehydration of sorbitol produces isosorbide and sorbitan. Isosorbide is used differently in medical fields like in regulating pressure in brain tumor (decreasing intracerebral pressure) and glaucoma (increasing intraocular pressure), and as medicine for Meniere's disease and angina pectoris (Parker and Parker 1998; Kobayashi and Fukuoka 2013). By hydrogenolysis, sorbitol can be converted into lower alcohol like glycerol,

propylene glycol, ethylene glycol, ethanol, and methanol. These lower alcohols further can be utilized to get other value-added products (Zhang et al. 2013).

5.1.8. 3-hydroxypropionic acid

Hydroxypropionic acid (3-HPA) is formed by fermentation of glycerol. On catalytic hydrogenation of 3-HPA produces 1,3-propanediol whereas heating of aqueous 3-HPA produces acrolein and acrylic acid. They are polymerized and used as an absorbent in diapers, hygiene products, coatings, adhesives, carpets, and fabrics. Traditionally, 3-HPA was produced from propylene (product of crude oil refining) oxidation but now it is produced as bio-based production (Bozell and Petersen 2010; Biotechnology Innovative Organization 2016).

5.1.9. Xylitol

Xylitol is a five-carbon sugar alcohol, produced commercially by catalytic hydrogenation of xylose (Bozell and Petersen 2010) found naturally in some fruits and vegetables with fewer calories (Star-Colibri 2010). Xylitol metabolism is not dependent on insulin thus it can be used in the therapeutic sector as a sugar substitute for diabetes individual (Lugani et al. 2017). Microbial production of xylitol from yeast is very attractive because the high-quality product is produced and is cost-efficient. In addition, high pressure and temperature, and xylose purification are not needed to produce xylitol from yeast (Guo et al. 2013). Xylitol can be converted into ethylene glycol and propylene glycol. Ethylene glycol is further used as an anti-freeze and a precursor of polyester. Similarly, propylene glycol can be used as an anti-freeze, brake fluid, cosmetic and food additive, and food emulsifier (Kobayashi and Fukuoka 2013).

5.2. Lignin derivable polymers

Lignin is considered as the key aromatic resource of the bio-based economy. Lignin contains different functional groups like methoxy, phenolic, hydroxyl, and aldehyde thus can produce

different chemicals particularly aromatic compounds and fuels. Some compounds derived from lignin are syringic acid, naphthalin methylnapthaline, p-coumaric acid, ferulic acid, caffeic acid, methanol, syringaldehyde, 4- hydroxybenzaldehyde, BTX (benzene, toluene, xylene), phenol and phenolics, benzoic acid, terephthalic acid, styrene, 4-hydroxybenzoic acid, eugenol, vanillin, vanillic acid, cinnamaldehyde, cinnamic acid, catechol, muconic acid etc. (Vanarasi et al. 2013; Isikgor et al. 2015). These polymers are used for the production of aromatic monomers and polymers, as the bio-absorbents for heavy metal ions in wastewater purification, as a roughage or fiber in food to prevent colon cancer, as the anti-viral, antioxidant, additives, chemical fuels, in tissue engineering, as the vehicles for gene delivery into human cells, as a dispersing agent while using pesticides and herbicides (Bozell et al. 2007; Agrawal et al. 2014).

5.2.1. Bioenergy/Electricity

The fossil energy is depleting continuously; thus, renewable energy source development is becoming essential. Potocnik (Potočnik 2007) mentioned solar energy and biomass energy are two important energy sources. Technologies like solid oxide fuel cells and microbial fuel cells can convert biomass to electricity (Scacchi et al. 2010). Photocatalytic conversion of LB can also generate electricity. It is mentioned that the International Energy Agency suggests bioenergy has the potential of providing 10% of the world's energy supply by 2035, and biofuel can replace 27% of world transportation fuel by 2050 (Wang et al. 2017). Microbial fuel cells (MFCs) are bio-electrochemical systems for electric power generation. MFCs can function at low temperatures, low electric power output, harsh reaction conditions, and limited lifetime hinder applications (Rosenbaum et al. 2010). MFC is composed of *anode* and *cathode*. Organic compounds are oxidized at the anode, and electrons are liberated, which move through an external circuit to the

cathode. At the cathode, electrons combine with an electron acceptor to generate electricity (Wang et al. 2017; Rosenbaum et al. 2010).

Algae biomass has the potential to produce sustainable and low-cost electricity (Velasquez-Orta et al. 2009). Activated sludge is reported to contain various types of electricity-producing bacteria such as *Alcaligenes faecalis*, *Enterococcus gallinarum*, *Pseudomonas aeruginosa*, and *Shewanella* sp. Thus, these bacteria help in waste treatment and electricity generation (Rashid et al. 2013). Electricity production from biomass using bio-electrochemical systems is also challenging because a single organism may not hydrolyze the biomass. However, binary culture or mixed culture of microbes like *Clostridium cellulolyticum* and *Geobacter sulfurreducens* can produce electricity (Ren et al. 2007).

5.2.1.1. Ethanol

From LB, ethanol can be produced by the thermal gasification process and biochemical fermentation process following different steps like pretreatment, saccharification and hydrolysis, fermentation, and ethanol recovery (Saini et al. 2015; Kumar et al. 2016). The thermal gasification process includes biomass conversion into syngas via partial oxidation at high temperature (500-800° C), purification of syngas as the impurities affects the process, and conversion of syngas into ethanol (Subramani and Gangwal 2008) as shown in the equations below.

$$nC_6H_{10}O_5 + nH_2O \rightarrow 6nCO + 6nH_2 \quad (1)$$

$$C.O. + 2H_2 \rightarrow CH_3OH \quad (2)$$

$$2CH_3OH \rightarrow C_2H_5OH + H_2O \quad (3)$$

From equations (1) and (2), carbon monoxide and hydrogen are produced in the same ratio, but methanol formation does not require the same ratio of carbon monoxide and hydrogen. Thus,

carbon monoxide reacts with steam and adjusts the proportion of them (Wyman et al. 2017) as in equation (4).

$$CO + H_2O \text{---}\rightarrow CO_2 + H_2 \quad (4)$$

Ethanol can be produced from syngas either by chemical catalysis process (Subramani and Gangwal 2008) or biological fermentation process (Abubackar et al. 2011). In the biological process, syngas produces ethanol, as shown in equations 5 and 6 (Wyman et al. 2017).

$$6CO + 3H_2O \text{------}\rightarrow C_2H_5OH + 4CO_2 \quad (5)$$

$$6H_2 + 2CO_2 \text{-----}\rightarrow C_2H_5OH + 3H_2O \quad (6)$$

Biological fermentation process is more specific and produces a higher yield of the desired product compared to the chemical catalytic process (Bozell and Peterson 2010) and benefits from operating at ambient pressures and temperatures (Wyman et al. 2017). Dehydration and polymerization of ethanol can produce polyethylene which can further produce polyvinylchloride (Bozell and Petersen 2010). Bioethanol can act as renewable building blocks to produce ethylene, propylene, and butadiene synthesis. Additionally, bioethanol can also be converted into other chemical products such as acetaldehyde, ethyl acetate, and acetic acid. The rapidly increasing production of bioethanol is an important replacement for fossil fuel (Takkellapati et al. 2018).

5.2.1.2. Biodiesel

Biodiesel is another product that can be produced from LB. It is non-toxic, safe to use, eco-friendly, cost-effective, has excellent lubricity, and exhibits a higher flash point than petroleum diesel. The waste residues can be feed to oleaginous microbes such as *Myxozyma melibiose*, *Lipomyces* sp., *Candida freyschussii*, *Aspergillus* sp., *Rhizopus*, *Mycobacterium* sp., *Haematococcus pluvialis*, *Euglena gracilis*, *Crypthecodinium cohnii,* etc. to produce biodiesel (Gujjala et al. 2019). Those microbes produce oil which is also known as single cell oil containing fatty acid very similar to

vegetable oil. This prospect is beneficial as it can be potential alternatives for petroleum diesel. However, the production of biodiesel from LB can be impressive economically and technically. The biomasses such as maize cobs, rice bran, and sugar cane bagasse are used as raw material for microbial oil production, which is finally used for biodiesel production (Yousuf 2012). Thushari and Babel produced biodiesel by utilizing coconut waste and nonedible used oil employing solid acid catalyst one step procedure using different types of reactors (Thushari and Babel 2020).

5.2.2. Biochar

LB can also be converted into carbonaceous solid material, i.e., biochar or activated carbon upon different processes like slow, intermediate, and fast pyrolysis, gasification, hydrothermal carbonization, or flash carbonization (Meyer et al. 2011) and various activation processes (chemical or physical treatments) (Srinivasan et al. 2015). The properties of biochar vary with the type of biomass, temperature differences for example living, colorful biomass having low surface area produces black biochar with a high surface area and no cellular structure. Biochar is used to mitigate greenhouse gas emissions, climate change and mostly use in carbon sequestration. In the agricultural view, they are used to increase soil pH, decrease aluminum toxicity, remove pollutants, decrease soil tensile strength, improve soil conditions for earthworm populations, and improve the fertilizer use efficiency (Xiao et al. 2018).

5.2.3. Enzymes

Industrially important and highly demanded enzymes are cellulases, xylanases, ligases, pectinases, and proteases. Different substances present in LB favors different enzymes of microorganisms during solid-state fermentation or submerged fermentation. Functions of different enzymes have been already described briefly in previous pages. Those enzymes are applied by various industries such as fuel, food, wine and brewery, animal feed, textile and laundry, pulp and paper, and

agriculture (Kuhad and Singh 2007; Kuhad et al. 2011; Mtui 2012). Hence to congregate the increasing demand for industrially important enzymes and to comprehend the enzymes potentials in different fields, different integrative research on basic and applied aspects is important and must be continued (Kuhad et al. 2011).

5.2.4. Single-cell protein

The world would need to produce millions of tons of meat and dairy products per year by 2050 to meet global demand for animal-derived protein as per current consumption levels (Ritala et al. 2017). The developing countries in the world are reportedly having a major protein deficiency concern and hence there is a need for an increased protein animal feed and human food supply. The protein demand can be fulfilled by single-cell protein (SCP) as an alternative to global food problem (Mondal et al. 2012). SCP can be produced from inexpensive waste materials like food and beverage processing industries, as well as directly from forestry and agricultural sources (Anupama and Ravindra 2000; Anbuselvi et al. 2014). Algae, fungi including filamentous fungi, yeast and bacteria can all be used as SCP (Anupama and Ravindra 2000). SCP derived from fungi provide vitamins primarily from the B-complex group (thiamine, riboflavin, biotin, niacin, pantothenic acid, pyridoxine, choline, glutathione, folic acid, and p-aminobenzoic acid). The cell walls of fungi being rich in glucans also provide fiber to the diet. Low-density lipoprotein cholesterol has been reduced when mycoprotein from *Fusarium venenatum* was consumed (Turnbull et al. 1992).

5.2.5. Syngas

Syngas is the synthetic gas produced when biomass is heated over 430° C/860° F in the presence of oxygen or gas. This process is also known as gasification. Syngas thus produced from a renewable source like biomass can be used as the source for power production or can be converted

into lower alcohol; methanol, ethanol, fuel and chemical products; ammonia, dimethyl ether (NSF 2008). Polyhydroxyalkanoates like biodegradable polymer can be produced from the fermentation of syngas by microbes and this can replace petrochemical plastics since this process is economical and feasible (Choi et al. 2010).

5.2.6. Bioactive compounds

Fruit and vegetable biomass contain various health beneficial natural compounds known as bioactive compounds. These bioactive compounds can be extracted by different methods like solvent-based, solid-liquid, liquid-liquid, ultrasound-assisted, microwave-assisted, and enzyme-assisted extraction. Biocatalysts such as cellulase, pectinase, and hemicellulase improve in the extraction and recovery of these bioactive compounds. Some of these compounds are carotenoids, flavonoids, phenolic acids (Choi et al. 2010). Such bioactive compounds are used as the antioxidant, anti-inflammatory, anti-allergic agents and are used in the production of beauty products, prevention, and treatment of various diseases (Choi et al. 2010; Saini et al. 2019).

The Carotenoids group includes zeaxanthin, lutein, cryptochrome, lycopene β-carotene, β-cryptoxanthin, neochrome, α-carotene, and β-carotene. Carotenoids are commonly found in colored fruits and vegetables like tomato, different citrus peels, carrot waste and shellfish. These compounds are found in different photosynthetic organisms like cyanobacteria, algae, higher plants, some non-photosynthetic bacteria, yeast and fungi (Misawa 2009; Jaswir et al. 2011).

Flavonoids include luteolin, sinensetin, naringin, hesperidin, neohesperidin, diosmin, rutin, kaempferol, quercetin which are present in different citrus peels (Lou et al. 2014). Different flavonoids have different functions like protecting from ultraviolet radiation and phytopathogens, signaling during nodulation, male fertility, auxin transport, coloration of flower attracts pollinators (Bradshaw and Schemske 2003).

Phenolics include gallic acid, sinapic acid, ferulic acid, hydroxybenzoic acid, caffeic acid-O-glucoside, tryptophane, anthocyanins, flavonol glycosides, catechin, myricetin which are present in different peels and seeds of fruits (Saini et al. 2019). The source of phenolics are fruits, vegetables, and beverages like coffee, tea, wine, and fruit juices. Phenolics protect human tissue against oxidative stress, have different health benefits and avoid the development of chronic diseases (Minatel et al. 2017).

5.2.7. Nanocellulose

LB contains high fiber cellulose-rich waste material that can be converted into a novel and advanced material known as nanocellulose. Nanocellulose has unique properties like high specificity, high surface area, liquid crystalline behavior, barrier properties, surface chemical reactivity, biocompatibility, biodegradability, and lack of toxicity. Depending upon morphology, dimension and size, nanocellulose are grouped into 3 types; cellulose nanocrystals (CNC), cellulose nanofibrils (CNF), and bacterial cellulose (Garcia et al. 2016; Cho et al. 2019). CNC and CNF are produced from cellulosic biomass after pretreatment and purification whereas BC is produced mainly from bacteria (*Gluconacetobacter xylinus*) that can use a variety of carbon sources (Garcia et al. 2016). Nanocellulose has a wide range of application; it can be used as a capacitor in the energy storage device, for controlled drug delivery, repairing connective tissue and congenital heart defects, constructing contact lenses and protective barriers, thermal insulation and fire retardation, ultrafiltration, and packaging (Bacakova et al. 2019).

5.2.8. Dye dispersants

There are different dyes like direct dyes, reactive dyes, disperse dyes, sulfur dyes, basic dyes, acid dyes, and solvent dyes. All those dyes are difficult to disperse in water without dispersants (Yang et al. 2015). Dye dispersants have good thermal stability, are environment-friendly, can be renewed

and improve the dispersion performance. Therefore, dye dispersants are attracting great attentions. Dye dispersants are synthesized from lignosulfonate and alkali lignin which are byproducts produced from lignin industry (Qin et al. 2018). Sodium lignosulfonate, naphthalene sulfonate formaldehyde condensates, sulfonated alkali lignin, and hydroxypropyl sulfonates alkaline lignin are 4 types of lignin-based dye dispersants. Among these 4 lignin-based dye dispersants, hydroxypropyl sulfonated alkaline lignin is the best due to its high molecular weight (11.02 kDa), higher temperature stability, and high dye uptake (85.3 %). Sulfonated alkali lignin is used as dye dispersant has dark color and severe staining problem which is main obstacle for its application. This staining problem was reduced effectively by removing the chromophores and made applicable as dye dispersant (Qiu et al. 2018).

5.2.9. Bioplastic

Bioplastic can be produced by using lignin because lignin has glass transition temperatures, is renewable and has thermoplastic properties. Bioplastics are competent with petroleum-based plastics in both cost and performance and are sustainable, cost effective and biodegradable. However, lignin is rigid in nature, has relatively low molecular weight and high polydispersity that hinders the development of high-performance thermoplastic materials. Plasticizers or polymers having low glass transition temperatures can be added as addictive to improve the thermoplasticity of lignin i.e., flexibility, processability and durability (Wang et al. 2016).

6. Concluding remarks

In this study, the properties of LB and their components (cellulose, hemicelluloses, lignin, and pectin) have been discussed. In addition, more emphasis is given for the pectinolytic enzyme, and its application. Further, different valuable products such as biochemicals, single-cell protein, biochar, nanocellulose, bioactive compounds, dyes dispersants, bioplastics, and electricity

production have briefly been discussed and their importance have also been listed. In literature, it is known that lignocellulosic materials contain several high-value substances such as polysaccharides, minerals, and protein but when they are discarded in the environment may cause serious environmental problems and loss of value-added substances to the soil or landfill. Therefore, emphasis should be made to reuse these wastes as raw materials in the production of industrially relevant compounds like enzymes via fermentation, single-cell proteins, and many other products with appropriate technologies. For example, microbial enzymes can be produced by utilizing low-cost substrate like agro-wastes and can be used in decomposition of agro-waste in turn. The lignocellulolytic enzymes have humongous applications in different avenues, thus there is a need for a green and economical process for maximum enzymes production. However, the primary consideration is the stability of the enzyme in a wide range of industrial environmental conditions and to make the process cost-effective. The stability of enzymes over a wide range of temperature and pH is the most crucial factor that gives an additional advantage to a microbial strain. So, new microbes with high enzyme activity, stable over a wide range of temperature and pH for a more extended period, along with their cost-effective production, need to be emphasized. Immobilization and re-immobilization of enzymes onto low-cost material can have great potential for making the process more cost-effective; hence further research should be focused on this area as well. More powerful and versatile enzymes are needed to be developed through protein engineering and recombinant DNA technologies and discover the combined effects of different enzymes. New and exciting enzymes which drastically decrease the production cost for specific applications are beneficial in many areas, lower energy consumption, and enhance the quality of products. Besides, the technologies applied need to be environment-friendly for waste

management. More extensive research is required for the commercial production of various valuable products by a resourceful, assimilated, friendly, and low-cost biotransformation process.

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Chapter 3

Isolation, Identification, and Characterization of Pectinase Producing Bacteria from Soil

A part of this chapter (Chapter 3A) has been published in '**Fermentation**' (IF 5.123) and another part (Chapter 3B) is ready to submit in a journal.

3A Screening and Molecular Identification of Novel Pectinolytic Bacteria from Forest Soil

Abstract： Pectinases are a group of enzymes that have been used for different purposes such as plant fiber processing, pectic wastewater treatment, paper pulping, fruit juice extraction and clarification, etc. Due to the increasing demand for pectinase in numerous industries, it is essential to isolate the organisms producing a high pectinase with a wide range of stability factors like temperature and pH. In this study, 17 out of 29 bacteria (58.62%) from forest soil samples were pectinolytic. However, only 4 bacteria (S-5, S-10, S-14, and S-17) showing high pectin hydrolysis zones were processed for identification. The pectin hydrolysis zone ranges from 0.2 cm to 1.7 cm. These 4 bacteria were identified based on colony morphology, microscopic characterization, biochemical characteristics, and 16S rDNA sequencing. They were designated as *Streptomyces* sp. (S-5, S-14), *Cellulomonas* sp. (S-10), and *Bacillus* sp. (S-17). Interestingly, bacteria showed cellulase and xylanase activity in addition to pectinase. The quantitative assay for pectinase activity of those four isolates aided to prove that they were pectinase producers and can be potential candidates for industrial uses. The crude enzyme extracts of those bacteria were applicable in oil and juice extraction from sesame seeds and apples, respectively.

Keywords: Pectinase enzyme; soil; screening; identification; oil extraction; juice extraction

1. Introduction

Pectinases catalyze the degradation of pectic polysaccharides into simpler molecules like galacturonic acids (Pedrolli et al., 2009). Pectinases are found in bacteria, fungi, yeasts, plants, and insects (Frati et al., 2006). The biotechnological potentials of pectinase from microorganisms have become of great interest due to its broad substrate specificity and versatility (Pedrolli et al., 2009). Pectinases are widely used for industrial applications such as processing fruits and vegetables, production and clarification of juice, fermentation of tea and coffee. In addition,

pectinases are exploited for bleaching pulp and recycling wastepaper, animal feed, vegetable oil extraction, and pretreatment of wastewater produced from different fruit juice industries. Moreover, pectinases are applied for bioethanol production, liquefaction and saccharification of biomass, bio-scouring of cotton fiber, retting and degumming plant fiber, oil extraction, etc. (Kashyap et al., 2001; Garg et al., 2016; Kubra et al., 2017). A recent study showed that pectinase-treated fruit juice has inhibitory effects on colorectal cancer proliferation (Cho et al., 2019). Due to the humongous applications of pectinase in different avenues, the demand for pectinase enzymes is increasing continuously (Oumer, 2017). To mitigate the need for pectinase, it is necessary to isolate and characterize new pectinase-producing bacteria. Also, different microbes, including bacteria, fungi, yeast, insects, are habituated in soil, and it is the most readily available source for microbes (Aislabie and Deslippe, 2013). Thus, the present study focuses isolating and identifying the pectinase-producing bacteria from the forest soil, know their phylogenetic relationship with each other, and study their application in oil extraction from sesame seeds and juice extraction from apples.

2. Materials and methods

2.1. Collection of samples

The soil sample was collected during end of spring and beginning of summer from the nearby three forests of Thunder Bay, Ontario, Canada. The topsoil was dug by a sterile spatula, kept in a clean zip lock bag, and transferred to the laboratory as soon as possible.

2.2. Isolation of pure bacterial strains and preservation

Initially, 5 grams of soil sample was taken in a sterilized Erlenmeyer flask (250 mL), and 45 mL of 0.9% (w/v) autoclaved NaCl solution in distilled water was added. The homogenized samples were agitated for an hour at 120 rpm in a shaking incubator and then serially diluted until 10^{-4}

and 10^{-5}. For the isolation of a single pure bacterium, a sample of 0.1 mL aliquots from each dilution was spread onto sterilized and solidified nutrient agar (NA) plates with the help of sterile disposable spreaders. Then these plates were cultured at 35°C for 24- 48 hours. Different colonies from countable plates were selected and sub-cultured on NA plate by streak plate technique until a pure isolated colony was observed. Pure culture of microorganisms was stored at 4°C after streaking in NA slant for further studies.

2.3. Screening of isolates for the pectinase activity

The isolates were primarily screened for pectinase activity by point inoculation on 1% (w/v) pectin agar, i.e., pectinase screening agar medium (PSAM), pH 5.5 ± 0.5, and incubated at 35°C for 24 to 48 hours. After 48 hours of incubation, when colonies of 2-4 mm were observed, the plates were flooded with 50mM iodine potassium iodide solution. A transparent halo zone around the colonies indicated the isolates could produce pectinase (Takcı and Turkmen, 2016). Therefore, the halo around the colonies was noted, and further different tests were performed on those bacteria. Similarly, the screening for cellulase, xylanase, and amylase activity was performed by flooding congo red and iodine solution over the colonies isolated in the agar plate containing 1% (w/v) CMC, 1% (w/v) xylan, and 1% (w/v) starch respectively. The halo or clear zone is the indication of pectin degradation. Pectin degradation index (PDI)% is calculated as PDI%= (colony diameter + clear zone diameter)/ clear zone diameter (Haile and Kang, 2019). The composition of different media described in Supplementary materials.

2.4. Identification

After selecting bacteria with pectinolytic properties, they were streaked on NA plates and incubated at 35°C to get an isolated colony. From the pure individual growth, colony morphology, biochemical studies, and molecular analysis were performed.

2.4.1. Colony morphology and biochemical studies

Once the pure isolated colonies were observed in NA agar plates, size, shape, elevation, color, consistency, and transparency like characteristics of each colony were noted. From each different colony, the biochemical tests such as Gram's staining, catalase, oxidase, indole, methyl red (MR), Voges-Proskauer (VP), citrate utilization, DNase, urease, hemolysis, etc. were performed. The additional information of the tests mentioned in the Supplementary materials.

2.4.2. Genomic DNA extraction, 16S rDNA amplification, and extraction of DNA from gel

Pure isolated colonies were picked up from NA plate with a sterile toothpick and resuspended in 50 mL of Luria-Bertani (LB) broth and incubated at 35°C for 18-24 hours. From this suspension, the genomic DNA of selected isolates was extracted by the freeze-thawing cycles method as described by Chen et al. (Chen et al., 2020). The 16S rDNA gene of the isolates was amplified by Taq DNA polymerase with a universal eubacterial primer set, (Forward Primer) 27F-5′-AGAGTTTGATCCTGGCTCAG-3′ and (Reverse Primer) 1492R-5′-GGTTACCTTGTTACGACTT-3′.

The amplification system contained 2 × *Taq* PCR Master Mix of 10 µL (10 x Taq DNA polymerase buffer, 10 mM dNTPs, 25 mM of $MgCl_2$, 1 U of *Taq* DNA polymerase), 1 µL of 10 µM forward and reverse primers respectively, 1 µL of the genomic DNA template, and 7 µL of distilled water making a total volume of 20 µL. The PCR reaction conditions used were; denaturation at 94°C for 5 min and the cycle starting at 94°C for 30 sec, followed by annealing at 55°C for 30 sec, extending at 72°C for 1.5 min for 33 cycles, and finally extending at 72°C for 10 min. The PCR products were determined by 1% (w/v) agarose gel electrophoresis. The target

fragments from the gel were cut and DNA was extracted from gel by using gel extraction minipreps kit (Biobasic) and then sent for DNA sequencing.

2.4.3. Gene sequencing and phylogenetic analysis

The 16S rDNA sequences of those isolates were compared with the known sequences found in the GenBank operating the basic local alignment search tool (BLASTn) of the National Center for Biotechnology Information (NCBI). Isolates were identified based on the percentage similarity with the known species sequences in the database. New sequences of those isolates were deposited in GenBank (accession numbers MW547427- MW547430). For molecular analyses, the accessible sequence data for all related species of *Cellulomonas* sp., *Streptomyces* sp., and *Bacillus* sp. were downloaded from the NCBI database. All the sequences were congregated and parallelized using the Clustalw module in BioEdit v. 7.0.9.0 (Hall, 1999) with default settings. Phylogenetic analysis was constructed using Neighbor-Joining (NJ) tree with 1000 bootstrap using MEGA 7 (Kumar et al., 2016).

2.5. Growth at different temperature and pH

Those selected isolates were cultured in NA and incubated at different temperatures ranging from 25℃ to 50℃. Also, they were cultivated in NA with different pH (5 to 9) and at 35℃. The colonies of the isolates were observed and noted after 24 hours of incubation.

2.6. Quantitative determination of pectinase enzyme activity

The concentration of reducing sugar was estimated by the dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction products after the degradation of pectin by a pectinase were reducing sugars. The quantity of reducing sugars obtained by the sample was calculated with the standard graph curve obtained from different galacturonic acid concentrations versus absorbance

following the same procedure (Miller, 1959). The enzyme activity (U/mL) was calculated according to the following equation (Kavuthodi et al., 2015):

$$\text{Enzyme activity (U/ml)} = \frac{\mu\text{g galacturonic acid released x V}}{\text{v} \times 194.1 \times \text{t}}$$

Where 'V' is the total volume of solution, 'v' is the volume of the crude enzyme used in the assay, '194.1′ is the molecular weight of galacturonic acid, and 't' is the reaction time in min. One unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1µ mol of reducing sugars per minute (Miller, 1959).

For enzyme activity assay, 1 mL of freshly grown culture was taken and centrifuged at 10,000 rpm for 5 mins. The supernatant obtained represented as the crude enzyme extract, and 1% (w/v) pectin in phosphate buffer (0.1M, pH 7) served as the enzyme assay substrate. From the prepared substrate, 20µl was taken into a well, and 10 µl of enzyme extract was added, then it was kept in a water bath (50°C) for 10 mins. The mixture temperature was then brought down to room temperature, 60µl of DNS reagent was added to the mixture and the solutions were kept in a boiling water bath for 5 mins. Finally, the absorbance of the solutions was measured at 540 nm using a spectrophotometer. Enzyme blank and reagent blank were also measured for quantitative analysis of pectinase.

2.7. Application in oil and juice extraction

Oil extraction was performed as described by Demir et al. with slight modification (Demir et al., 2014). Sesame seeds were dried, cleaned, and stored at room temperature. Two grams of sesame seeds were weighed and ground with mortar and pestle. When the seeds were ground, 2 mL of the crude enzyme was added, whereas, for control, distilled water was added instead of enzyme extract. The paste formed was transferred into 50 mL centrifuge tube by adding 10 mL of water.

The mixture was incubated at 50℃ for 1 hour and centrifuged at 4000 rpm for 20 mins. The floating layer of emulsified oil was collected in another test tube, and the volume was measured. For juice extraction, an apple was washed, cut into small pieces, and weighed (25 grams). Thereafter, apple pieces were ground with mortar and pestle by adding 4 mL of crude enzyme. The apple paste was transferred into a 50 mL centrifuge tube by rinsing the mortar and pestle with 10 mL of distilled water. The mixture was incubated at 50℃ for 1 hour and centrifuged at 5000 rpm for 20 mins. The amount of clear supernatant was measured and collected in another tube. The percentage juice recovery, clarity, and relative viscosity of the recovered apple juices were analyzed. The clarity of apple juice was analyzed by taking absorbance at 660 nm using a UV visible spectrophotometer. The relative viscosity of the juice was measured at room temperature using an Ostwald viscometer. The percentage of juice recovery was calculated as '(apple weight – total solid waste) X 100/ apple weight'.

2.8. Statistical analysis

The tests in this study were performed in triplicates, and the values were expressed as mean with standard deviation. The statistical significances were assessed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer comparison. The P-value of the experimental data less than 0.05 is regarded as statistically significant.

3. Results

3.1. Isolation of pure bacterial strain and preservation

The forest soil was serially diluted, spread on NA plates, and incubated at 35℃ for 24 to 48 hours. After incubation, we observed different isolates with different colony morphologies. From various colonies, 29 different isolates were selected based on their color, elevation, consistency, transparency, edges, etc. They were subcultured on the NA plate till the pure colonies were

isolated. The screening of pectin hydrolyzing activity was detected based on the halo zones around the microbial colonies. Among 29 isolates, only 17 (58.62%) isolates gave the halo zone around the colonies showing the pectin hydrolyzing properties. Those bacterial strains were preserved by streaking on the nutrient slant and storing them at 4°C for further study.

3.2. Screening and identification of pectinolytic isolates

Of 17 pectin hydrolyzing isolates, four isolates (S-5, S-10, S-14, and S-17) showing the high hydrolysis zone were selected for the identification (in supplementary materials; Figure S 1). The isolates were identified based on the colony morphologies, biochemical tests, and 16S rDNA sequencing. After reading the colony characteristics, they were subjected to different tests, and the results, along with the colony characteristics and their PDI% were observed as shown in Table 1.

Table 1 Different tests result of the selected 4 isolates having pectinolytic properties

Different tests	Isolate S-5	Isolate S-10	Isolate S-14	Isolate S-17
Colony characteristics	Small circular rough (grey powdery) colony having raised convex elevation with hard consistency	Small yellowish glistering smooth, raised colony with soft consistency	Small circular rough (white powdery) colony having raised convex elevation with hard consistency	Small whitish noncircular, flat with not smooth edge (jagged edge)
Pectin hydrolyzing zone (D-d) [PDI%]	22-5=17 [129.41]	15-3=12 [125.0]	16-2=14 [114.28]	12-2=10 [120.0]
Gram staining	+	+	+	+
Cell morphology	rods	rods	filamentous	rods
Spores	+	-	+	+
Capsule	-	-	-	-
Biofilm	-	-	-	-

Catalase		+	+	-	+
Oxidase		-	-	-	-
Indole		-	-	-	-
MR		+	+	-	-
VP		-	+	-	-
Citrate		-	-	-	-
H_2S		-	-	-	-
TSI		alkaline/alkaline	acid/alkaline	alkaline/alkaline	acid/acid
DNase		+	+	+	+
Urease		-	-	-	-
Hemolysis		-	-	-	-
Starch hydrolysis		+	-	-	++
Gelatin hydrolysis		-	-	-	-
Lactose		-	-	-	-
VRB		-	-	-	-
Cellulase (D-d)		+ (15)	+ (3)	+ (9)	-
Xylanase (D-d)		+ (8.5)	-	+ (7)	+ (4)
Amylase (D-d)		+ (2)	-	-	+ (5)
Growth in different temperatures	25°C	+	-	+	+
	30°C	+*	+	+*	+
	35°C	+*	+*	+*	+*
	40°C	+*	+*	+*	+
	50°C	-	-	-	+
Growth in different pH	pH 5	-	-	-	-
	pH 6	+	-	+	-
	pH 7	+*	+	+*	+
	pH 8	+*	+*	+*	+*
	pH 9	+	+*	+	+*
Antibiotic susceptibility test		Zone of inhibition (mm)			

Antibiotics used	Ampicillin	0 (R)	18.3±1.5 (S)	0 (R)	>14 (S)
	Bacitracin	18.3±0.6 (S)	49.3±2.1 (S)	18.3±2.1 (S)	>11 (S)
	Penicillin	0 (R)	27.7±0.6 (R)	0 (R)	>29 (S)
	Novobiocin	27.7±0.6 (S)	44.3±1.2 (S)	24.7±0.6 (S)	>16 (S)
	Chloramphenicol	23.7±0.6 (S)	41.7±1.2 (S)	25.3±1.5 (S)	>18 (S)
	Erythromycin	18.3±0.6 (I)	37.0±1.7 (S)	16.3±1.5 (I)	>23 (S)
	Tetracycline	17.7±1.5 (I)	29.7±2.5 (S)	15.3±0.6 (I)	>19 (S)

MR: methyl red, VP: Voges-Proskauer, H_2S: hydrogen sulfide, TSI: triple sugar iron, VRB: violet red bile, -: Negative/absent/no growth, +: Positive/present/growth, +*: Maximum growth, D: total diameter (mm) of hydrolysis area with colony, d: diameter (mm) of colony, R: resistant, S: susceptible, I: intermediate, data are presented as mean ± standard deviation

The screening test for cellulase and xylanase of those four isolates was performed by flooding congo red over the colonies. Similarly, the screening test for amylase was done by flooding with iodine solution. The results of the screening tests were revealed in Table 1.

The sequence similarity of isolates was analyzed, blasted, and compared with the known probable sequences in the NCBI database. The phylogenetic tree of 16s rDNA sequences was constructed using the Neighbor-joining algorithm, as shown in Fig. 1. The phylogenetic results showed the isolates S-5, S-10, S-14, and S-17 were identified as *Streptomyces* sp., *Cellulomonas* sp., *Streptomyces* sp., and *Bacillus* sp. respectively.

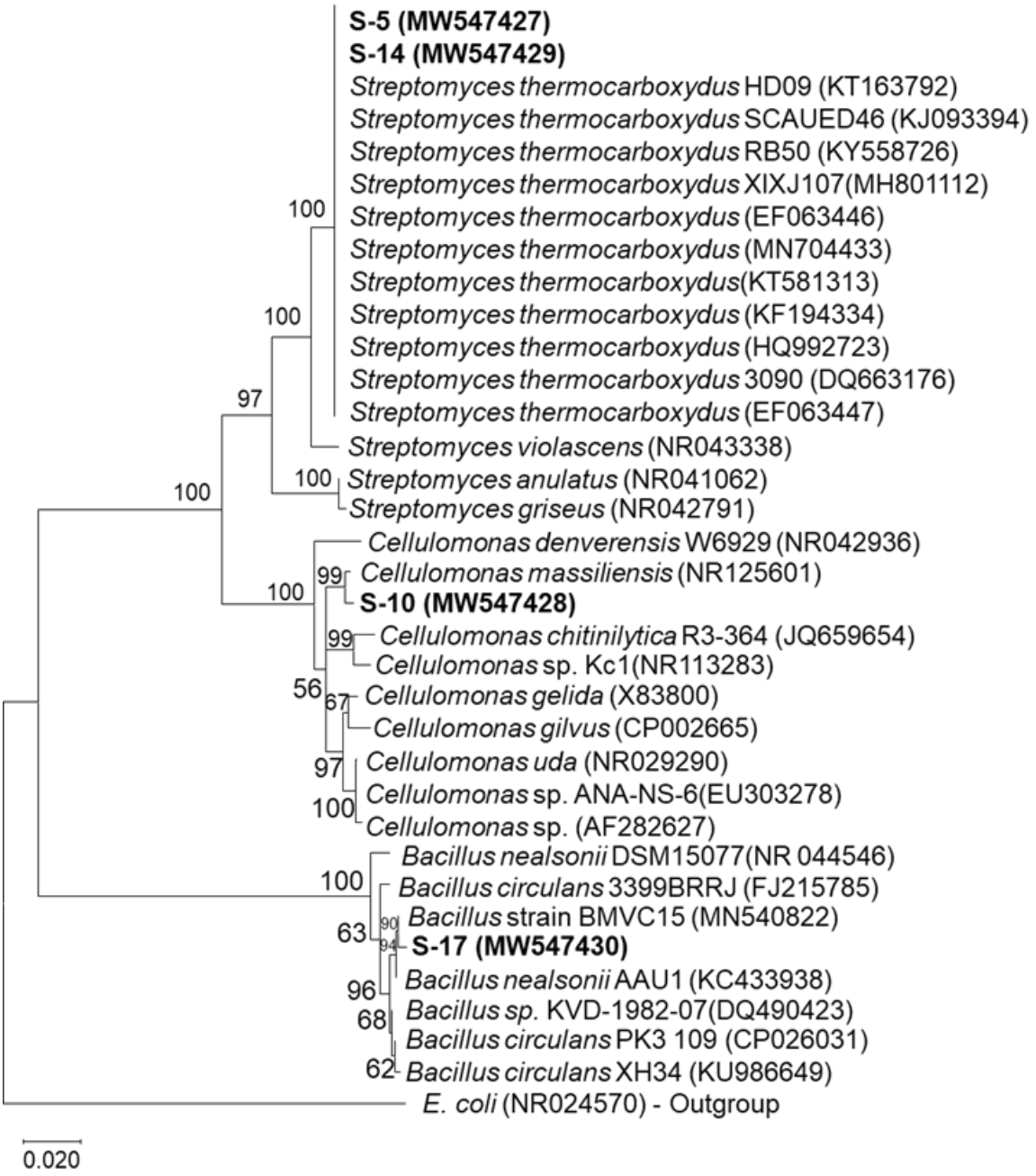

Fig. 1 Neighbor-Joining (NJ) tree based on DNA sequences of the 16S rRNA gene showing the phylogenetic relationships. Numbers present on branches of the NJ tree are bootstrap support values.

3.3. Isolation of isolates at different temperature and pH

When those isolates were cultured at different temperatures (25 to 50℃) and pH (5 to 9), all the bacteria were able to grow at 35℃ and pH 7, as depicted in Table 1.

3.4. Quantitative analysis of pectinase enzyme activity of different isolates

For quantitative analysis of pectinase activity, all isolates were grown at 35℃, pH 7, and for 120 hours in pectinase production media. Every 24 hours, 1 mL of the cultured broth was taken out aseptically and centrifuged to get cell-free crude enzyme extract for quantitative analysis of enzyme activity. The bacterial growth (OD_{600}) and enzyme activity of the isolates were studied, as shown in Fig. 2.

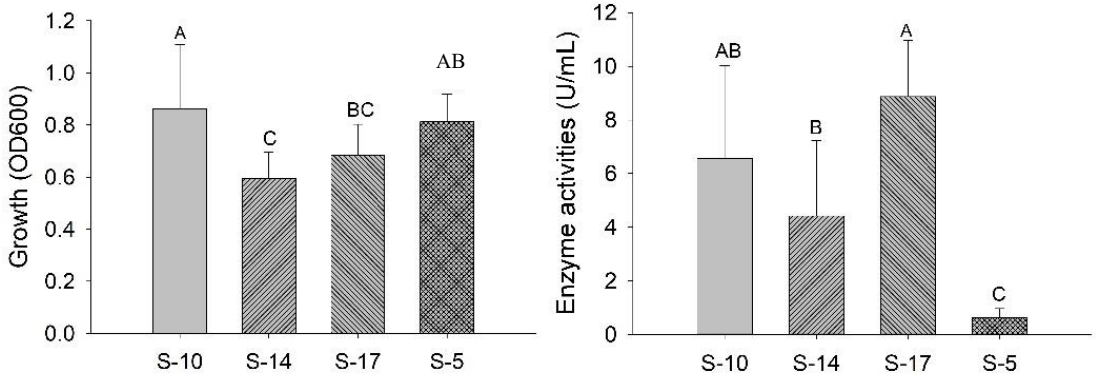

Fig. 2 Bacterial growth and enzyme activity produced by isolates. The bar represents the mean of bacterial growth and enzyme activity. The statistic was derived from one-way ANOVA followed by Posthoc Tukey comparison test. The different letter above the bar represents they are statistically significant at $P<0.05$

3.5. Application of pectinase in oil extraction and juice extraction

The crude enzyme extract of 2 mL was added after the sesame seeds were ground and transferred into the 50 mL centrifuge tube, adding 10 mL of water. After incubation at 50℃ for 1 hour and centrifugation, the emulsified oil appeared as the floating layer at the top in the tube was

collected and measured. The emulsified oil extracted from enzyme extract of isolate S-10 was significantly higher than others (Table 2).

Similarly, the crude enzyme extract was added for juice extraction, incubated, and centrifuged. The amount of juice extracted was measured and compared with control (with no enzyme extract), as shown in Table 2. The highest percentage juice recovery was achieved from isolate S-10. Moreover, apple juice extracted by treating with the crude enzyme extract from isolate S-10 had a much lower relative viscosity compared to the control. The clarity of apple juice was affected by the enzymatic treatment, which was determined in terms of absorbance and transmittance at 660 nm.

Table 2 Application of pectinase produced from the isolates in oil and juice extraction

Sample	Oil extraction (mL)	Juice extraction (mL)	% Juice recovery	Clarity (%transmittance)	Relative viscosity
Control	1.11 ± 0.2^{b}	0.33 ± 0.3^{c}	41.00 ± 0.2^{e}	30.22 ± 1.0^{d}	2.99 ± 0.1^{a}
S-5	1.23 ± 0.0^{ab}	2.00 ± 1.0^{bc}	44.22 ± 0.1^{c}	33.12 ± 1.89^{cd}	2.44 ± 0.0^{b}
S-10	1.66 ± 0.1^{a}	4.99 ± 0.9^{a}	51.77 ± 0.1^{a}	48.88 ± 1.0^{a}	1.99 ± 0.0^{d}
S-14	1.11 ± 0.1^{b}	1.66 ± 0.9^{bc}	43.55 ± 0.1^{d}	35.77 ± 1.8^{bc}	1.77 ± 0.0^{e}
S-17	1.11 ± 0.1^{b}	2.66 ± 0.9^{b}	46.22 ± 0.1^{b}	38.88 ± 1.6^{b}	2.33 ± 0.0^{c}

^{a,b,c,d,e} the different superscripted letters in the same column denoted the data were significantly different ($P\leq0.05$), and responses represented as mean±standard deviation

4. Discussions

Pectinase has been exploited in fruit and juice industries since many years ago, but in the present biotechnological era, the application of pectinase and market demand are in increasing order.

Thus, it is necessary to isolate novel microorganisms having higher pectinolytic properties with stability to a wide range of temperature and pH. In our study, 58.6% of isolates having pectin hydrolyzing properties were isolated from forest soil samples, and most of them were gram-positive cocci. Aislabie and Deslippe (Aislabie and Deslippe, 2013) mentioned that soil contains various microorganisms that contribute to different soil services such as recycling wastes, nitrogen cycle, detoxification of pollutants, etc. The types and mass of the microorganisms in the soil depend upon soil properties and the carbon source(s) available for energy and cell synthesis. Aaisha and Bharate (Aaisha and Barate, 2016) isolated 51.4% of pectinolytic bacteria from different soil samples and observed *Bacillus sp.* as the prominent pectinase-producing isolates. In another study, ten bacteria were isolated from agricultural waste dump soil, and 3 out of 10 (33.3%) were pectinolytic bacteria (Karthik et al., 2011). Similarly, the study performed by Oumer and Abate (Oumer and Abate, 2018b) isolated 31.6% of isolates with pectinase activity from the coffee pulp, and Ajobiewe et al. (Ajobiewe et al., 2019) isolated 5 pectinolytic bacteria from soil containing decaying fruits and vegetables.

The pure culture of isolates was obtained only after several subculturing on NA. The pure culture of isolates was considered if there is no contamination, and the colonies were all similar concerning color, size, shape, elevation, consistency, etc. After getting the pure culture of isolates, further studies were possible such as screening tests, identification of isolates, DNA extraction, and more.

The screening test for pectin hydrolysis was performed within the screening agar plate containing pectin, and the hydrolysis zone was observed after the addition of potassium iodide solution (Supplementary Figure 1). The hydrolysis area looked clear because the pectinase produced by bacteria hydrolyzed the pectin and made the pectin incapable of binding with iodine

that formed a complex. The highest hydrolysis area was indicated to have the most pectinase activity (Takcı and Turkmen, 2016). In this study, the hydrolysis zone ranged from 2 mm to 17 mm. However, the four isolates having a hydrolysis zone of over 10 mm were chosen for further studies, including identification.

The xylanase and cellulase enzymes were present in addition to pectinase in isolates S-5 and S-14. The isolate S-10 had pectinase and cellulase, while isolate S-17 had pectinase, and amylase. Beg et al. (Beg et al., 2000a) isolated *Streptomyces* sp. having thermostable pectinase and xylanase. Similarly, Kaur et al. (Kaur et al., 2017) isolated *Bacillus pumilus* that produced xylanase and pectinase from soil contaminated with paper and pulp industry effluents. Production of more than one commercial enzyme from a single microorganism is scarce but is economical and feasible for industrial applications. The combination of xylanase and pectinase effectively removes bark from wood, processing plant fibers (Beg et al., 2000a), and bio-scouring fibers in textile industries (Singh et al., 2020), etc. Thus, detailed studies need to undergo for the utmost production of various industrially essential enzymes from a single microbe for commercial benefit and their industrial applications.

The detailed morphological characteristics and different biochemical tests result of those isolates tentatively helped to identify them based on Bergy's Manual of Systemic Bacteriology. Additionally, 16S rDNA sequencing was performed to determine the isolates. The genomic DNA of 4 isolates gave one distinct band on an agarose gel. The 16S rDNA amplification of those genomic DNA was subjected to PCR by using universal primers. The amplified 16S rDNA was extracted from the gel by Gel extraction minipreps kit (Bio Basic) and sent for sequencing. The sequence results were analyzed and blasted with the probable sequences in NCBI.

The phylogenetic tree was constructed with the closest sequences found within the NCBI GenBank and *E. coli* as an out-group. The phylogenetic analysis indicated that the *Cellulomonas* strain (isolate S-10) and *Bacillus* sp. (isolate S-17) were clustered with other strains of *Cellulomonas massiliensis* and *Bacillus* strain, respectively, with high bootstrap support value. Similarly, *Streptomyces* strains (isolates S-5 and S-14) were grouped with *Streptomyces thermocarboxydus* (Fig. 1).

In the study conducted by Bharadwaj and Udupa (Bharadwaj and Udupa, 2019), 50℃ and pH 4 was the optimal growth temperature and pH for *Streptomyces thermocarboxydus* isolated from soil. However, the maximum enzyme activity of partially purified pectinase was found to be maximum at 60℃ and neutral to alkaline pH (Bharadwaj and Udupa, 2019). In another study, *Streptomyces fumigatiscleroticus* VIT-SP4 showed the optimum values of the incubation period-48 hours, pH 6, and temperature 35℃ (Govindaraji and Vuppu, 2020). Thus, in our study, the four isolates were cultured in broth media containing 1% (w/v) pectin at 35℃ and pH 7 for the quantitative analysis of pectinase activity. Also, the temperature and pH were selected after observing the results of the isolates cultured in different temperatures and pH (Table 1). Our study showed the isolate S-17 produced higher pectinase activity followed by S-10 within the mentioned conditions as in Fig. 2. However, the enzyme activity of S-10 and S-17 was not statistically different but was statistically different from other isolates ($P<0.05$). Similarly, the bacterial growth was higher for S-10, and their growth was statistically significant ($P<0.05$). The result revealed both enzyme activity and bacterial growth were higher in isolate S-10. The enzyme activity and growth depend on various factors, including microorganisms, carbon and nitrogen sources, incubation temperature, pH, hours, fermentation process, etc. Thus, further

detailed study for optimization of the cultural conditions of the isolates must be explored to maximize pectinase production.

The antibiotic susceptibility test was conducted against those isolates and observed that most of them are susceptible to the seven commonly used antibiotics (supplementary materials: Table 1). Furthermore, those isolates were tested for hemolysis, capsule, and biofilm production (Supplementary materials) and found they were non-capsular, non-hemolysis, and biofilm non-producers (Table 1).

Cell-free supernatants (crude enzyme extracts) were used to study their application in oil and apple juice extraction. The amount of emulsified oil obtained from aqueous extraction of the crude enzyme-treated sesame seeds and apple juice extracted was measured and considered to know the enzyme's ability to extract oil and juice, respectively. Table 2 demonstrated that enzymes stimulated oil extraction from sesame seeds and accelerated the juice extraction from apples. The oil and juice were extracted in higher volume compared to control without enzyme extract. Of the four bacteria, the emulsified oil volume and juice amount were higher from isolate S-10 followed by S-17, and the extraction was significant statistically ($P<0.05$). The pectinase facilitates oil extraction from sesame seeds and juice extraction from apples by breaking pectin present in seeds and apples (Demir et al., 2014). The synergistic action of different hydrolytic enzymes such as pectinase, cellulase, xylanase, and amylase plays an important role in extracting and clarifying juice (Sharma et al., 2016). Enzyme treatment degraded pectin present in fruit, leading to a decrease in the water holding capacity of pectin, and water was released to the system, increasing juice yield (Kashyap et al., 2001). Moreover, a combination of enzymes increases clarity, juice recovery and decreases viscosity and turbidity of juice and oil. The enzymatic extraction of fruit and oil is a novel, green and beneficial

technology (Sharma et al., 2016; Mwaurah et al., 2020). The study infers the isolates isolated from forest soil can extract oil from sesame seeds and juice from apples. The pectinase produced by those isolates may have potential in various industries besides fruit juice and oil extraction.

5. Conclusions

The novel pectinase-producing bacteria were isolated from the forest soil of Thunder Bay, Ontario. Four bacteria showing high pectinolytic activity on the screening test were identified as *Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Streptomyces* sp. (S-14), and *Bacillus* sp. (S-17). The identification was based on the morphology of the bacteria, various biochemical tests, and molecular analyses. The bacteria gave gram-positive reaction, rod-shape, and spore former except S-10. The enzymes produced from those isolates have the potential for oil and juice extraction, and those isolates could be alternatives for commercial pectinase production. However, further studies such as optimization of cultural conditions for enzyme production, optimization of oil and juice extraction conditions to maximize oil production and juice yield need to be studied. The characterization of enzymes, oil, and juice and their physicochemical properties are essential to study for economic advancement. The isolates having multi-enzyme activities indicated that these isolates are potential candidates for various industrial applications and need to be explored.

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3B Characteristics of Pectinase-producing Soil Bacteria and Strain Improvement by UV Radiation and Ethidium bromide

Abstract: The pectinase-producing soil bacteria studied in this study were Gram-positive, non-hemolytic, not proteolytic, non-capsulated, and lipase producers. The auto-aggregation capacity of the isolates increased with time and exhibited the maximum auto-aggregation capacity in 24 hours in an order S-5 (66.67%) > S-14 (56.86%) > S-17 (49.77%) > S-10 (27.06%). Further, the bacteria were found to be weak biofilm producers and non-hydrophobic. *Streptomyces* sp. (S-5) illustrated the highest macerating capacity for both potato and cabbage (18.49 and 42.59%, respectively) among the isolates, and increased pectinase activity when exposed to UV radiation. Therefore, the findings of this study can conclude that soil bacteria are not pathogenic, have the potential to be used as probiotic, and UV radiation mutagenesis can increase pectinase activity.

Keywords: soil bacteria, auto-aggregation, maceration, pectinase, strain improvement

1. Introduction

Soil has enormous bacteria with diversity, and soil bacteria can self-immobilize via auto-aggregation and biofilm formation (Ning et al., 2021b). The exopolysaccharides and proteins produced by the bacteria help in the auto-aggregation of bacteria, forming multicellular clumps. This mechanism protects bacteria against environmental stresses such as oxygen availability, and temperature change, or host responses (Trunk et al., 2018). Further, a polymeric matrix including proteins, polysaccharides, extracellular DNA, and lipids form biofilms that act as a shield for bacterial cells embedded and protects the bacteria (Trunk et al., 2018). Soil bacteria can become harmful or pathogenic to humans, animals, and plants (Mogrovejo et al., 2020). Bacteria can produce various virulence factors, mainly depending on environmental conditions, by exhibiting several mechanisms: (1) adherence, auto-aggregation, and colonization (2) biofilm formation (3)

secretion of extracellular enzymes such as protease, lipase, hemolysin (Trunk et al., 2018; Mogrovejo et al., 2020; Ning et al., 2021b). Auto-aggregation is the first step in biofilm formation (Trunk et al., 2018). The non-hemolytic bacteria having strong auto-aggregation and hydrophobicity have the potential to be applied as probiotics (Panda et al., 2017). In addition, pectinase-producing bacteria are industrially important, mainly in phytochemical extraction and fruit juice and wine industries, as they can macerate the plant tissues, rupture the cell wall, and extract fluid from the cell (Tanabe and Kobayashi, 1987; Martos et al., 2013b; Sharma et al., 2018).

Besides, microorganisms are considered a good source of industrial enzymes due to their fast growth and ease of culture. They can be easily exploited for genetic manipulations for maximum enzyme production (Singh et al., 2016). The applications of microbial enzymes are widespread in various avenues and are continuously in high demand (Singh et al., 2016). Therefore, microorganisms are being utilized for industrially essential enzymes such as pectinase, cellulase, xylanase and protease productions. The improvement of bacterial strains is necessary for increasing enzyme and other varieties of product production (Parekh et al., 2000; Heerd et al., 2014). There are various strategies for improving enzyme production. The classical physical strain development technique is simple. Exposing the organism to ultraviolet radiation and ethidium bromide can enhance enzyme production (Parekh et al., 2000; Shrestha et al., 2021c). Therefore, this study investigated bacterial virulence factors, the ability to produce some enzymes with maceration activity and strain improvement by exposing bacteria to UV radiation and ethidium bromide to increase pectinase activity.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The pectinase-producing bacteria used in this study were isolated from forest soil near Lakehead University, Thunder Bay, Ontario, Canada and stored at -80°C. The bacteria exploited in this study were *Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), Streptomyces sp. (S-14), and *Bacillus* sp. (S-17), and their identification was based on 16S rDNA sequence analysis (Shrestha et al., 2021b). The bacteria were revived by inoculating bacteria in LB broth and incubated overnight at 35°C. Then, 1% v/v of overnight culture was inoculated onto pectin containing media and incubated at 35°C and 180 rpm.

2.2. Screening for hemolytic, proteolytic, lipase activities, and capsule production

Hemolytic, proteolytic, and lipase activities were screened using blood agar, gelatin agar, and Tween-80 agar, respectively. Overnight grown bacterial cultures were point inoculated in agar plates and incubated at 35ºC for 48 hours. After incubation, the hydrolyzed clear zone around the bacterial colony indicated the presence of proteolytic and lipase activities. A greenish-grey or brownish discoloration, the clear zone, and the absence of coloration zone around the colony revealed α-hemolysis, β-hemolysis, and γ-hemolysis (α- hemolysin production, β-hemolysin production, and no blood cell lysis), respectively (Mogrovejo et al., 2020).

Screening for capsule production was performed using Congo red agar as described in Lamari et al. (2018) (Lamari et al., 2018). The Congo red agar is tryptic soya agar supplemented with 0.8 g/L Congo red, 36 g/L sucrose, and 1% NaCl. Overnight grown bacterial culture was inoculated on Congo red agar plate and incubated at 35ºC for 24 hours. After incubation, the color of the colony was observed; the black colony indicated capsule producers, while the red colony was the non-capsule producer.

2.3. Auto-aggregation capacity

The auto-aggregation capacity of bacteria was performed as mentioned in Escamilla-Montes et al. (2015) (Escamilla-Montes et al., 2015). This settling or sedimentation method is the simplest quantitative method for auto-aggregation assay (Trunk et al., 2018). In short, the overnight grown bacterial culture was collected, centrifuged at 5000×g for 15 min, washed twice, and suspended in phosphate-buffered saline (PBS, pH 7.4). Then 4 mL of bacterial cell suspensions were vigorously mixed by vortexing for 10 secs and incubated at room temperature for 24 hours. After 1, 2, 4, 18 and 24 hours, the absorbance of the upper suspension at 600 nm (OD_{600}) was measured, keeping PBS as a blank.

Auto-aggregation (%) = $1 - (A_t/A_0) \times 100$, where A_t represented the absorbance at time t and A_0 the initial (0 min) absorbance.

2.4. Biofilm-forming capacity and hydrophobicity

The biofilm-forming capacity of bacterial strains was carried out by adhesion to polystyrene plate as mentioned in Sandasi et al. (2010) and Chaieb et al. (2011) (Sandasi et al., 2010; Chaieb et al., 2011). Isolates were grown in tryptic soy broth (TSB) at 30ºC and then diluted to 1:100 w/v (in TSB with 2% glucose). Aliquots of cell suspensions (200 µL) were transferred to 96-well microtiter plates and incubated at 35ºC for 24 hours. Plates were washed twice with PBS and dried. The well with sterile TSB alone was used as a control. Adherent strains were fixed with 95% ethanol and stained with 100 µL of 1% w/v crystal violet solution for 15 min. Microplates were washed with distilled water, air-dried, and biofilm-forming capacity was measured at 570 nm. Biofilm formation was interpreted as; highly positive ($OD_{570} \geq 1$), moderately to weakly positive ($0.1 \leq OD_{570} > 1$), or negative ($OD_{570} \leq 0.1$).

The hydrophobicity of bacteria is the bacterial adherence ability and can be determined by bacterial adherence to the hydrocarbon (BATH) test (Latha et al., 2016). Four mL of the bacterial

suspension having OD_{600} of 0.55-0.60 was taken in a clean and sterile test tube. Then, 1 mL of n-octane (non-polar solvent) was added to the test tube, vortexed vigorously for 1 min and allowed to stand for 60 mins. The aqueous upper layer was separated gently, and optical density at 600 nm was measured. The hydrophobicity (%) is calculated according to the formula, Hydrophobicity (%) = $1 - (OD_b/OD_a) \times 100$, where OD_a represents the initial absorbance before mixing and OD_b absorbance of the suspension after mixing. The isolates are classified into three categories: not hydrophobic (< 20%), moderate (20-50%), and strong (> 50%).

2.5. Culture preparation and exopolysaccharides (EPS) production

The bacteria were cultured in nutrient broth at 30℃, 180 rpm for 48 hours, followed by centrifugation at 5000 rpm for 30 mins. The EPS quantification was done following the procedure described in other studies ((Torino et al., 2001; Mendi and Aslım, 2014). Then the supernatant was collected, mixed with 95% ethanol (1:3) and incubated overnight at 4℃. The next day, the pellet formed after centrifugation was washed twice with distilled water and placed at -80℃ for 24 hours. The obtained pellet was dried and weighted, which is the crude EPS. Carbohydrate content in EPS was determined by the phenol-sulphuric acid method. In brief, 1 mg of EPS was suspended in 30 µl of distilled water, and 150 µl of concentrated sulphuric acid was added. The mixture was incubated at 90℃ for 15 mins, followed by adding 30 µl of phenol and incubated at 25℃, at 120 rpm for 5 mins. The absorbance was then measured at 595 nm. All the measurements were obtained in triplicates.

2.6. Maceration test

This test was performed using potato and cabbage following the method described in Bhat (2012) and Golanowska et al. (2017) (Bhat et al., 2012; Golanowska et al., 2017). Potatoes were washed with water, sterilized with 10% bleach, and again washed with distilled water. Then, potato peel

was removed, cut into pieces, and 0.6 mm diameter holes were made with a sterile borer in each piece. Similarly, cabbage was also cleaned and cut into pieces, almost making square in shape and fitting them in Petri plates. The weight of potatoes and cabbage pieces were taken and kept on the Petri plates containing the filter paper. An equal volume of distilled water was added to the filter paper on all plates to maintain moisture. Then 50 µl of cultured broth was inoculated in the holes made in potatoes, whereas the sterile pin was dipped into the broth and stabbed 8-10 times in the cabbage piece. Then they were incubated at 30℃ for 48 hours, and their weight was again taken. The percentage of tissue macerated was calculated by using the change in weight divided by the original weight and multiplied by 100. The weight of the un-inoculated control was deducted as the loss in weight due to drying.

2.7. Strain improvement by UV radiation and ethidium bromide exposure

The overnight cultured bacterial suspensions having an OD_{600} value of approximately 0.6 were exposed to UV rays and ethidium bromide (Et-Br) for a few hours. At every hour of exposure, they were streaked on an agar plate and incubated at 35℃ for 18-24 hours. Once they were grown on the nutrient agar plates, they were cultured in pectinase-producing liquid media and enzyme activity was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959) as described in a previous study (Shrestha et al., 2021b).

3. Results and discussion

3.1. Screening for hemolytic, proteolytic, lipase activities, and capsule production

The different properties of the isolated bacteria were screened for characterizing them, and the results are shown in Table 1. All the bacteria studied were Gram-positive, non-hemolytic, not proteolytic (based on gelatin hydrolysis), non-capsulated, and lipase producers.

Table 1 Characteristics illustrated by isolated bacteria [*Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Streptomyces* sp. (S-14), and *Bacillus* sp. (S-17)]

Isolates	Gram staining	Hemolysis test	Proteolytic test	Lipase test	Capsule
S-5	+	-	-	+	-
S-10	+	-	-	+	-
S-14	+	-	-	+	-
S-17	+	-	-	+	-

* -: negative, +: positive

The Gram-positive bacteria are more resistant to drying; this may be the reason for observing Gram-positive isolates from the soil bacteria in our study. In addition, our findings are supported by other studies, and the bacteria are found to be nonpathogenic since they are non-hemolytic, non-proteolytic and non-capsulated (Lee and Liu, 2000; Culp and Wright, 2017). These environmental bacteria can be pathogenic under stressful environments or if they acquire virulence genes from other microorganisms (Marshall et al., 2009). However, those Gram-positive environmental bacteria are more susceptible to antibiotics because of their cell wall structure. Also, Gram-positive bacteria lack an outer lipopolysaccharide layer and thick peptidoglycan layer (Alhumaid et al., 2021). Besides, metabolites produced by *Streptomyces* sp. can inhibit biofilm formation by other bacteria and be used as an alternative trick against bacterial resistance (Amorim et al., 2020).

3.2. Auto-aggregation

The auto-aggregation capacity of the isolates increased with time and exhibited the maximum auto-aggregation capacity in 24 hours. The highest auto-aggregation was shown by S-5 (66.67%), followed by S-14 (56.86%), S-17 (49.77%), and S-10 (27.06%).

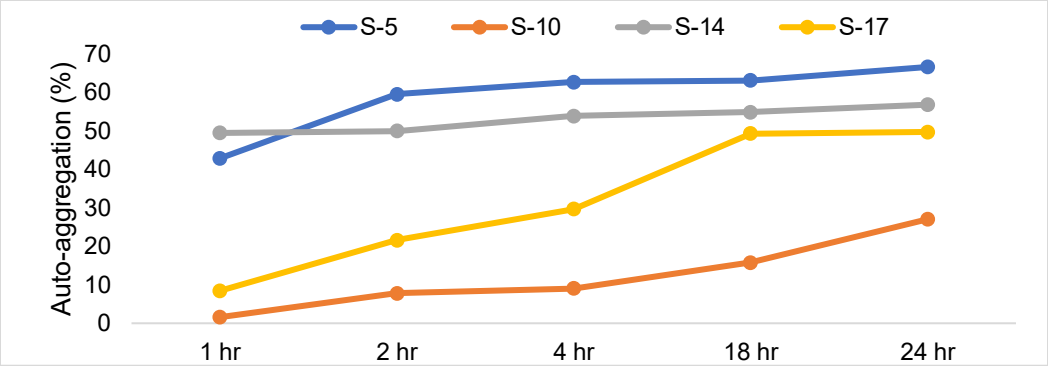

Fig. 1 Auto-aggregation of the isolates [*Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Streptomyces* sp. (S-14), and *Bacillus* sp. (S-17)]

Bacteria auto-aggregate for their protection from environmental stress and sometime for competing bacteria. Auto-aggregation also helps in biofilm formation and adhesion to the surface. The hydrophobicity of the bacterial cell may influence the cell adhesion to the surface and auto-aggregation, too (Escamilla-Montes et al., 2015; Trunk et al., 2018). The other study concluded that the non-hemolytic bacteria with high hydrophobicity and auto-aggregation could be a probiotic strain (Panda et al., 2017). The *Bacillus* sp. has the potential to be used as probiotic bacteria and illustrated to have 32.6±2.77% auto-aggregation (Patel et al., 2010). Another study illustrated that the tested actinobacterial isolates (strains of *Streptomyces* sp.) had auto-aggregation in the range of 84.4 to 90.2% and belonged under strong and mixed auto-aggregation phenotypes (Latha et al., 2016). The auto-aggregation capacity is strain specific and might be due to species-specific surface proteins (Latha et al., 2016).

3.3. Biofilm forming capacity and hydrophobicity

This study illustrated that the bacteria were weak biofilm producers because the OD_{570} values were less than 1 and could not show hydrophobic capacity, as shown in Table 2.

Table 2 Biofilm producing capacity and hydrophobicity of the isolates [*Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Streptomyces* sp. (S-14), and *Bacillus* sp. (S-17)]

Isolates	Biofilm (OD_{570})	Hydrophobicity (%)
S-5	0.17±0.04	14.86
S-10	0.09±0.01	5.45
S-14	0.09±0.02	9.61
S-17	0.13±0.04	12.85

In our study, the isolates illustrated weak biofilm producing capacity as the OD_{570} was within the range of 0.09 to 0.17. However, in another study, different strains of *Streptomyces* sp. illustrated OD_{570} values in the range of 1.55 to 3.28, indicating good biofilm producers and their ability to colonize on a new surface (Homero et al., 2021). The highest hydrophobic capacity in our study was 14.86%, which is less than 20%, so all the isolates were non-hydrophobic. Another study illustrated the hydrophobicity of the bacteria ranging from 25 to 48%, which is higher than our study (Panda et al., 2017). Similarly, a study demonstrated the cell surface hydrophobicity of actinobacterial isolates in the range of 13.2 to 89.3% for chloroform, toluene, and ethyl acetate (Latha et al., 2016). The low hydrophobicity exhibited by the bacteria in our study may be due to the different solvents used. In our study, we used a non-polar solvent which might not interact with the cell. However, other studies used chloroform, toluene, and ethyl acetate. The strong hydrophobicity of the isolates indicates that the bacteria have strong interactions with cells on the surface in the presence of hydrophobic molecules (Latha et al., 2016).

Table 2 results may indicate that there is a correlation between biofilm and hydrophobicity. In parallel to our result, another study reported a positive correlation between biofilm formation and cell surface hydrophobicity. They showed the bacteria with a high biofilm-forming ability have a high hydrophobic surface affinity (Ning et al., 2021b). Some species of *Streptomyces* sp. are known to produce biofilm and cause water quality deterioration. At the same time, biofilm has

shown biotechnological importance in water purification systems and soil and water bioremediation (Homero et al., 2021).

3.4. EPS determination

Among the four bacteria, *Cellulomonas* sp. (S-10) illustrated the highest crude EPS and *Bacillus* sp. (S-17) highest total carbohydrate based on glucose.

Table 3 EPS and total carbohydrate exhibited by the isolates [*Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Streptomyces* sp. (S-14), and *Bacillus* sp. (S-17)]

Isolates	Crude EPS (g/L)	Total carbohydrate (g/L)
S-5	0.27	6.52
S-10	0.98	13.29
S-14	0.68	13.05
S-17	0.72	16.99

EPS has antitumor and free radical scavenging activity; therefore, actinobacteria are becoming famous for producing EPS. Also, EPS from *Streptomyces* sp. illustrated cytotoxic effects on cancer and tumour cells (Homero et al., 2021). Torino et al. (2001) illustrated 49-248.8 mg/L of EPS from *Lactobacillus helveticus,* and acidic pH favored the EPS production (Torino et al., 2001). Similarly, another study demonstrated a higher EPS production by *Bifidobacterium breve* (122 mg/L) and showed that EPS production depends on the bacterial strain (Mendi and Aslım, 2014).

3.5. Maceration test

In the study, *Streptomyces* sp. (S-5) illustrated the highest macerating capacity for both potato and cabbage (18.49 and 42.59%, respectively) among the isolates. The maceration capacity of the isolates was in the order *Streptomyces* sp. (S-5)> Cellulomonas sp. (S-10)> *Streptomyces* sp. (S-14)> and *Bacillus* sp. (S-17) for both cabbage and potatoes.

Table 4 Maceration test of the isolates [*Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Streptomyces* sp. (S-14), and *Bacillus* sp. (S-17)]

Isolates	Maceration (%)	
	Cabbage	Potato
S-5	42.59	18.49
S-10	31.27	18.31
S-14	20.74	10.45
S-17	11.35	9.59

Microbial maceration is the process of softening tissues by breaking them and leaching out the compound present inside the cells with the help of microorganisms. Most commonly, microbial maceration has been applied in wine industries and in bioactive compound extraction from fruits, vegetables and agricultural waste (Sharma et al., 2018). The microbial maceration also depends on the temperature, time, concentration of microbial inoculum, pH, and humidity (Sharma et al., 2018). In contrast, maceration of the plant tissue is mainly due to microbial pectinolytic enzymes (Tanabe and Kobayashi, 1987; Martos et al., 2013a; Golanowska et al., 2017). The study reported that the yeast, *Wickerhanomyces anomalus*, isolated from citrus fruit peels, produced pectinolytic enzymes and was able to macerate potato tissues (Martos et al., 2013a). *Dickeya solani* isolated from potato plants caused potato tuber maceration (Golanowska et al., 2017). *W. anomalus* having cassava maceration property is highly important in producing dehydrated mashed cassava instead of mechanical cellular disruption (Martos et al., 2013a).

3.6. Strain improvement by UV radiation and ethidium bromide (Et-Br) exposure

The traditional strain improvement method includes UV radiation and Et-Br exposure. When the isolates were exposed to UV radiation and Et-Br for different hours, they illustrated their capacity to grow in NA, as in Table 5.

Table 5 Growth observed after exposure to UV radiation and ethidium bromide (Et-Br)

Exposure hour	S-5		S-10		S-14		S-17	
	UV	Et-Br	UV	Et-Br	UV	Et-Br	UV	Et-Br
0	++++	++++	++++	++++	++++	++++	++++	++++
1	++++	++	++++	+++	+++	+++	++++	+++
2	++++	+	++++	++	+++	-	++++	+++
3	++++	-	++++	++	+++	-	++++	++
4	++++	-	++++	+	+++	-	++++	++

[Note; ++++: highly intense growth, +: lowest intense growth, -: no growth]

The bacteria were observed to be more resistant to UV radiation than ethidium bromide (Et-Br). The isolate S-5 did not grow after 2 hours of Et-Br exposure while S-14 after 3 hours, whereas the isolates S-10 and S-17 illustrated their growth, but the intensities were decreased. The bacteria were sub-cultured on new NA plates from the plates where they showed their growth after exposure. For example, the bacteria exposed to UV were sub-cultured from the plates exposed for 4 hours because they all showed their good growth even after 4 hours of exposure. The bacteria from these new NA plates were exploited for pectinase activity determination.

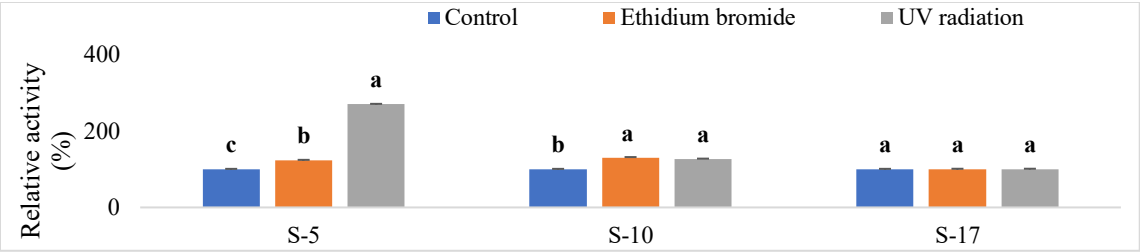

Fig. 2 Relative pectinase activity illustrated by bacteria after exposure to UV radiation and ethidium bromide

Fig. 2 depicted that the pectinase activity was significantly increased when the isolates S-5 and S-10 were exposed to UV radiation and Et-Br. However, isolate S-17 did not show significant change in enzyme activity after exposure to UV and Et-Br. The isolates S-14 and S-5 were both

identified as *Streptomyces* sp. from 16S rDNA analysis, and the growth after exposure to mutants showed similar result, so the pectinase activity of S-14 was not studied.

The physical mutant (UV radiation) induces pyrimidine dimerization and cross-linking in DNA. At the same time, chemical mutant (Et-Br) results in frameshift by intercalating the base pairs between nucleotides (Parekh et al., 2000; Heerd et al., 2014). UV radiation may produce constitutive expression of pectinase and significantly increase pectinase activity (Alazi et al., 2019) which may be the reason for isolate S-5. Similar to our study, a study developed the strain of *Aspergillus sojae* by UV radiation and observed the increased pectinase activity (Heerd et al., 2014). In contrast, another study illustrated the chemical mutant to be superior to UV radiation and showed an increased polygalacturonase activity when *A. tamatii* was treated with sodium azide. The chemical mutant, in this case, may attach to the precursors and enhance the expression of polygalacturonase, resulting in increased activity (Munir et al., 2020).

4. Conclusions

The bacteria studied in this research were Gram-positive, non-hemolytic, not proteolytic, non-capsulated, and lipase producers. They all exhibited the maximum auto-aggregation capacity in 24 hours, and all were weak biofilm producers. These characteristics play an important role in their survival in the environment. The highest auto-aggregation and maceration were shown by *Streptomyces* sp. (S-5) among four pectinase-producing bacteria. Also, the pectinase activity of the bacteria can be enhanced simply by mutagenesis like UV radiation and Et-Br exposure. However, further detailed studies, such as the mechanism of auto-aggregation, maceration, and mutagenesis, are necessary.

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Chapter 4

Optimization of Cultural Conditions for Maximum Pectinase Production

A part of this chapter, Chapter 4A is published in '**Microbial Physiology**' and Chapter 4B is published in '**Frontier in Microbiology**' (IF: 6.064).

4A Optimization of Cultural Conditions for *Streptomyces* sp. for Pectinase Production and Characterization of Partially Purified Enzyme

Abstract

The cultural parameters of *Streptomyces* sp. for pectinase production were optimized using the Box-Behnken design. The maximum pectinase production was obtained after 58 hours at 35℃ and pH 7 upon submerged fermentation in yeast extract-containing media. The enzymes were partially purified with acetone precipitation and the analysis by SDS-PAGE and zymogram revealed that *Streptomyces* sp. produced two proteins with molecular weights of about 25 and 75 kDa. The pectinase activity was detected in a wide range of temperatures (30℃ to 80℃) and pH (3 to 9) with maximum pectinase activities observed at 70℃ and pHs 5 and 9. The enzymes retained about 30 to 40% of their activities even after incubating the enzyme at different temperatures for 120 mins. The pectinase activities of *Streptomyces* sp. were enhanced in the media containing 1.5% pectin, 1% casein as a nitrogen source, 0.5 mM $MgSO_4$, and 5 mM NaCl. Further, the addition of Tween-20, amino acids, and vitamins to the media also enhanced the pectinase activity. Moreover, the bacterium illustrated the ability to decolorize crystal violet dye efficiently. The decolorization rate ranged from 39.29 to 53.75% showing the highest bacterial decolorization in the media containing 2 mg/mL crystal violet at 144 hours. Therefore, the bacterium has the potential in treating wastewater produced by industries like textile industries.

Keywords: *Streptomyces* sp., pectinases, response surface methodology, decolorization

1. Introduction

Pectinases are a group of complex enzymes of the polysaccharidases family, that degrade the complex pectin and are leading commercial enzymes (Kavuthodi and Sebastian, 2018; Shrestha et al., 2021b). Insects, nematodes, plants, and microorganisms are the sources of pectinases, but microorganisms are the leading sources of industrial pectinase production. The microorganisms are easy to grow, can be manipulated genetically, and efficiently study different mechanisms like phyto-pathogenesis, plant-microorganisms symbiosis, and the decomposition of organic matter (Hoondal et al., 2002; Kohli and Gupta, 2015).

The demand and applications of pectinases are continuously increasing. The pectinases are used in the production of functional foods (Khan et al., 2013), retting and degumming of fibers in the textile industry (Chiliveri et al., 2016), and the production of good quality paper (Ahlawat et al., 2009). In addition, pectinases are applied in the production of animal feed, liquefaction and saccharification of biomass, bio-scouring of cotton fiber, oil extraction, coffee and tea fermentation (Kashyap et al., 2001; Jayani et al., 2005; Kubra et al., 2017; Shrestha et al., 2021b), treatment of pectic wastewater, and bioethanol production (Kashyap et al., 2001; Wang et al., 2019).

Due to the increasing demands of pectinases, it is necessary to isolate and identify pectinase-producing bacteria and optimize the cultural conditions for maximum pectinase production. The forest soil bacterium, *Streptomyces* sp. showed different enzymatic activities while studying its biochemical characteristics (Shrestha et al., 2021b). Also, many species of *Streptomyces* have the potential to remove dyes via different mechanisms like biosorption and biodegradation (Adenan et al., 2021), and the biological methods of eliminating the dyes are being considered. Crystal violet is a commonly used dye in textile industries, which harms the environment due to

its mutagenic and mitotic poisoning properties (Roy et al., 2018a). The textile industries use different dyes, around 60 to 70%, and release the effluent into the environment or water or rarely in the treatment system. Such wastewater is harmful, lead pollution, the mutation in the environment, and may cause severe problems in the world (Roy et al., 2018a; Kishor et al., 2021). Thus, decolorization or degradation of crystal violet is necessary to reduce or eliminate the adverse effect on the environment. Bacteria able to grow in harsh conditions can be helpful for this task. Therefore, this study exploited the soil bacterium that can grow in a wide range of temperatures and pH (Shrestha et al., 2021b), for decolorizing crystal violet and minimizing the negative impact of dyes. The various species of this bacterium are found predominantly in environmental samples (Adenan et al., 2021).

This study aims to optimize the fermentation conditions of the pectinolytic bacteria isolated from forest soil for maximum pectinase production. Also, the study explored the effects of some additives in pectinase production when added to media and characterized the pectinase in various ranges of temperature and pH. The molecular mass of the enzyme was determined. Eventually, the application of *Streptomyces* sp. in the decolorization of crystal violet was studied to know its efficiency in treating wastewater generated from dye using industries.

2. Materials and methods

2.1. Bacterium strain and inoculum preparation

The bacterium exploited in this study was isolated, screened, and identified as *Streptomyces* sp. (Shrestha et al., 2021b) and stored at -80°C in the lab as a bacterium stock. The bacterium was revived by inoculating 1% v/v (40 µL) of preserved bacterium stock into 4 mL of LB broth and incubated at 35°C for 18 hours. For further studies, this overnight cultured broth was taken and proceeded.

2.2. Optimization of fermentation conditions

The overnight cultured *Streptomyces* sp. of 0.5 mL was inoculated in an Erlenmeyer flask containing 50 mL of yeast extract pectin media (YEP) composed of 0.3% w/v yeast extract, 1% w/v pectin, 0.2% w/v KH_2PO_4, and 0.2% w/v K_2HPO_4 in distilled water. The inoculated flasks were incubated at 35°C, 200 rpm for 4 days. Samples from inoculated flasks were collected at regular intervals of 24 hours, and enzyme activities were assayed and compared. Pectinase activity was calculated by measuring the reducing sugar content released from the substrate following the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), as mentioned in a previous study (Shrestha et al., 2021b). Each enzymatic activity was expressed as the amount of enzyme that releases 1 μmol of galacturonic acid in 1 min under the mentioned conditions.

The various parameters that affect enzyme production were optimized by one variable at a time while all other factors were kept constant. The experiments carried out in this study were performed in triplicates.

2.2.1. Effect of incubation period, temperature, and pH in pectinase production

The *Streptomyces* sp. was cultured in pectinase production media (YEP) for 120 hours. At different incubation periods, the cultured broth was aseptically taken out and the cell-free supernatant was used for enzyme activity assay. The bacterial growth was monitored by measuring the optical density (OD) at 600 nm using the pallet resuspended in distilled water. The pectinase activities at different incubation temperatures (30°C, 35°C, 40°C, and 45°C) and pH (5, 6, 7, 8, 9, and 10) were also studied.

2.2.2. RSM design for optimization of pectinases production by Streptomyces sp.

Three factors and a three-level Box-Behnken design was employed to produce maximum pectinase and understand the interactions of different variables. The study considered three

variables: incubation time, temperature, and pH of cultural conditions and the range of variables were determined from optimization one variable at a time method. The designation of the experiment (15 runs with three replicates of center points) and analysis of results were performed using Minitab software. The response variable (pectinase activity) was fitted by a second-order model to relate the response variable to the independent variables. The maximum pectinase activity was obtained from optimizing these cultural conditions and the statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

2.3. Factors affecting pectinase production

2.3.1. Effects of different pectin concentrations, carbon, and nitrogen sources for pectinase production

Different concentrations of pectin (0.5%, 1%, 1.5%, and 2% w/v), carbon sources (glucose, fructose, xylose, lactose, mannitol, sorbitol, and pectin), and nitrogen sources (peptone, casein, yeast extract, ammonium sulfate, ammonium nitrate, and ammonium chloride) were supplemented as individual components to the basal media to know their effects on pectinase production.

2.3.2. Effect of different metal ions in chloride and sulfate forms for pectinase production

The effect of various metal ions was also studied for pectinase enzyme activity by using 0.1 mM, 0.5 mM, 1 mM, or 5 mM of each metal ion, such as Fe_2SO_4, Na_2SO_4, $(NH_4)_2SO_4$, $ZnSO_4$, $MnSO_4$, and $MgSO_4$ in sulfate forms. Similarly, in chloride forms, $MnCl_2$, $CaCl_2$, KCl, $CoCl_2$, $MgCl_2$, NaCl, and NH_4Cl were studied to know their effects on pectinase production.

2.3.3. Effect of different surfactants and chemicals for pectinase production

Different surfactants such as SDS, Triton X-100, Tween-20, and chemicals like mercaptoethanol and H_2O_2 were added to the culture media in 1%, and their effect on pectinase activity was studied.

2.4. Partial purification and characterization of pectinase

2.4.1. Precipitation and molecular weight determination of pectinase

The crude enzyme extract obtained after culturing the bacterium in the optimized cultural conditions and centrifugation was precipitated by 50 to 70 % saturated ammonium sulfate, acetone, or ethanol. All the mixtures were kept at 4°C overnight and centrifuged at 12,000 rpm for 20 min. The residue after centrifugation was suspended in a minimal amount of 0.1 M phosphate buffer at pH 7 and kept at 4°C for future use. The pectinase activity of the partially purified enzyme was assayed by the method described earlier.

The molecular weight of the produced pectinase was decided by running sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out in a 3 mm slab using 5% (w/v) stacking and 12 % (w/v) separating gels. For this, samples were heated for 5 min at 95°C in the sample loading buffer before loading on wells. After running the gel for a specific time, it was stained with Coomassie brilliant blue and destained by keeping it in a destaining solution. The standard board range protein marker was used to compare the molecular weight of the enzyme sample.

For zymogram, the gels after electrophoresis were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in phosphate buffer (pH 7.4) for 30 min, flooded with 2% pectin and kept at 45°C for 60-90 mins. Then the gel was stained with 0.05% (w/v) potassium iodide solution for 10 min and washed with water until bands became visible.

2.4.2. Effects of temperature and pH on pectinase

The reaction mixture of the partially purified enzyme and substrate was incubated at different temperatures (25°C, 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C) for 10 min to determine the effects of temperature on pectinase activity. Similarly, the effect of pH on pectinase activity was accessed using different pH buffers such as citrate buffer (pH 3 to 4), potassium-phosphate buffer (pH 5 to 8), and glycine-NaOH buffer (pH 9) and incubating for 10 min at 50°C.

2.5. Ability of the bacterium to decolorize crystal violet

The bacterium was positive for catalase, DNase, cellulase, xylanase, amylase screening test and spore former. Further, the bacterium was grown in a wide range of temperatures and pH (Shrestha et al., 2021a), so it was exploited to determine its ability to decolorize crystal violet. The overnight grown bacterium was inoculated in YEP media supplemented with crystal violet (1 mg/mL, 2 mg/mL, or 3 mg/mL) and incubated at 35°C. The uninoculated medium with crystal violet was used as a control. Every 24 hours, the decolorization rate was determined by observing the absorbance at 680 nm. The decolorization efficiency was evaluated as the percentage ratio of change in OD by initial OD based on the equation (Roy et al., 2018a):

$$\text{Decolorization (\%)} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

2.6. Statistical analysis

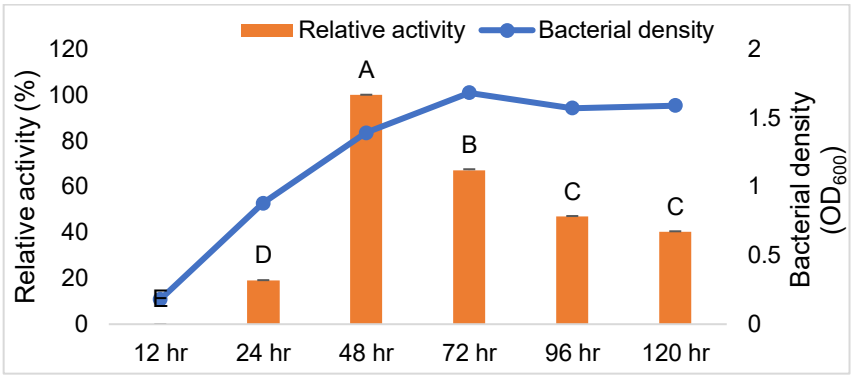
All the experiments in this study were performed in triplicates, and the data were analyzed using ANOVA. The level of significance of results was determined by Tukey's post hoc test with a 95% confidence level and considered significant if $P < 0.05$.

3. Results

3.1. Effects of incubation time, temperature, and pH

The results of incubation time revealed the maximum enzyme activity was observed in 48 hours of incubation (Fig. 1a) in a YEP medium. Similarly, the maximum pectinase activity was observed at 35℃ (Fig. 1b) and there was a significant decrease in pectinase activity below or above 35℃.

a)



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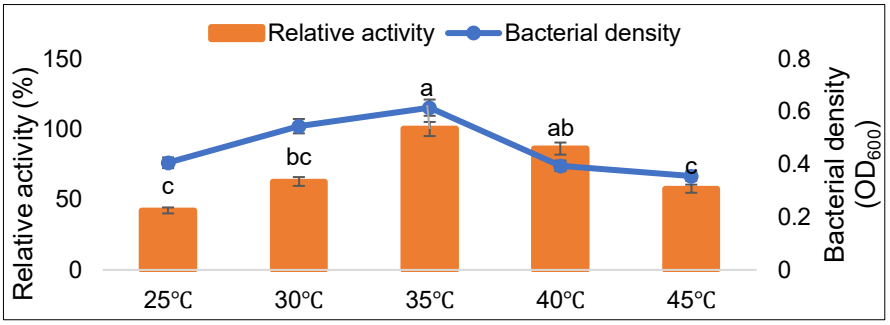


c)

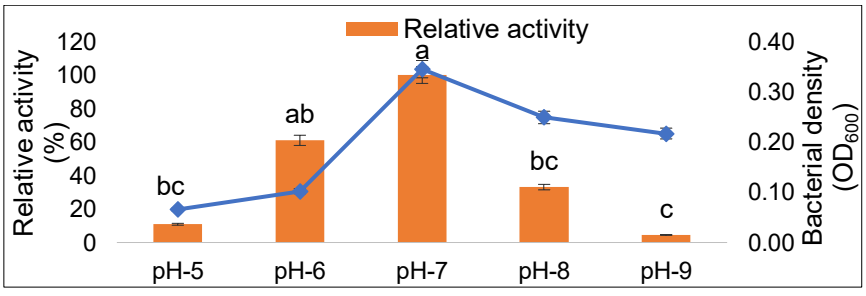


Fig. 1 The effects of **a)** incubation period, **b)** temperatures, and **c)** pH on pectinase production by *Streptomyces* sp. (different lowercase letters above the bar indicate the significant difference between experimental conditions)

The present study elicited the maximum enzyme activity at pH 7 (Fig. 1c), followed by pH 6 and 8. However, the pectinase activity was observed in a wide range of pH (5 to 9).

3.2. Optimization of cultural conditions using response surface methodology (RSM)

Table 1 depicts pectinase activity exhibited by *Streptomyces* sp. when the three factors were selected with three levels. The design included three replicates at the center point to provide a measure of process stability and inherent variability.

Table 1 Experimental results of Box-Behnken design for pectinase activity (U/mL)

Run	Incubation temperature (°C)	pH	Incubation time (hr)	Pectinase activity (U/mL)
1	30	6	48	1.41±0.07
2	40	6	48	1.73±0.09
3	30	8	48	1.65±0.08
4	40	8	48	1.44±0.07
5	30	7	24	0.46±0.02
6	40	7	24	0.42±0.02
7	30	7	72	1.49±0.07
8	40	7	72	2.14±0.11
9	35	6	24	0.41±0.02
10	35	8	24	0.55±0.03
11	35	6	72	1.70±0.09

12	35	8	72	2.30±0.12
13	35	7	48	2.79±0.14
14	35	7	48	2.16±0.11
15	35	7	48	2.72±0.14

*Analysis was done using coded units and the values of pectinase activity was mentioned as mean±standard deviation

The BBD experimental results were fitted with a polynomial equation using a multiple regression technique and expressed as: $y = -57.0253 + (1.8879 \cdot A) + (6.2229 \cdot B) + (0.1331 \cdot C) - (0.0259 \cdot A \cdot B) + (0.0005 \cdot A \cdot C) + (0.0048 \cdot B \cdot C) - (0.0243 \cdot A^2) - (0.3900 \cdot B^2) - (0.0016 \cdot C^2)$

where y is pectinase activity, A is temperature, B is pH, and C is incubation time.

The accuracy and statistical significance of each term in the model were evaluated using Analysis of variance (ANOVA) and shown in Table 2. The analysis of factors elucidated F-value for the regression model of pectinase activity as 12.75, which showed that the model of this study was significant.

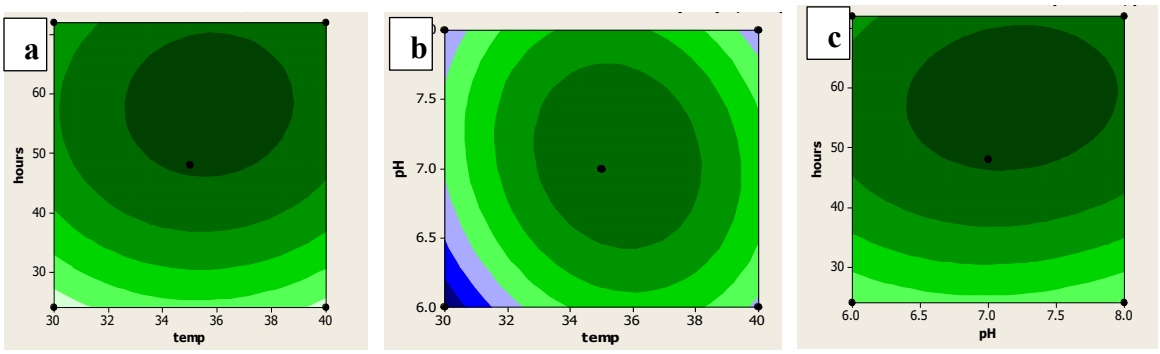
Table 2 Analysis of variance of Box-Behnken design for pectinase activity

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value	
Regression Model	9.69	9	1.08	12.75	0.0060	Significant
Linear	5.03	3	1.67	19.83	0.003	Significant
A-Temp	0.16	1	0.16	1.89	0.2275	
B-pH	0.06	1	0.06	0.69	0.4440	

C-Hours	4.81	1	4.81	56.92	0.0006	Significant
Interaction	0.14	3	0.05	0.55	0.671	
temp*pH (AB)	0.07	1	0.07	0.79	0.4129	
temp*hours (AC)	0.02	1	0.02	0.21	0.6629	
pH*hours (BC)	0.05	1	0.05	0.63	0.4628	
Square	1.51	3	1.51	17.86	0.004	Significant
temp*temp (A^2)	1.37	1	1.37	16.20	0.0101	Significant
pH*pH (B^2)	0.56	1	0.56	6.65	0.0495	Significant
hours*hours (C^2)	3.16	1	3.16	37.40	0.0017	Significant
Residual	0.42	5	0.08			
Lack of Fit	0.18	3	0.06	0.52	0.7093	Not significant
Pure Error	0.24	2	0.12			
Cor Total	10.12	14				

R^2 = 95.82%, predicted R^2 = 65.41%, adjusted R^2 = 88.31%

2D contour and 3D response plots were constructed to illustrate the interactive effect of each independent variable on pectinase activity for maximum pectinase production. Here, the effects of the two variables were shown and the third variable was fixed (Fig. 2).



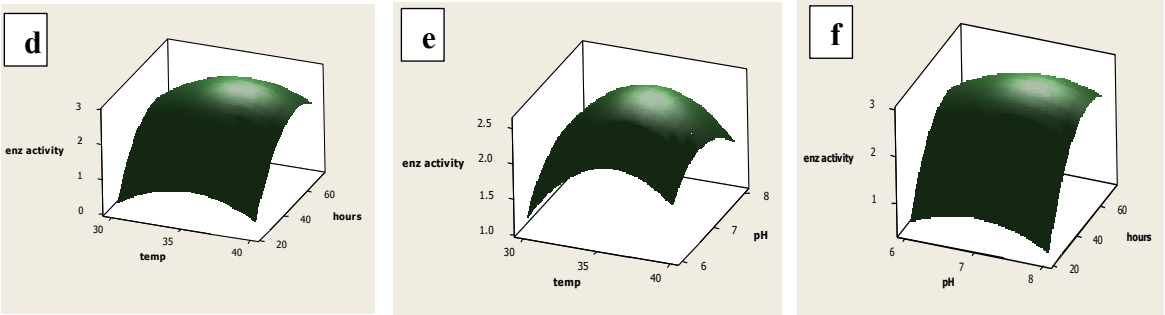


Fig. 2 2D contour plots and 3D response surface plots showing the effect of incubation temperature and hour **(a**, **d)**, temperature and pH (**b**, **e**), and pH and hour (**c**, **f)** for the pectinase activity

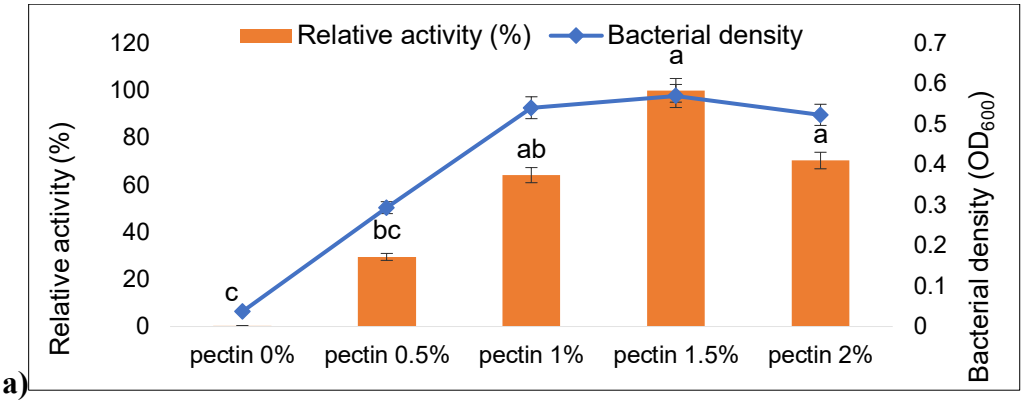
The RSM optimizer found the optimal conditions; 35.62℃, pH 7.15, and 58.40 hours for pectinase production (Supplementary Fig. 1). The bacterium was cultured in the mentioned optimal conditions and enzyme activity was calculated for verification. The calculation showed that the measured value did not ideally agree with the value predicted by the response model. However, the value (2.37 U/mL) was close to the predicted value (2.74 U/mL). Therefore, the optimal cultural condition for pectinase production by *Streptomyces* sp. was 35℃, pH 7 and 58 hours of incubation.

The counter plot figures 2a and 2d showed that the pectinase activity increased with an increase in incubation temperature and time to a certain level and decreased afterwards. Similar patterns were observed for the effects of temperature and pH, and pH and incubation time, as shown in Fig. 2b, e and c, f, respectively.

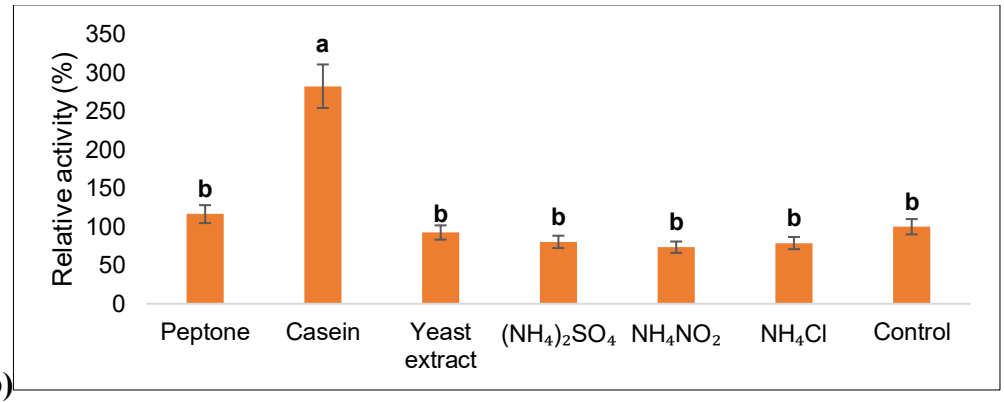
3.2.1. Effects of different pectin concentrations, nitrogen and carbon sources on pectinase production

The fermentation media containing 1.5% pectin was observed to show the highest enzyme activity, followed by 2% pectin when used as carbon source. Although the fermentation medium containing 1.5% observed the highest enzyme activity, there is no significant difference between

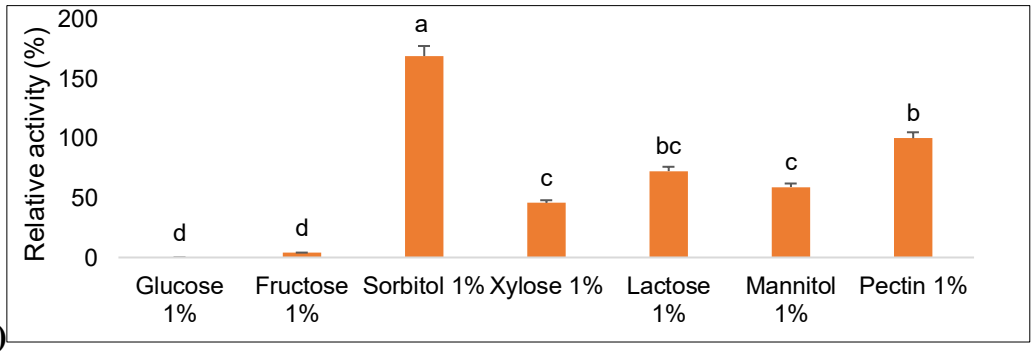
1.5% and 2% pectin-containing media when analyzed statistically (Fig. 3a). Thus, 1.5% of pectin was used in further studies.



a)



b)



c)

Fig. 3 Effects of different **a)** pectin concentrations **b)** nitrogen and **c)** carbon sources on pectinase production (different lowercase letters above the bar indicate the significant difference between experiments)

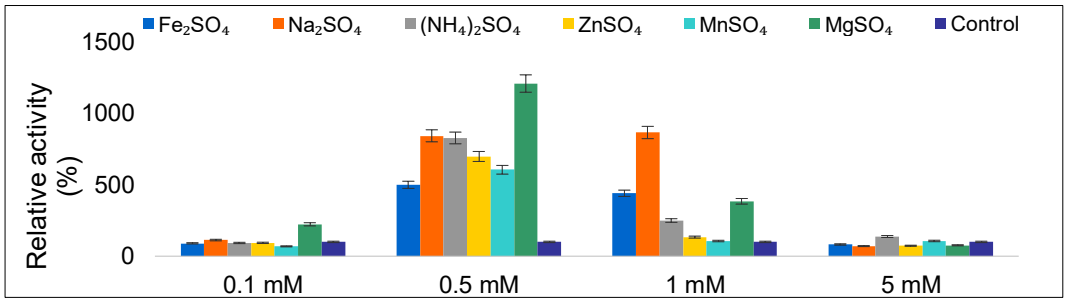
Among different nitrogen sources studied, casein significantly increased pectinase production (Fig. 3b). The present study illustrated that the growth of *Streptomyces* sp. and pectinase activity

was influenced by the carbon sources added. Pectin after sorbitol exhibited the maximum pectinase activity and acted as the best carbon source for pectinase production. The addition of glucose inhibited the pectinase activity. In contrast, fructose, xylose, lactose, and mannitol decreased pectinase production significantly (Fig. 3c).

3.2.2. Effects of different metal ions in pectinase production

The different trends in pectinase activities were observed when various concentrations of metal ions in sulfate and chloride forms were added to the fermentation media (Fig. 4). The result revealed that pectinase production was higher with 0.5 mM of various metal ions in sulfate forms (Fig. 4a). Similarly, NaCl exhibited higher pectinase activity than control in various concentrations among the chlorides of varying metal ions supplemented in basal media. $CaCl_2$ exhibited significantly higher activity in 1 mM but lower in other concentrations (Fig. 4b).

a)



b)

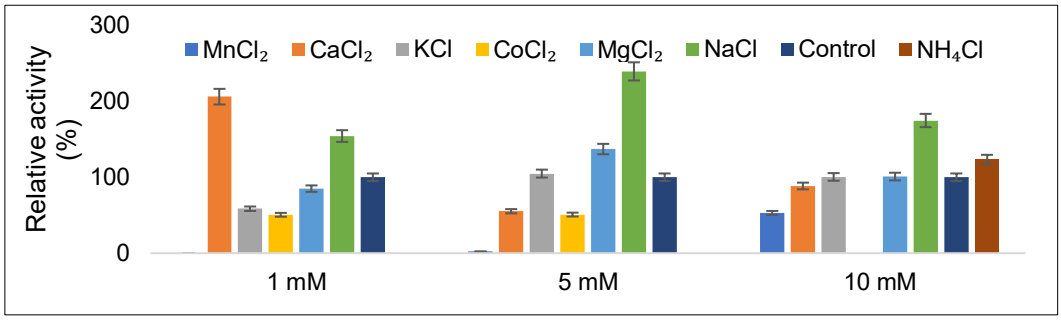


Fig. 4 Effects of different metal ions in **a)** sulfate and **b)** chloride forms for pectinase production by *Streptomyces* sp.

3.2.3. Effects of different surfactants and chemicals on pectinase production

Different surfactants and additives may affect pectinase production. All the surfactants and chemicals added to this research inhibited the pectinase production except Tween-20 (Fig. 5).

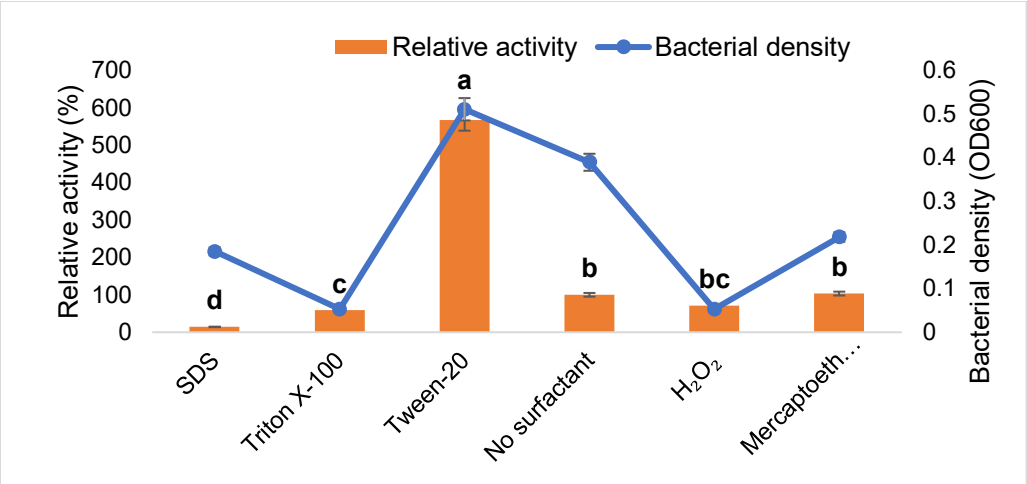
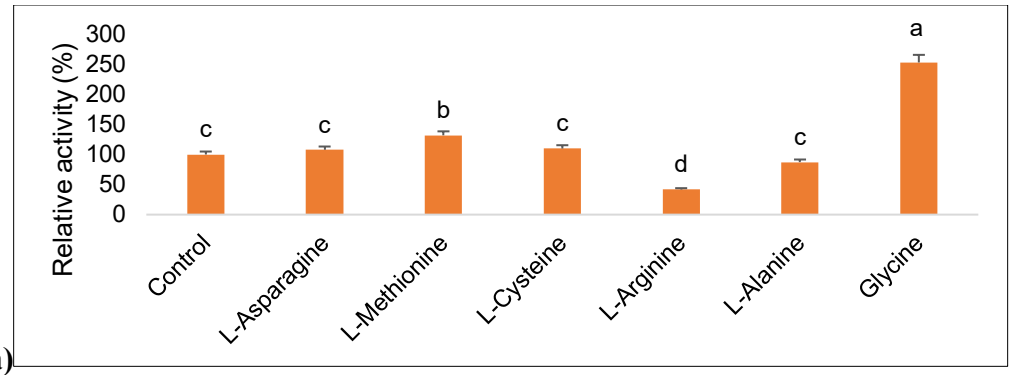


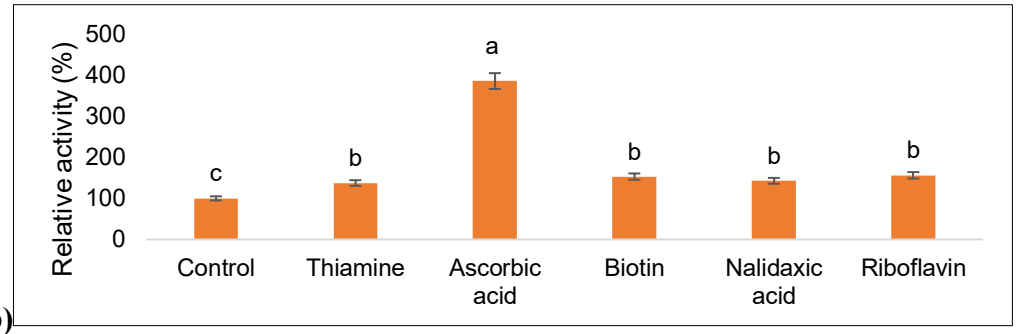
Fig. 5 Effects of different surfactants and chemicals on pectinase production by *Streptomyces* sp. (different lowercase letters above the bar indicate the significant difference between experiments)

3.2.4. Effects of amino acids and vitamins on pectinase production

The different amino acids and their analogues showed the pectinase activity as in Fig. 6a; arginine and alanine inhibited the activity compared to control. However, asparagine, methionine, and cysteine slightly increased the activity, but glycine stimulated the activity significantly (Fig. 6a).



a)



b)

Fig. 6 Effects of different **a)** amino acids and **b)** vitamins on pectinase production by *Streptomyces* sp. (different lowercase letters above the bar indicate the significant difference between experiments)

The pectinase production by *Streptomyces* sp. was enhanced by adding vitamins in the fermentation media (Fig. 6b). Ascorbic acid significantly increased the pectinase production and there was a slight increase in pectinase activity by thiamine, biotin, nalidixic acid, and riboflavin.

3.3. Partial purification and characterization of pectinase

The cell-free supernatant was partially purified using ammonium sulfate, acetone, and ethanol precipitation method. The acetone precipitated enzyme illustrated maximum enzyme activity followed by ethanol and ammonium sulfate precipitation (Fig. 7). Also, the result revealed that a higher concentration of solvents decreased the enzyme activity. Indeed, the enzyme was inhibited in presence of acetone and ethanol higher than 70% and ammonium sulfate higher than 60%.

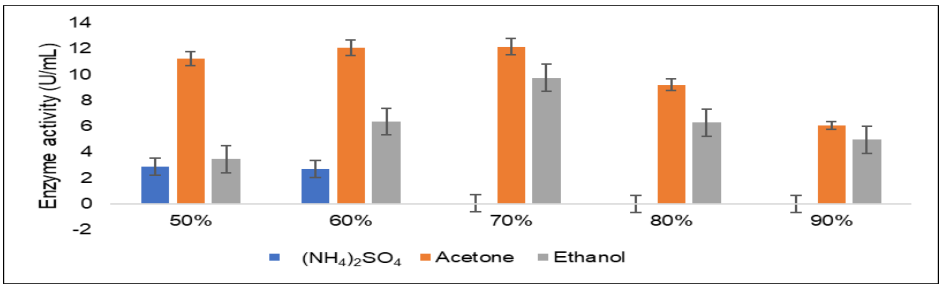
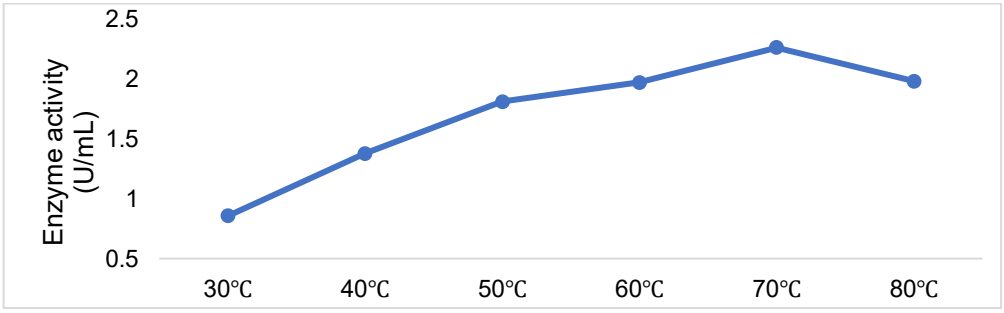


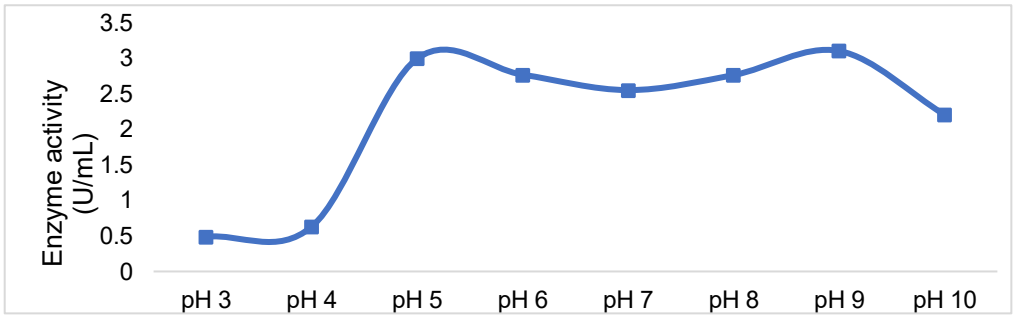
Fig. 7 Partial purification by different solvents in different concentrations

The activity of partially purified pectinase continuously increased till 70°C, showing its maximum activity at 70°C and declined at a higher temperature above 70°C (Fig. 8a). However, the enzyme showed a different trend when the enzyme extract reacted at different pH. The activity increased from pH 3 to 5, decreased from pH 5 to 7, and again increased continuously from pH 7 to 9. The pH above 9 decreased the activity showing maximum activity at pH 5 and 9 (Fig. 8b). The pectinase from *Streptomyces* sp. was observed to have residual activity of approximately 30-40% after 120 mins of exposure to different temperatures (Fig. 8c).

a)



b)



c)

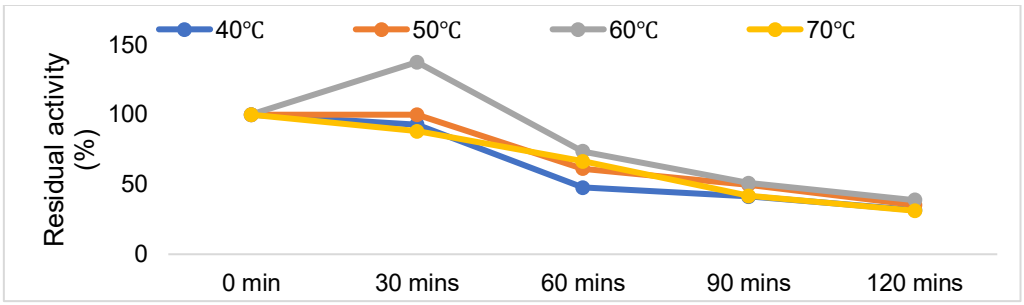


Fig. 8 Effects of different **a**) temperature, **b**) pH on enzyme activity, and **c**) stability at different temperatures of partially precipitated enzyme

3.4. SDS and zymogram analysis

The partially purified enzyme was electrophoresed on a 12% SDS-PAGE. The multiple protein bands were observed around molecular weight 20 to 70 kDa (Fig. 9). The zymogram revealed two clear bands at approximately 25 and 70 kDa, which indicated the presence of pectinases produced by *Streptomyces* sp.

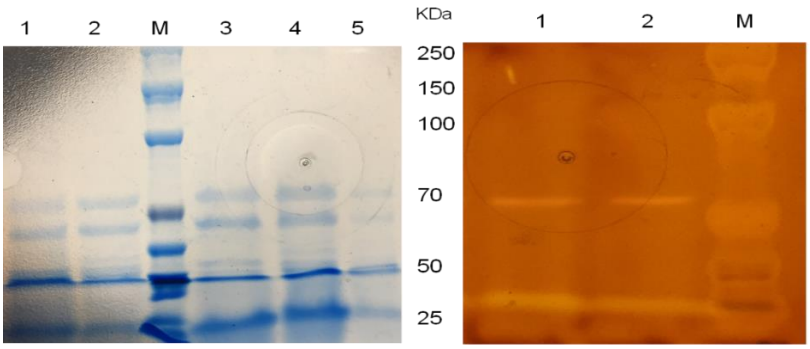


Fig. 9 SDS-PAGE (1and 5: crude enzyme (supernatant before precipitation), 2: ammonium sulfate precipitated, 3: ethanol precipitated, 4: acetone precipitated pectinase, M: marker) and zymogram (1: crude, 2: acetone precipitated, M: marker) of pectinase

3.5. Decolorization of crystal violet dye

The experimental result revealed the decolorization of crystal violet dye by *Streptomyces* sp. ranged from 39.29 to 53.75%. The highest decolorization rate of crystal violet dye (53.75%) and

the lowest (39.29%) was observed in the medium containing 2 mg/mL and 1 mg/mL of crystal violet at 144 and 24 hours, respectively (Table 3).

Table 3 Decolorization of crystal violet by *Streptomyces* sp. at different concentrations and incubation hours

Crystal violet	**Decolorization rate (%)**					
	24 hours	**48 hours**	**72 hours**	**96 hours**	**120 hours**	**144 hours**
1mg/mL	39.29	43.26	43.32	44.39	44.06	44.09
2mg/mL	43.52	46.12	48.11	51.33	52.51	53.75
3mg/mL	41.52	43.12	44.01	45.06	45.30	46.13

4. Discussions

The present study exploited *Streptomyces* sp. that was isolated from forest soil (Shrestha et al., 2021b) to determine the optimum fermentation conditions for maximum pectinases production and to characterize the enzyme produced. The bacterium *Streptomyces* sp. illustrated significantly higher enzyme activity (Supplementary Fig. 2) in yeast extract pectin (YEP) so this media was used as the pectinase production media in this study. Another study supported the result as yeast extract is a good source of essential nutrients and organic nitrogen (Kaur et al., 2016). Temperature is one of the influencing factors in bacterial growth, metabolic and enzymatic activities. The maximum pectinase activity was observed at 35℃ so it was considered as the optimum temperature for pectinase production by this bacterium. Similar findings were reported for *Chryseobacterium indologenes* strain SD (Roy et al., 2018b) and *Bacillus* species (Aaisha and Barate, 2016). However, *Saccharomyces cerevisiae* illustrated the maximum enzyme activity at 30℃ for PG and PL (Poondla et al., 2015). The decrease in activity at low temperatures may be due to insufficient energy. In contrast, the low activity at high temperatures

may be due to denaturation or some modification in enzyme structure (Rehman et al., 2015; Ibrahim et al., 2021).

The pH of the fermentation media plays a vital role in enzyme production. The maximum microbial growth and enzyme activity in our study were observed at pH 7. Previous studies reported that the *Bacillus* sp. produced a high amount of pectinase between pH 7.5 and 8 (Aaisha and Barate, 2016; Oumer and Abate, 2018a; Roy et al., 2018b). The result from this study inferred that very low and very high pH conditions are not favorable for the growth of the organism, which might decrease the growth and enzyme activity of the organism.

The P-values from analysis of BBD were used to ensure the significance of each coefficient and interaction strength between each independent variable. The P-value less than 0.05 ($P<0.05$) observed in analysis indicated the model term is significant. The quality of fit of the second-order model equation was expressed by the coefficient of determination (R^2), and an F-test determined its statistical significance (Gonçalves et al., 2012; Handa et al., 2016). In this analysis, C, A^2, B^2, and C^2 were significant model terms as the P-values were less than 0.05. And the coefficient of determination (R^2) was calculated as 0.984, indicating that this model could explain 98.4% of the variability in the response. The R^2 value closer to 1.0 predicts a better and stronger model. Similarly, the Lack of Fit F-value of 0.52 and P-value of 0.7093 implied the Lack of Fit was not significant relative to the pure error, and there was a 70.93% chance that a large P-value could occur due to noise. Non-significant lack of fit was what we wanted, and this indicated the model was well fitted to the experimental data (Gonçalves et al., 2012; Handa et al., 2016).

In the present study, pectin was observed as a stimulator for pectinases activity because in the absence of pectin there was no pectinase activity (Fig. 7a). Ramírez-Tapias et al. (2015)

demonstrated that 1% pectin gave the maximum polygalacturonase activity (Ramírez-Tapias et al., 2015) which was similar to the results reported by Ketipally and Ram (2018) (Ketipally and Ram, 2018). Our study result illustrated the maximum activity at 1.5% pectin although the activity was not significantly different between 1, 1.5, or 2% pectin. The result indicated that a higher concentration of citrus pectin in the fermentation media could have an antagonistic effect on pectinase production (Ramírez-Tapias et al., 2015).

Nitrogen is required for microbial growth because it is an essential protein constituent and different nitrogen sources influence the growth of microorganisms and metabolites production. The inorganic nitrogen sources inhibited enzyme production compared to the control. Hence, the organic nitrogen source is more beneficial than the inorganic source. Furthermore, our study results supported that organic nitrogen sources increased pectinase activity.

The carbon present in the fermentation media plays a crucial role in providing energy for bacterial growth and enzyme production. *Streptomyces* sp. produced maximum pectinases when sole pectin and sorbitol were added to the media because they may act as a suitable carbon source. Whereas other carbon sources decreased pectinase activity when added to the basal media similar to other studies (Kaur and Gupta, 2017; Oumer and Abate, 2018a). The pectinase production decreased slightly when glucose, lactose, and xylose were added to the media (Kaur & Gupta, 2017). Furthermore, other studies illustrated that adding glucose inhibited the PG production by *Aspergillus* sp., and maximum PG was observed in the absence of glucose (Runco et al., 2001). This may be because glucose has a repressive impact on the catabolism of enzymes or a negative effect of glucose on enzyme production. In contrast to our result, Kuhad et al. (2004) found higher pectinase activity in glucose-supplemented media and the highest pectinase yield in the media containing pectin and glucose (Kuhad et al., 2004). *Bacillus subtilis* produced

maximum activity when lactose was added, whereas maltose acted as the best carbon source for *Bacillus amyloliquefaciens* (Arekemase et al., 2020). The inconsistency in the maximum enzyme activities may be due to the genetic differences between microorganisms. In addition, the study illustrated that the addition of glucose or sucrose did not reduce pectinase activity in solid-state fermentation but strongly decreased in submerged state fermentation (Solís-Pereira et al., 1993). Thus, the nature and concentration of carbon sources play a significant role in enzyme production. The pectinase production media containing glucose or sucrose like carbon depends on the types of fermentation too.

The high concentration of metal ions inhibited enzyme production, possibly due to the blockage of protein secretion into the external medium (Ahlawat et al., 2009). The presence of metal ions affects the enzyme's catalytic properties by inhibiting or activating the enzyme activity that affects the enzyme's active site and the protein molecules' stability. The lower concentration of Mg^{2+} (2 mM or 4 mM) acts as the activator, but the higher concentration (6 mM, 8 mM, or 10 mM) inhibited the enzyme activity (Anggraini et al., 2020). Similar can be found for $CaCl_2$, Fe_2SO_4, $MgSO_4$, $MnSO_4$, and $ZnSO_4$ in this study.

The surfactants may affect cell membrane permeability leading to blockage in the pectinases secretion, or the surfactants may denature the enzyme (Ahlawat et al., 2009). The surfactants generally interact with proteins and alter particular regions affecting the structure of proteins and enzyme activities (Zohdi and Amid, 2013). Another study demonstrated that the addition of Tween-20 significantly enhanced the pectinase yield by *Bacillus subtilis* (Ahlawat et al., 2009). In contrast, pectinase secretion by *Aspergillus* sp. was inhibited by Tween-20, Tween-80, Triton X-100 and enhanced significantly by polyethylene glycol (Li et al., 2015). In this study, Tween-

20 enhanced the enzyme activity, which might be due to this surfactant providing a suitable membrane structure for enzymes to bind with the substrate and increase the activity.

Amino acid and vitamin also affect the pectinase activity. In a prior study, DL-isoleucine, DL-norleucine, L-lysine monohydrochloride, L-leucine, casamino acid, and DL-b-phenylalanine showed a stimulatory effect on pectinase production by *Streptomyces* sp. QC-11-3 (Beg et al., 2000). Proline, L-tyrosine, L-cysteine, DL-aspartic acid, L-cystine monohydrochloride, and DL-threonine inhibited pectinase yield, but DL-alanine, L-histidine monohydrochloride, DL-b-phenylalanine, and L-lysine monohydrochloride stimulated the pectinase production by *Streptomyces* sp. RCK-SC (Kuhad et al., 2004). *Aspergillus terrus* increased polygalacturonase production when leucine, tyrosine, and methionine were added to the production media (Runco et al., 2001). Another study showed vitamins like ascorbic acid, riboflavin, nicotinic acid, and biotin enhanced the pectinase production by *Streptomyces* sp. RCK-SC and thiamine reduced pectinase production (Kuhad et al., 2004). In our study, glycine and ascorbic acid may act as modifiers for fitting the pectinase precisely in the active sites changing the configurations. Also, ascorbic acid might function as a coenzyme, and the glycine charges play critical roles in the catalytic reactions, increasing enzymatic activities.

The acetone precipitated enzyme illustrated maximum enzyme activity which possibly may be because the enzyme favors the organic compound rather than the inorganic compound (ammonium sulfate). The partially precipitated pectinase was explored to study the effect of temperature and pH on its activity and stability at different temperatures. This findings revealed that pectinase produced by *Streptomyces* sp. was thermostable and not neutral (can resist both acidic and alkaline pH but not very low and very high pH). This characteristic of the enzyme was unique and beneficial for various industrial applications. The decrease in activity after 70°C and

in very high and low pH may be due to the denaturation of enzymes and changes in the native structure of pectinases. The higher temperature increases the collision between the reactant molecules and kinetic energy. The higher kinetic energy after a certain limit may denature the enzyme and changes the structure of the enzyme (Rehman et al., 2015). The study conducted by Ramírez-Tapias et al. (2015) observed the optimum catalytic conditions of PG produced by *Streptomyces halstedii* at 50℃ and pH 12. They stated that the alkaline PG is applicable in the depectinization of wastewater from the pulping mill and paper-making industries (Ramírez-Tapias et al., 2015). In another study, the maximum activity of pectin lyase (PNL) was observed at 60℃ and pH 8 (Demir et al., 2014).

The result of zymogram showed that *Streptomyces* sp. produced enzymes able to degrade citrus pectin and have molecular weights around 25 kDa and 70 kDa. This result is in harmony with another study which revealed two bands of ethanol precipitated pectinases with a molecular weight of 60 and 64 kDa (Takcı and Turkmen, 2016), and PG with molecular weights of ~36 kDa and ~72 kDa (Paudel et al., 2015). In addition, Poondla et al. (2015) found 2 bands of PG isoenzymes having molecular weights around 47 kDa and 50 kDa (Poondla et al., 2015) and ammonium sulfate precipitated pectinase from *Aspergillus foetidus* illustrated 2 bands at 34 and 44 kDa (Kumar et al., 2012). Ramírez-Tapias et al. (2015) revealed that the molecular weight of alkaline PG produced by *Streptomyces halstedii* was approximately 48 kDa (Ramírez-Tapias et al., 2015), and Demir et al. (2014) observed the molecular weight of pectin lyase of about 36 kDa (Demir et al., 2014). The molecular weight of pectinases varies with different microorganisms; for example, PG from *Bacillus licheniformis* was observed to be 153 kDa (Rehman et al., 2015), pectin lyase of 25 kDa in *Bacillus pumilus* (Güllüce and Demir, 2010), and polygalacturonase of 153 kDa in *Bacillus licheniformis* (Rehman et al., 2015). The

polygalacturonase from *Rhizopus pusillus* was purified by two chromatographic steps (Sephadex G-200 and Sephacryl S-100) gave a band around 32 kDa in SDS-PAGE (Siddiqui et al., 2012). The degradation of the dye depends on different factors such as pH, temperature, concentrations of dye, incubation time, and inoculum size. Roy et al. (2018) illustrated the complete (100%) crystal violet degradation after optimizing the environmental parameters and *Enterobacter* sp. effectively decolorized the dye of textile effluents (Roy et al., 2018a). Similarly, *Pseudomonas aeruginosa* isolated from textile industry wastewater was exploited to decolorize methyl orange dye. *P. aeruginosa* decolorized 99% of methyl orange in 100 mg/L and showed *P. aeruginosa* could be effectively used to detoxicate industrial wastewater (Kishor et al., 2021). Our study illustrated that the highest decolorization rate was ~54% which is lower than other studies. This may be because this study was performed without optimization of environmental parameters.

5. Conclusions

Thus, the bacterium (*Streptomyces* sp.) isolated from forest soil revealed to have the potential for pectinase production and crystal violet decolorization. The bacterium has the optimum fermentation conditions at 35℃, pH 7 and 48 hours of incubation for maximum pectinases production. The partially purified pectinase showed activity at a wide range of temperature and pH, but the optimum was 70℃ and pH 5 and 9. The present study demonstrated pectinases produced are moderately thermostable, can resist low and high pH, and has possibilities in various industries where the temperature and pH might be a limiting factor. The approximate molecular weight of enzyme was observed at around 70 kDa and 25 kDa from SDS and zymogram analysis. Additionally, the bacterium was found to decolorize crystal violet dye of 2 mg/mL concentration up to 53.75% showing its application in textile industries' wastewater treatment. Further, a detailed study such as purification of enzyme, stability, different

applications of the produced pectinases, and the oxidizing enzyme from *Streptomyces* sp. able to decolorize dye are recommended. Moreover, the pectinase production in solid-state fermentation needs to be compared with submerged fermentation, and differences in pectinase characteristics comparison need to be explored too.

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4B Optimization of Multiple Enzymes Production by Fermentation using Lipid-producing *Bacillus* sp.

Abstract

The present study identified the pectinase-producing bacterium isolated from the contaminated broth as *Bacillus* sp. on 16S rDNA sequence analysis. The bacterium illustrated water-like droplets on the colony grown on the Sabouraud dextrose agar plate. It also exhibited multi-enzymes activities, such as pectinase, polygalacturonase, xylanase, and cellulase by using various agro-wastes as low-cost substrates. The orange peel was observed to be the best substrate among the agro-wastes used for maximum multi-enzymes (pectinase, polygalacturonase, xylanase, and cellulase). However, the bacterium demonstrated its capability to produce different enzymes according to the different substrates/agro-wastes used. The Plackett-Burman design was used to determine the essential influencing factors, while the Box-Behnken design response surface methodology was for optimizing cultural conditions. At their optimal conditions (40℃ incubation temperature, 24 hours of incubation period, 1% w/v orange peel, and 2% v/v inoculum volume), the bacterium exhibited the maximum pectinase (9.49±1.25 U/mL) and xylanase (16.27±0.52 U/mL) activities. Furthermore, the study explored the ability of the bacterium to produce bacterial lipids and observed about 25% bacterial lipid content on a dry weight basis. Therefore, the bacterium is a good candidate for producing important multi-enzymes and subsequent agro-waste degradation controlling the environment and facilitating waste management. Also, the bacterium can be a potential feedstock in producing renewable biofuel.

Keywords: *Bacillus* sp., multi-enzymes, agro-wastes, optimization, lipid content

1. Introduction

Agro-wastes are low-cost, renewable, and sustainable resources for industrially important enzyme production (Bharathiraja et al., 2017; Ravindran et al., 2018; Shrestha et al., 2020). Agricultural waste is usually produced during processing, pre-harvesting, post-harvesting, marketing procedures, and household activities (Kodagoda and Marapana 2017; Shrestha et al. 2021a). The loss of agricultural products during these processes may decrease the products and increase production costs. Additionally, the agro-wastes pollute the environment and create disposal problems (Sadh et al., 2018; Shrestha et al., 2021a). Population growth and waste generation are directly proportional and it is expected the waste generation per year will increase by 83% by 2050 (https://datatopics.worldbank.org/what-a-waste/trends_in_solid_waste_management.html). Dumping or landfill is the common method of waste disposal. However, it is not safe. The air near or surrounding the area of the dumping site may have air pollution due to the gases produced and the suspended particles in the air (Siddiqua et al., 2022). The leachate from landfill may contain toxic byproducts and may contaminate water bodies or soil which finally may affect living things including human and aquatic life (Parvin and Tareq, 2021; Siddiqua et al., 2022). At the same time, there is a continuous rise in demand for enzymes, and the pure carbon and nitrogen source used in enzyme production is expensive (Ravindran et al., 2018; Shrestha et al., 2022). Therefore, these agro-wastes can be used as low-cost substrates to produce polysaccharides hydrolyzing enzymes as they contain different polysaccharides such as cellulose, xylose, and pectin in different compositions (Sadh et al., 2018; Mellinas et al., 2020; Shrestha et al., 2020, 2022).

Besides, the multi-enzymes biocatalyst technology and production of enzyme cocktails from a single organism are gaining interest (Amadi et al., 2022). The most common and commercially

important enzymes are cellulase, pectinase, and xylanase, used for broad biotechnological applications. Pectinases and xylanases have been used in wastewater treatment, brewing technology, animal feed preparation, textile, pulp and paper industries, and food processing industries (Thite et al., 2020; Nawawi et al., 2022). Cellulolytic enzymes and other enzymes apply to cell wall disruption, juice extraction, and lipid extraction (Guo et al., 2017). In addition, the multi-enzymes help protects the environment by degrading various plant and agro-wastes (Ravindran et al., 2018; Thite et al., 2020; Amadi et al., 2022; Shrestha et al., 2022). Furthermore, enzymes production depends on the microorganisms exploited, and there are only a few studies for multi-enzymes productions from a single bacterium (Laothanachareon et al., 2022; Ozzeybek and Cekmecelioglu, 2022; Shrestha et al., 2022). Producing multi-enzymes by a single bacterium using different agro-wastes is cost-effective and time-efficient because using a single pure carbohydrate as substrate can only induce a single specific enzyme which is expensive and time-consuming (Wang et al., 2019; Thite et al., 2020; Nawawi et al., 2022; Shrestha et al., 2022).

Furthermore, the uninterrupted increase in the world's population is diminishing fossil fuel reserves, so there is a need to explore alternative energy sources (Yusuf, 2019). In this aspect, some oleaginous microorganisms are natural oil producers. They accumulate about 20% w/v of lipid on a dry weight basis and are the most promising feedstock for lipid and oleochemical production (Patel et al., 2020). Therefore, this study focuses on the isolation and identification of the bacterium that increased the pectinase activity significantly. Also, the current study exploits the bacterium for multi-enzymes production by utilizing different agro-wastes and optimization of the fermentation conditions. Furthermore, the bacterium was exploited to know its capability

of producing lipids due to the unique colony of the bacterium with water-like droplets on Sabouraud dextrose agar plate.

2. Materials and methods

2.1. Isolation and identification of bacteria

The bacterium was isolated from the contaminated broth. A loopful of the contaminated broth was streaked on Sabouraud dextrose agar (SDA) and nutrient agar (NA) plates and incubated at room temperature and 35°C, respectively, for 2-3 days. After the growth of colonies, a single colony was sub-cultured many times on SDA and NA plates to get the pure isolated colonies. Once the pure isolated colonies were observed on the agar plate, genomic DNA was extracted following the SDS-CTAB/NaCl method.

The 16S rDNA genes of the isolate were amplified by Taq DNA polymerase with universal primer sets: 16S rDNA forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-GGTTACCTTGTTACGACTT-3. The amplification systems for the 16S PCR reaction mixture contained 2 × *Taq* PCR master mix of 12 µL (10 x Taq DNA polymerase buffer, 10 mM dNTPs, 25 mM of $MgCl_2$, 1 U of *Taq* DNA polymerase), 3 µL of 10 µM forward and reverse primers, 2 µL of the genomic DNA template, and 4 µL of distilled water, making a total volume of 24 µL. Furthermore, the PCR reaction conditions used were denaturation at 94°C for 5 min and the cycle starting at 94°C for 30 sec, followed by annealing at 54°C for 30 sec, extending at 72°C for 1.5 min for 33 cycles, and finally extending at 72°C for 10 min. Then the PCR products were determined by 1% (w/v) agarose gel electrophoresis. The 16S rDNA target fragments from the gel were cut, DNA was extracted using a gel extraction minipreps kit (Bio Basic), and the extracted DNA was sent for DNA sequencing.

2.2. Gene sequencing and phylogenetic analysis

The 16S rDNA sequences of the isolate provided by the sequencing company were compared with the known sequences found in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLASTn). The isolate was identified based on the percentage similarity with the known species sequences in the database. All the sequences were collected and parallelized using the Clustalw module in BioEdit v. 7.0.9.0 (Hall, 1999) with default settings. Phylogenetic analysis was performed using a Neighbor-Joining (NJ) tree with 1000 bootstraps using MEGA 7 (Kumar et al., 2016).

2.3. Screening tests

Different screening tests for pectinase, cellulase, xylanase, and amylase were performed by culturing the bacterium on agar plates containing pectin, cellulose, xylan, and starch, respectively. After the growth of the bacterium, potassium iodide solution for pectinase, Congo red for cellulase and xylanase, and iodine solution for amylase screening test were flooded. The clear halo zone around the colonies indicated the presence of respective enzymes (Meddeb-Mouelhi et al., 2014; Takcı and Turkmen, 2016; Al Mousa et al., 2022).

2.4. Pectinase activity

The speck of pure isolated colony, which showed the clear pectinolytic zone on screening pectin agar plate, was subjected to prepare seed culture. Then, 1% v/v seed culture was inoculated into a flask with 50 ml of yeast extract pectin media (YEP) composed of 0.3% yeast extract, 1% pectin, 0.2% KH_2PO_4, and 0.2% K_2HPO_4 in distilled water. The inoculated flasks were incubated at 35°C for 4 days at 200 rpm. Samples from inoculated flasks were collected at regular intervals of 24 hours, and enzyme activities were assayed and compared. Pectinase activity was calculated by measuring the reducing sugar content released from the substrate following the 3,5-

dinitrosalicylic acid (DNS) method (Miller, 1959), as mentioned in a previous study (Shrestha et al., 2021b). In brief, 10 µL of crude enzyme extract was added to 20 µL of 1% citrus pectin solution as a substrate solution in the wells of a microplate, incubated in a 50℃ water bath for 10 min, cooled, and 60 µL of DNS reagent was added. Then the microplate was covered and heated in boiling water for 5 mins, followed by cooling down to room temperature. To the mixture, 200 µL of distilled water was added, and the absorbance was recorded at 540 nm to calculate the amount of reducing sugar released. Each enzymatic activity was expressed as the amount of enzyme that releases 1 µmol of galacturonic acid in 1 min under the mentioned conditions.

2.5. Effect of incubation period, temperature, pH, inoculum volume, and pectin concentration in pectinase production

The bacterium was cultured in YEP, pectinase production media, for different incubation periods (24 to 120 hours). At different incubation periods, the cultured broth was aseptically taken in a sterile Eppendorf tube and centrifuged. The cell-free supernatant was used for enzyme activity assay. The fermentation condition for the bacteria regarding incubation temperatures and pH was studied by culturing at different incubation temperatures (30°C, 35°C, 40°C, and 45°C) and pH (5, 6, 7, 8, 9, and 10). Similarly, the different inoculum volume (0.5%, 1%, 2%, 3%, and 4% v/v), and different pectin concentrations (0.5%, 1%, 1.5%, and 2% w/v) were added to the media, to study the enzyme activity.

2.6. Agro-waste preparation and multi-enzyme production

Different agro-wastes which are easily and locally available were selected. Orange peel, banana peel, pomegranate peel, and pumpkin pulp+seeds were from the waste of those fruits and vegetables bought from the market. Barley straw and maple leaf were commercially available. Canola straw was accumulated from a local farm, and brewer's spent grains from a local brewing

company (Sleeping Giant Brewing Co., Thunder Bay, Ontario, Canada). All those agro-wastes were dried, ground in a coffee grinder, and washed with hot water several times to remove contaminant and simple sugar. The presence of reducing sugar was determined by DNS method. Once the samples were free of reducing sugar, they were dried on a hot air oven at 50℃ for 48 hours (till constant weight). The dried agro-waste powders were kept in airtight containers for further use.

For multi-enzymes production using agro-wastes, the bacterium was cultured on the media containing 1% w/v agro-wastes as the carbon source instead of 1% w/v pectin in the YEP pectinase production media. Polysaccharidases such as pectinase, polygalacturonase (PGase), xylanase, and cellulase activities were determined every 24 hours. The citrus pectin, polygalacturonic acid, beechwood xylan, and carboxymethyl cellulose (CMC) were used as the respective substrates.

2.7. Optimization of cultural conditions for the maximum enzyme production

Orange peel was illustrated as the best substrate among the agro-wastes used in this study, so optimization of enzyme production was performed by using orange peel. Plackett-Burman design included 7 different factors at two levels; incubation temperature, pH, incubation period, $MgSO_4$, NaCl, $FeSO_4$, and $(NH_4)_2SO_4$ to screen the main influencing factor, including 15 experimental runs with three central points. The high level (+1) indicates the maximum concentration, and the low level (-1) indicates the minimum concentration of the variables (Table 1). The Box-Behnken design (BBD) response surface methodology was used to optimize the cultural conditions for maximum enzyme production using the most significant factors from the Plackett-Burman design. Here, enzyme activity was considered response variable, whereas incubation period (hour), orange peel concentration (% w/v), and inoculum volume (% v/v) were three independent

variables. The BBD used all the factors at three levels assigned as -1, 0, and +1 for the lowest, central, and highest value (Table 2). Both Plackett-Burman and BBD experimental designs were generated by using the Minitab 16 software.

2.8. Determination of lipid content

The bacterium was cultured in yeast extract peptone (YEP') culture media (consisting of 0.25, 0.25, 0.15, and 2 % w/v of yeast extract, peptone, $MgSO_4$, and dextrose, respectively) and Mineral salt medium (MSM) culture media (consisting of 0.9, 0.15, 0.02, 0.01, 0.00012, 0.002, and 0.005% w/v of Na_2HPO_4, KH_2PO_4, $MgSO_4$, $(NH_4)_2SO_4$, ferric citrate, $CaCl_2$, and $NaHCO_3$, respectively). Every 24 hours, lipid content was determined by Bligh and Dyer (1959) (Bligh and Dyer, 1959) with little modification. In brief, the biomass was first harvested by centrifugation at 10,000g for 10 min, and 1 gm of the pellet was sonicated and then homogenized with a 20 mL mixture containing chloroform, methanol, and water (1:2:0.8 ratio), followed by 20 mins of shaking in an orbital shaker at ambient room temperature. The homogenate was centrifuged at low speed (2000g) to separate into two phases. The upper phase was siphoned off, and the lower chloroform phase containing lipids was evaporated. Finally, the lipids extracted were quantified by weighing, and lipid content (%) was calculated as,

$$\text{Lipid content (\%)} = \frac{\text{Weight of lipid per mL}}{\text{Dry weight of bacteria per mL}} \text{ X } 100$$

Furthermore, lipid content was determined at two different media (YEP' and MSM), different incubation periods (24 to 96 hours), temperature (30 to 45°C) and pH values (6 to 9) to optimize the lipid content from the bacterium.

2.9. Statistical analysis

All the tests were performed in triplicates, and the data were expressed as mean with standard deviation (mean ± SD). One-way analysis of variance (ANOVA) was used to assess the statistical significance, followed by the Tukey-Kramer comparison. The data is regarded as statistically significant when the P-value of the experimental data is less than 0.05.

3. Results

3.1. Isolation and identification of the bacterium

After subculturing many times, pure and isolated colony was observed. The colony morphology of an isolated colony on the Sabouraud dextrose agar (SDA), Casein starch agar (CSA), and Nutrient agar (NA) plates were observed (Supplementary Fig. 1). The colony of the bacterium was pale in color, irregular in shape, and had many colorless waterlike droplets on SDA agar. The peripheral of the colony was soft to pick, whereas the inner part was hard. The unique colony characteristics with the occurrence of waterlike droplets suspected the bacterium of producing lipids.

Then the bacterium was screened for pectinase, cellulase, xylanase, lipase, and amylase enzymes. The clear zone around the colony was observed, indicating the bacterium was strongly positive for pectinase and amylase screening tests (Fig. 1a and b), whereas weakly positive for cellulase, xylanase and lipase.

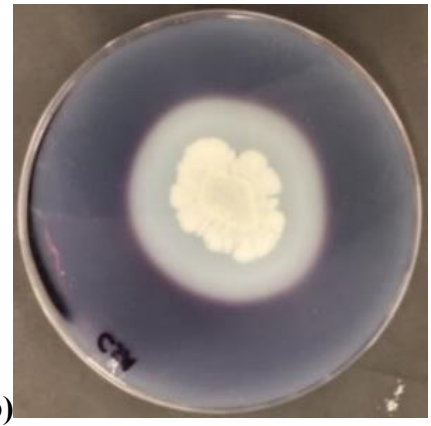
a)  b) 

Fig. 1 Screening test for a) pectinase, and b) amylase

The bacterium was identified as *Bacillus* sp. after isolation of the bacterium, DNA extraction, gel electrophoresis, 16S rDNA sequencing, and phylogenetic analysis. The 16S rDNA amplification and detection were performed as mentioned in the method, and the result of the 16S rDNA after gel electrophoresis depicted the fragments at around 1500 bp (Supplementary Fig. 2). The sequence similarity of the isolate was compared with the known species and similar sequences in the NCBI database. The phylogenetic tree of 16S rDNA sequences based on the Neighbor-Joining algorithm was constructed, as shown in Fig. 2. The constructed phylogenetic tree depicted the studied bacterium (*Bacillus* sp. C19) is closely related to the other *Bacillus* sp. (*Bacillus megaterium, Bacillus siamensis, Bacillus amyloliquefaciens,* and *Bacillus velezensis*) (Fig. 2).

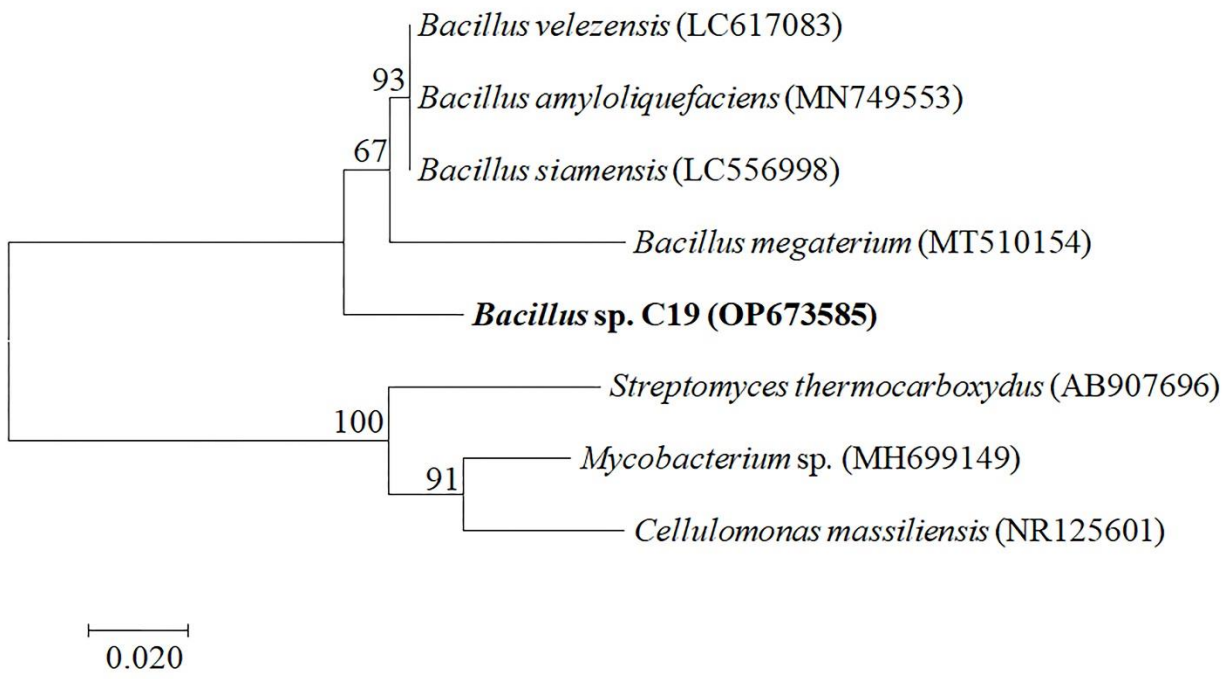


Fig. 2 Phylogenetic relationships of the bacterium constructed by Neighbor-Joining (NJ) tree based on 16S rDNA gene sequences. Numbers present on branches of the NJ tree are bootstrap support values.

The uncorrected genetic divergence between the bacterium and topotypic *Bacillus siamensis*, *Bacillus velezensis*, and *Bacillus amyloliquefaciens* was 3.5%, whereas *Mycobacterium* sp., *Cellulomonas massiliensis*, and *Streptomyces thermocboxydus* were 20, 21, and 21% respectively (shown in Supplementary Table 1).

3.2. Effect of incubation period, temperature, inoculum volume, pH, and pectin concentration in pectinase activity

The maximum pectinase activity was observed in incubation period of 24 hours, 40℃, 1% v/v inoculum volume, alkaline pH (7 and 9), and pectin 2% w/v (Fig. 3). The values observed were statistically significant ($P \leq 0.05$) when analyzed with one-way analysis of variance (ANOVA) followed by Tukey's comparisons.

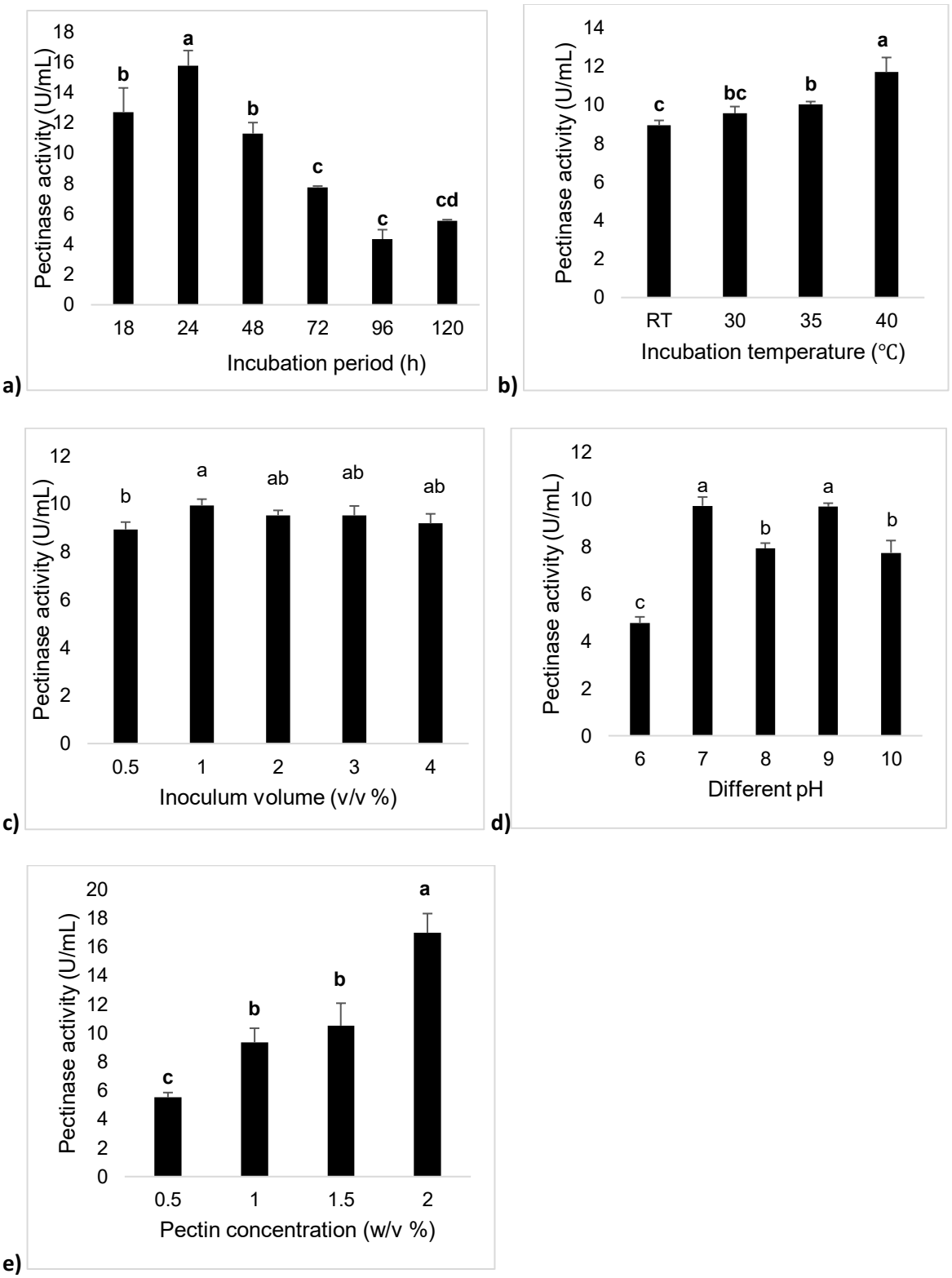


Fig. 3 Pectinase activity at different **a**) incubation period (h), **b**) temperature (℃), **c**) inoculum volume (% v/v), **d**) pH and **e**) pectin concentration (% w/v). The bar represented the mean

pectinase activity with the standard deviation error bar at different conditions. Lowercase alphabets indicate that the values are statistically significant at P≤0.05.

3.3. Potential of *Bacillus* sp. to produce multi-enzymes using agro-wastes

Fig. 4 depicts the pectinase activity by *Bacillus* sp. using eight agro-wastes. The bacterium could not show xylanase activity from barley straw and pumpkin pulp+seeds, while cellulase activity was only observed from orange peel.

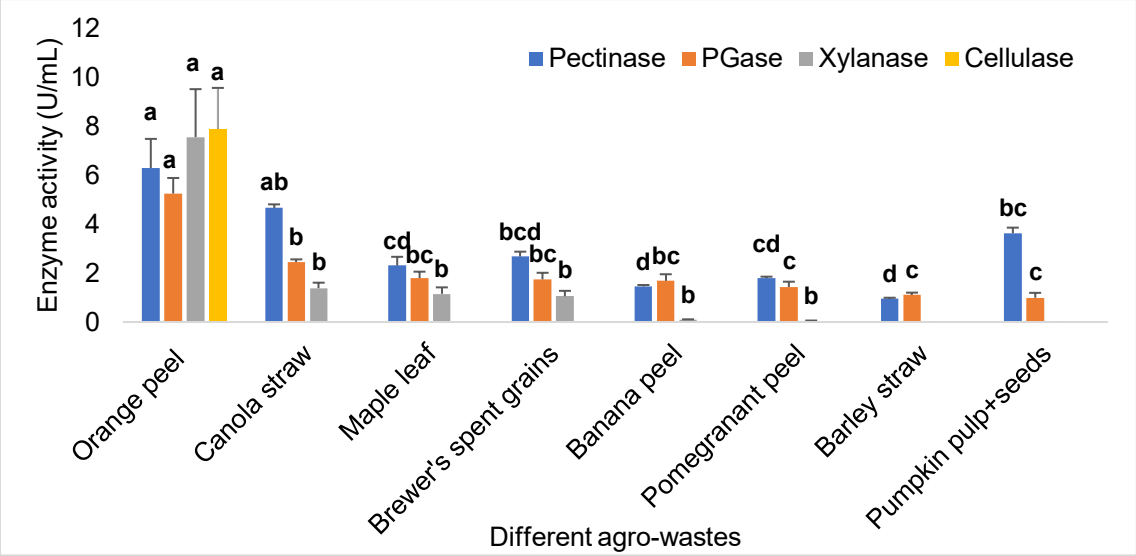


Fig. 4 Different enzyme activities exhibited by *Bacillus* sp. using different agro-wastes as carbon sources. The bar represented the mean enzyme activity with the standard deviation error bar. Lowercase alphabets indicate that the values are statistically significant at P≤0.05.

3.4. Optimization of the cultural conditions for maximum enzyme activities using orange peel

After the observations of different agro-wastes utilization to produce the enzymes, the orange peel was found to be the best substrate for multi-enzymes production. Therefore, the orange peel was selected to optimize the cultural conditions.

3.4.1. Plackett-Burman design

Plackett-Burman design was used for the initial statistical screening of seven cultural components (variables) and the enzyme activities (response), and the results are depicted in Table 1. The detailed analysis of the individual factors revealed that the incubation period as an influencing factor for pectinase, PGase, xylanase and cellulase activity with the lowest P-value. At the same time, all the six factors, like incubation period, pH, NaCl, Fe_2SO_4, $MgSO_4$, and $(NH_4)_2SO_4$ played important roles in cellulase activity. However, they were not significantly influential for other enzyme activities (Supplementary Table 2 and Supplementary Fig. 4).

Table 1 Experimental design and enzymes activities (U/mL) using Plackett-Burman factorial design

Run	Temperature (℃)	pH	Time (hour)	$MgSO_4$ (%w/v)	NaCl (%w/v)	Fe_2SO_4 (%w/v)	$(NH_4)_2SO_4$ (%w/v)	Pectinase (U/mL)	PGase (U/mL)	Xylanase (U/mL)	Cellulase (U/mL)
1	40	6	96	0.02	0.1	0.01	0.5	6.54	4.98	3.39	3.10
2	40	9	12	0.05	0.1	0.01	0.1	2.25	3.72	1.46	0.00
3	30	9	96	0.02	0.5	0.01	0.1	5.01	5.25	5.84	0.00
4	40	6	96	0.05	0.1	0.05	0.1	5.91	8.01	6.26	7.26
5	40	9	12	0.05	0.5	0.01	0.5	1.99	3.60	0.71	0.00
6	40	9	96	0.02	0.5	0.05	0.1	3.73	1.85	0.00	0.00
7	30	9	96	0.05	0.1	0.05	0.5	6.60	5.60	8.25	6.96
8	30	6	96	0.05	0.5	0.01	0.5	5.69	5.49	7.25	4.15
9	30	6	12	0.05	0.5	0.05	0.1	2.39	3.00	1.83	0.00
10	40	6	12	0.02	0.5	0.05	0.5	2.03	2.75	1.19	0.00
11	30	9	12	0.02	0.1	0.05	0.5	2.34	2.50	2.60	0.00
12	30	6	12	0.02	0.1	0.01	0.1	1.51	1.89	0.71	0.00
13	35	7.5	54	0.035	0.3	0.03	0.3	7.42	7.13	8.13	6.00
14	35	7.5	54	0.035	0.3	0.03	0.3	6.15	6.78	5.77	5.47
15	35	7.5	54	0.035	0.3	0.03	0.3	5.92	5.71	5.52	6.88

3.4.2. Box-Behnken design (BBD)

The optimization of cultural conditions was again designed using Box-Behnken design (BBD) and analyzed in Minitab 16 software. Enzyme activities exhibited by the bacterium using BBD illustrated in Table 2.

Table 2 Box-Behnken design for enzymes production by using orange peel as the substrate [-1, 0, and +1 are the codes for the variables]

Run	Time (h)	Orange peel (% w/v)	Inoculum volume (% v/v)	Pectinase (U/mL)		PGase (U/mL)	Xylanase (U/mL)		Cellulase (U/mL)
				Observed	Predicted	Observed	Observed	Predicted	Observed
1	12 (-1)	0.5 (-1)	2 (0)	1.71	2.60	1.25	0.96	1.27	0.00
2	36 (+1)	0.5 (-1)	2 (0)	7.41	8.33	5.65	8.59	8.41	0.00
3	12 (-1)	1.5 (+1)	2 (0)	4.58	3.66	7.98	1.03	1.21	13.68
4	36 (+1)	1.5 (+1)	2 (0)	6.53	5.64	6.12	7.09	6.77	17.22
5	12 (-1)	1 (0)	1 (-1)	2.34	2.21	0.16	2.67	2.52	0.00
6	36 (+1)	1 (0)	1 (-1)	5.52	5.37	5.87	8.68	9.02	8.8
7	12 (-1)	1 (0)	3 (+1)	1.01	1.16	1.48	0.00	0.00	0.00
8	36 (+1)	1 (0)	3 (+1)	5.57	5.70	7.27	5.72	5.87	13.69
9	24 (0)	0.5 (-1)	1 (-1)	5.24	4.47	8.24	6.57	6.41	7.04

10	24 (0)	1.5 (+1)	1 (-1)	4.49	5.54	4.62	8.23	8.20	17.34
11	24 (0)	0.5 (-1)	3 (+1)	7.04	5.99	7.43	6.03	6.05	7.51
12	24 (0)	1.5 (+1)	3 (+1)	2.54	3.30	0.11	2.38	2.54	0.00
13	24 (0)	1 (0)	2 (0)	10.62	9.49	13.83	16.79	16.27	19.54
14	24 (0)	1 (0)	2 (0)	8.15	9.49	10.66	15.75	16.27	17.90
15	24 (0)	1 (0)	2 (0)	9.69	9.49	11.25	16.27	16.27	18.72

The accuracy and statistical significance of each term in the model were evaluated using Analysis of variance (ANOVA). The BBD experimental results of the current study were fitted with a second-order polynomial equation using a multiple regression technique and expressed as:

Pectinase activity (U/mL) = $9.49 + (1.92 \bullet X1) + (-0.41 \bullet X2) + (-0.18 \bullet X3) + (-0.94 \bullet X1 \bullet X2) + (0.35 \bullet X1 \bullet X3) + (-0.94 \bullet X2 \bullet X3) + (-2.82 \bullet X1^2) + (-1.60 \bullet X2^2) + (-3.05 \bullet X3^2)$

PGase activity (U/mL) = $11.91 + (1.75 \bullet X1) + (-0.47 \bullet X2) + (-0.32 \bullet X3) + (-1.56 \bullet X1 \bullet X2) + (0.02 \bullet X1 \bullet X3) + (-0.92 \bullet X2 \bullet X3) + (-4.03 \bullet X1^2) + (-2.63 \bullet X2^2) + (-4.18 \bullet X3^2)$

Xylanase activity (U/mL) = $16.27 + (3.31 \bullet X1) + (-0.43 \bullet X2) + (-1.64 \bullet X3) + (-0.39 \bullet X1 \bullet X2) + (0.19 \bullet X1 \bullet X3) + (-1.33 \bullet X2 \bullet X3) + (-6.82 \bullet X1^2) + (-5.03 \bullet X2^2) + (-5.44 \bullet X3^2)$

Cellulase activity (U/mL) = $18.72 + (4.51 \bullet X1) + (4.33 \bullet X2) + (-1.83 \bullet X3) + (0.57 \bullet X1 \bullet X2) + (0.98 \bullet X1 \bullet X3) + (-5.36 \bullet X2 \bullet X3) + (-7.89 \bullet X1^2) + (-4.25 \bullet X2^2) + (-7.41 \bullet X3^2)$

X1 represents time, X2 orange peel concentration, and X3 inoculum volume.

The P-values for pectinase, PGase, xylanase and cellulase activity were 0.03, 0.35, 0.000, 0.35 and lack of fit 0.434, 0.128, 0.892, and 0.01, respectively. The R^2 values for pectinase, PGase,

xylanase and cellulase were observed as 0.91, 0.73, 0.99, and 0.72, respectively. The regression P-value and lack of fit observed from the analysis of variance revealed that the quadratic equations were relatively reliable for evaluating bacterium's pectinase and xylanase activities (Table 3).

Table 3 Analysis of Variance (ANOVA) for BBD quadratic model [Note: *Significant values at $p \leq 0.05$]

Response	Terms of model	Degree of freedom	Sum of square	Mean square	F-value	P-value
Pectinase	Constant	9	103.39	11.48	5.84	0.03*
PGase			171.79	19.09	1.47	0.35
Xylanase			444.04	49.34	350.86	0.00*
Cellulase			898.93	99.88	1.46	0.35
Pectinase	Incubation period (X1)	1	29.64	29.64	15.29	0.05*
PGase			24.62	24.62	1.90	0.23
Xylanase			87.61	49.34	623.01	0.00*
Cellulase			162.67	99.88	2.38	0.18
Pectinase	Orange peel % (X2)	1	1.33	1.33	0.68	0.45
PGase			1.76	1.76	0.14	0.73
Xylanase			1.46	1.46	10.36	0.02*
Cellulase			150.04	150.04	2.20	0.19
Pectinase	Inoculum volume (X3)	1	0.25	0.25	0.13	0.73
Pgase			0.85	0.85	0.07	0.81
Xylanase			21.39	21.39	152.10	0.00*
Cellulase			26.69	26.69	0.39	0.56
Pectinase	$X1^2$	1	23.15	29.43	14.97	0.01*
PGase			46.98	60.08	4.63	0.08
Xylanase			137.77	171.89	122.12	0.00*
Cellulase			186.27	23.24	3.37	0.37

Pectinase	$X2^2$	1	6.96	9.50	4.83	0.08
PGase			19.76	25.56	1.96	0.22
Xylanase			78.87	93.28	663.39	0.00*
Cellulase			50.35	66.75	0.98	0.13
Pectinase	$X3^2$	1	34.46	34.46	17.53	0.01*
PGase			64.64	64.64	4.98	0.08
Xylanase			109.15	109.15	776.25	0.00*
Cellulase			202.71	202.71	2.97	0.15
Pectinase	X1.X2	1	3.51	3.51	1.79	0.64
PGase			9.78	9.78	0.75	0.99
Xylanase			0.62	0.62	43.8	0.36
Cellulase			1.31	1.31	0.02	0.82
Pectinase	X2.X3	1	0.48	0.48	0.24	0.64
PGase			0.00	0.00	0.00	0.99
Xylanase			0.15	0.15	1.03	0.36
Cellulase			3.89	3.89	0.06	0.82
Pectinase	X1.X3	1	3.53	3.53	1.80	0.24
PGase			3.4	3.4	0.26	0.63
Xylanase			7.03	7.03	49.97	0.00*
Cellulase			114.99	114.99	1.68	0.25
Pectinase	Lack of Fit	3	6.73	2.24	1.45	0.43
PGase			59.24	19.75	6.96	0.13
Xylanase			0.16	0.05	0.20	0.89
Cellulase			340.18	113.39	168.51	0.01*
Pectinase	Pure error	2	3.10	1.55		
PGase			5.68	2.84		
Xylanase			0.54	0.27		
Cellulase			1.35	0.67		
Pectinase	R^2			0.91		
PGase				0.73		
Xylanase				0.99		

Cellulase	0.72

The Contour plot Fig. 5a and 5d showed the interaction of orange peel concentration and incubation period, 5b and 5e the interaction of inoculum volume and orange peel concentration, and 5c and 5f showed the interaction of inoculum volume and incubation period for pectinase and xylanase activities, respectively.

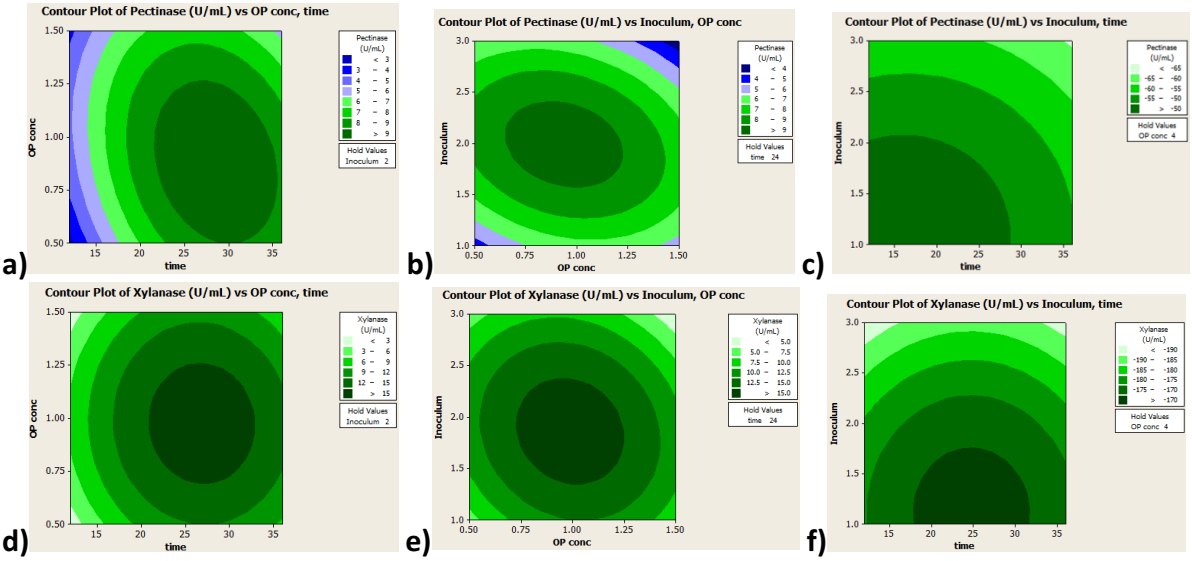


Fig. 5 Contour plot showing the interaction effects of orange peel concentration (OP conc), incubation period (hour), and inoculum volume (Inoculum) on pectinase (**a**, **b**, and **c**) and xylanase (**d**, **e**, and **f**) activities

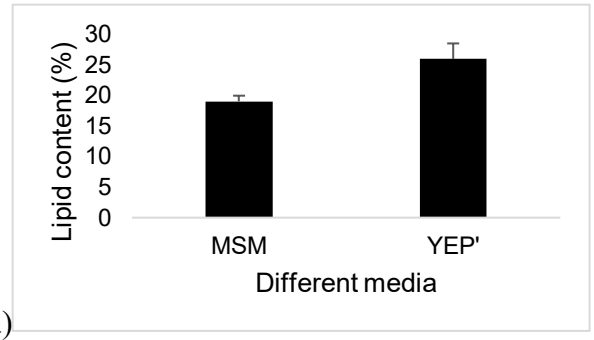
Fig. 5 depicted that both pectinase and xylanase activities increased with an increase in orange peel concentration and incubation period to certain values and decreased in activities after the optimal values. Fig. 5c and 5f illustrated a low inoculum volume and moderate incubation period gave the maximum pectinase and xylanase activities.

From the response optimizer, the optimal conditions observed for pectinase activity were incubation period of 29 hours, 1% w/v orange peel concentration, and 2% v/v inoculum volume. In contrast, the optimal conditions for xylanase activity were 27 hours, 1% w/v orange peel concentration, and 2% v/v inoculum volume (Supplementary Fig. 5).

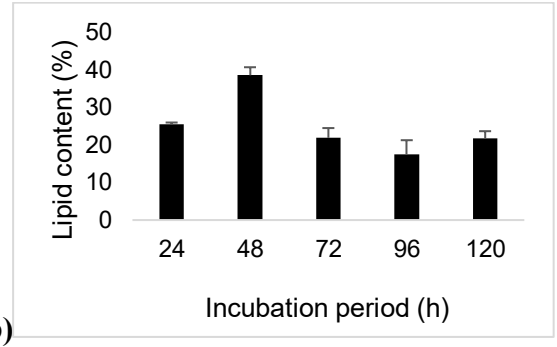
The model correctness was determined by performing the experiments under optimal conditions (from response optimizer), and the pectinase and xylanase activities observed were 8.62±1.56 U/mL and 15.02±1.23 U/mL, respectively. However, from BBD, 9.49 U/mL and 16.27 U/mL were revealed as the maximum pectinase and xylanase activities, respectively. At the same time, 9.90 U/mL and 16.76 U/mL were suggested for pectinase and xylanase by BBD optimizer. The enzyme activities thus observed were almost similar; thus, the models for pectinase and xylanase activities were valid and precise.

3.5. Lipid production by the bacterium at different conditions

The lipid content of the bacterium was around 25% when grown in yeast extract peptone (YEP') media. Further, the lipid content from the bacterium were studied using parameters like different incubation periods and pH values. The highest lipid contents by the bacterium were observed at incubation period of 48 hours, temperature 35°C and pH 8, as illustrated in figures (Fig. 6).



a)



b)

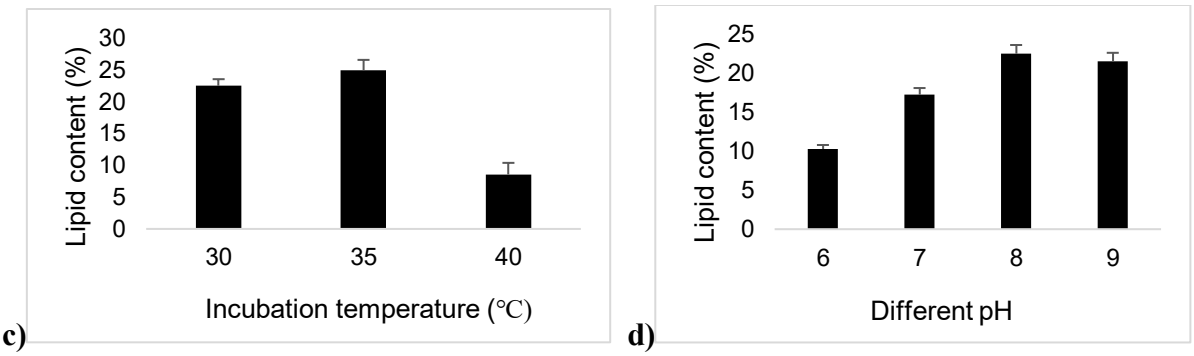


Fig. 6 Lipid content exhibited by *Bacillus* sp. at different conditions a) different media, b) different incubation period (h), c) temperature (°C) and d) pH

4. Discussions

The production of industrially important, novel, and robust enzymes for biotechnological applications is increasing. Therefore, there is a need to isolate and identify the bacteria from natural diversities and optimize the cultural conditions for maximum enzyme production. In our current study, we isolated the bacterium and screened for enzymes. The presence of a relative enzyme hydrolyzes the substrate around the colony, and a clear hydrolysis zone around the colony is observed. However, the substrate not hydrolyzed by relative enzymes forms a complex and does not show a clear zone (Soares et al., 1999; Meddeb-Mouelhi et al., 2014; Shrestha et al., 2021b; Al Mousa et al., 2022). On this basis, the bacterium was pectinase, amylase, cellulase, and xylanase producer.

The contaminant of the broth was identified as bacterium. Only 16S rDNA got amplified and showed the band at 1500 bp region. Further, the bacterium was identified as *Bacillus* sp. because the phylogenetic tree illustrated the bacterium closely related to *Bacillus* sp. (*Bacillus megaterium, Bacillus siamensis, Bacillus amyloliquefaciens,* and *Bacillus velezensis*). Furthermore, the uncorrected genetic divergence helped to confirm the bacterium as *Bacillus* sp.

Bacillus sp. are the dominant bacteria (Schallmey et al., 2004) and they have irregular colony (Logan and De Vos, 2015). Our study too revealed the irregular colony of *Bacillus* sp. Additionally, we observed clear water-like droplets on the colony which is unique character. Each bacterium has specific optimal conditions for the maximum enzyme activity. For instance, the study illustrated the optimal conditions by *Bacillus* sp. for the maximum xylanase activity were 50℃, 72 hours, pH 7.0 ± 0.2, and 1% xylan concentration (Lawrence et al., 2015). Another study recorded the maximum pectinase activity by *Aspergillus* sp. at 35℃, pH 6.5, 1% citrus pectin and after 192 hours of incubation period (Ketipally and Ram, 2018). Similarly, *Streptomyces thermocoprophilus* demonstrated maximum cellulase and xylanase activity at 40℃, pH 6.5, 120 hours, and 1% alkaline peroxide pretreated empty fruit bunch (Sinjaroonsak et al., 2020). And our study observed the highest pectinase activity by *Bacillus* sp. in 24 hours, 40℃, 1% v/v inoculum volume, alkaline pH (7 and 9), and pectin 2% w/v. The variation in the optimal conditions for enzyme activities may be due to the differences in the microorganisms used. The optimal conditions affect the growth and metabolic rate of the organisms exploited, and the maximum activity is illustrated at the most favorable conditions depending on the organisms.

The cheapest and readily available agro-industrial wastes can be used for industrially important enzyme production (Bharathiraja et al., 2017). The various agro-wastes, when used as the carbon source for enzyme production, the bacterium demonstrated the highest enzyme activities from orange peel. This may be because the bacterium must have found all the favorable conditions from orange peel only to demonstrate all the studied enzyme activities. Also, the orange peel may have a lower lignin concentration, making it accessible to pectin, hemicellulose and cellulose for the bacterium (Al Mousa et al., 2022). The bacterium exhibited xylanase and

pectinase activity from canola straw, maple leaf, brewer's spent grains, banana peel, and pomegranate peel. Further, the bacterium showed only pectinase activity from barley straw and pumpkin pulp+seeds. Thus, this study depicts the bacterium's ability to use orange peel more effectively and efficiently than other agro-wastes and demonstrated orange peel as the best substrate for *Bacillus* sp. Similarly, another study demonstrated that maximum pectinase and cellulase activity by *Mucor circinelloides* and *M. hiemalis* using tangerine peel due to the low concentration of lignin, tissue structure flexibility, and easy access to pectin, cellulose and hemicellulose (Al Mousa et al., 2022). Another study reported that banana peel with high pectin and starch content induced most of the target enzymes by *Aspergillus niger*. In contrast, cellulose-rich sugarcane bagasse induced beta-glucanase and xylanase. At the same time, starch-rich cassava pulp induced amylase and other enzymes but was comparatively lower than banana peel (Laothanachareon et al., 2022). Such variation might be due to the various chemical compositions and carbon sources of agro-wastes which induce the target enzyme production by a microorganism differently.

The different concentrations (0.5, 1, 1.5, and 2% w/v) of agro-wastes were studied for enzyme activities. The enzyme activities were increased with an increase in the concentration of almost all agro-wastes used (orange peel, barley straw, pumpkin pulp+seeds, banana peel, barley spent grains, canola straw, and maple leaf). In contrast, a high concentration of pomegranate peel decreased the pectinase and PGase activities (Supplementary Fig. 3A, B, and C). This result relates that the bacterium's ability to illustrate enzyme activity is directly proportional to agro-waste concentrations except for pomegranate peel. The higher the concentration of agro-wastes, the higher the chance of exposure to relative carbon source (pectin for pectinase, and hemicellulose for xylanase) present in that agro-waste.

Plackett-Burman design is a powerful and unique design to screen, identify and evaluate important variables that affect the response of the experimental tests. Thus, the present study used the Plackett-Burman design as an initial statistical screening of seven cultural components (variables) for enzyme activities (response). Our study revealed incubation period as an influencing factor for pectinase, PGase, xylanase and cellulase activity from this design, with the P-value less than 0.05. However, the cellulase activity was only affected by all six factors and was not significantly influential for other enzyme activities. Likewise, a study used the Plackett-Burman design and reported the pectinase activity by *Bacillus sonoresis* was strongly affected by the pectin mass fraction, pH, and $MgSO_4$ among eight different parameters studied (Mohandas et al., 2018).

Therefore, BBD was again used for optimizing the cultural condition considering incubation period, orange peel concentration and inoculum volume as the independent variables. Generally, the P-value less than 0.05 ensures the terms are statistically significant for each coefficient. The relationship between parameters was found to be significant for pectinase and xylanase but not for PGase and cellulase activities. Further, the statistical significance was checked by F-test to evaluate the coefficient of determination (R^2). The lesser R^2 value is not good, so the model was not too good for PGase and cellulase activity. However, the model was perfect for pectinase and xylanase activities.

Table 4 Enzyme activity and lipid content exhibited by various organisms

Organism	Enzyme activity	Lipid content (% dry weight basis)	Conditions	Reference

Bacillus sp.	Pectinase: 9.69±0.15 U/mL	-	40°C, 2% w/v pectin, 1% v/v inoculum volume, pH 9	This study
Aspergillus oryzae	Pectinase: 1.6 to 2.07 IU/mL		35°C, 1% w/v citrus pectin, pH 6	(Ketipally and Ram, 2018)
Bacillus sp.	Xylanase: 7.30 to 7.85 U/mL	-	50°C, pH 7, 1% w/v xylan	(Lawrence et al., 2015)
Mucor circinelloides	Pectinase: 38.02 U/mL	-	30°C, pH 7, 9 days, 3mL of inoculum volume, 5% w/v tangerine peel	(Al Mousa et al., 2022)
Mucor circinelloides	Cellulase: 37.20 U/mL	-	30°C, pH 7, 5 days, 3mL of inoculum volume, 5% w/v tangerine peel	(Al Mousa et al., 2022)
M. hiemalis	Pectinase: 39.72 U/mL	-	30°C, pH 5, 7 days, 3mL of inoculum volume, 5% w/v tangerine peel	(Al Mousa et al., 2022)
M. hiemalis	Cellulase: 33.82 U/mL	-	30°C, pH 7, 9 days, 3mL of inoculum volume, 5% w/v tangerine peel	(Al Mousa et al., 2022)
Bacillus sp.	Pectinase: 6.28±1.19 U/mL Xylanase: 7.54±1.96 U/mL	-	40°C, 1% w/v orange peel, 1% v/v inoculum volume	This study
Bacillus sp.	Pectinase: 9.49±1.24 U/mL Xylanase: 16.27±0.72 U/mL	-	BBD (24 h, 1% w/v orange peel, 2% v/v inoculum volume, 40°C)	This study

Bacillus sp.	-	22.51±1.12	48 h, 35°C, pH 8, and YEP' media	This study
Fusarium oxysporum	-	25.5 to 52.9	Containing different sugars (glucose, fructose, sucrose) alone and mixture of them	(Matsakas et al., 2017)
Fusarium oxysporum	-	17.7 to 22.0	Containing different concentrations of sweet sorghum	(Matsakas et al., 2017)
B. cereus	-	5 to 19	Containing palm oil mill wastewater	(Karim et al., 2019)
Cryptococcus sp.	-	30	When cultured in yeast malt agar	(Chang et al., 2015)

Due to the depletion of fossil fuel reserves, different alternate sources are being explored (Yusuf, 2019). The microorganisms have potential as an alternative for lipid production as they can accumulate the oil/lipid in them (Patel et al., 2020). The present study explored the capacity of bacterium to produce lipids because it depicted the water like droplets on the SDA media. The study illustrated that the lipid content was higher in YEP' media, possibly due to the higher cell growth, and the media favored the cell growth. In contrast, MSM inhibited the cell growth resulting in a decrease in lipid content. Our study illustrated that the lipid content continuously increased until 48 hours of incubation period and decreased onward, which may be related to cell growth. Similarly, the incubation temperature of 35°C and pH 8 favored the cell growth and resulted in higher lipid content at the respective temperature and pH. However, the bacterial lipid accumulation, lipid composition and even the cell membrane compositions of the same species vary with the environmental conditions of exposure (Sohlenkamp and Geiger, 2015). A study demonstrated that the lipid production was in the range of 25.5 to 52.9% of cell dry weight by *Fusarium oxysporum* using the synthetic media containing different sugars (glucose, fructose, and sucrose) alone and mixture of them (Matsakas et al., 2017). Whereas, *Bacillus cereus* accumulated lipid 5 to 19% on a dry weight basis using palm oil mill wastewater (Karim et al., 2019). Another study reported that *Lipomyces starkey* could potentially be used as a lipid source and accumulate lipid (12 to 29.5%) when cultured in oil mill wastewater-containing media (Yousuf et al., 2010). In addition, a study illustrated the microbial lipid from the yeast *Cryptococcus* sp. using corncob hydrolysate as a raw material (Chang et al., 2015). Therefore, microorganisms can be a potential source of lipid production by bioremediating the wastes. Further, studies relating to the isolation and identification of high lipid-containing

microorganisms, the optimization of the culturing condition, compositional analysis, and more are recommended for maximum microbial lipid production.

5. Conclusion

The pectinase-producing bacterium isolated from the contaminated broth was identified as *Bacillus* sp. from 16S rDNA sequence analysis. The bacterium produced different polysaccharides degrading enzymes, such as pectinase, polygalacturonase, xylanase, and cellulase. However, the different enzyme activities vary with agro-wastes used as low-cost substrates. The response surface methodology illustrated *Bacillus* sp. to exhibit maximum pectinase at 40℃, 29 hours of incubation period, 1% w/v orange peel concentration, and 2% v/v inoculum volume. Similarly, the optimal conditions for xylanase activity were 40℃, 27 hours, 1% w/v orange peel concentration, and 2% v/v inoculum volume. Furthermore, the bacterium has the potential to produce bacterial lipids. Therefore, the bacterium is a good candidate for producing biotechnologically important multi-enzymes and agro-waste degradation. Since the bacterium illustrates lipid content, it can be a potential feedstock in producing renewable biofuels and environmental resilience.

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Chapter 5

Valorization of Agro-wastes

A part of this chapter, Chapter 5A and 5B are published in '**Current Microbiology'**, and **'Bioresource Technology Report'**, respectively. Chapter 5C and 5E are submitted to **'International Journal of Environmental Science and Technology'**, and '**Waste Management Bulletin',** respectively. Whereas Chapter 5D is in final stage to submit in Journal.

5A Biomass-degrading Enzymes Production and Biomass degradation by a Novel *Streptomyces thermocarboxydus*

Abstract

Modern society has a great challenge to decrease waste and minimize the adverse effects of wastes on the economy, environment, and individual health. Thus, this study focuses on the use of eight agro-wastes (banana peel, barley straw, canola straw, pomegranate peel, orange peel, pumpkin peel+seeds, maple leaf, and brewer's spent grains) by a novel bacterium (*Streptomyces thermocarboxydus*) for enzymes production. Further, the study explored the subsequent degradation of those wastes by exploiting the bacterium. This bacterium was isolated from forest soil and identified as *Streptomyces thermocarboxydus* by 16S rDNA sequence analysis. The biodegrading capability of *S. thermocarboxydus* was determined by observing the clear zone around the colony cultured on the agar plate containing the different biomasses as sole carbon sources and calculating the substrate degradation ratios. Furthermore, scanning electron microscopy images of eight agro-wastes before and after bacterial treatment and weight loss of agro-wastes revealed the bacterium degraded the biomasses. The different trends of enzyme activities were observed for various wastes, and the maximum activity depended on the type of agro-wastes. Overall, *S. thermocarboxydus* was found to be a potential candidate for pectinase and xylanase production, and degrading agro-wastes. The enzyme production varies with the concentration of the biomasses.

Keywords: *Streptomyces thermocarboxydus,* biodegradation, agro-wastes/biomasses, enzyme activity

1. Introduction

The population in the world is growing at a rapid rate and it is increased by 0.83% from 2021 (Https://www.un.org/development/desa/pd/, 2022). This population growth rate is proportionate to the generation of waste. For example, the population of Canada is 37.742 million and generates approximately 35.5 million tons of garbage, including recyclable material (~30%) a year, which is about 2.7 kg of garbage per person on a daily basis (Yunis and Aliakbari, 2021). These enormous amounts of wastes cause a significant problem in terms of their disposal and impact on air, water, and soil contamination which may cause other health and environmental issues (Mihajlovski et al., 2020). Thus, they need to be appropriately managed or disposed of (Panda et al., 2016). Organic wastes, particularly horticultural and agricultural residues containing cellulose, pectin, lignin, xylan, and starch-like components, can be used as renewable resources (Ravindran et al., 2018; Shrestha et al., 2020, 2021c). These materials have attracted considerable attention as an alternative feedstock and energy source since they are abundantly available. Several microorganisms can use these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches. In addition, different valuable products, including bioactive compounds, organic acids, ascorbic acid, methanol, etc., can be produced from the organic wastes and, finally, the solution for the disposal of wastes (Mussatto et al., 2012; Singh et al., 2019b; Shrestha et al., 2020; Ning et al., 2021a).

The demand for microbial enzymes and their applications in various industries are continuously increasing (Oumer, 2017; Shrestha et al., 2021b). However, the production is limited because the production cost accounts for 30–40%. Therefore, exploring low-cost substrates and other alternatives is necessary (Bharathiraja et al., 2017). Currently, the utilization of these organic wastes by microorganisms to synthesize valuable products offers a good solution in terms of cost

reduction (Bharathiraja et al., 2017; Ravindran et al., 2018). On the one hand, these agro-wastes can be utilized as a carbon source for microbial growth and enzyme production hence providing a solution for the disposal of these wastes. And on the other hand, it helps in encountering pollution, greenhouse effect, and reduce production cost (Mussatto et al., 2012; Shrestha et al., 2020, 2021c). The present study describes the utilization of locally available agro-wastes (barley straw, orange peel, maple leaf, pomegranate peel, banana peels, canola straw, and pumpkin pulp+seeds) by a novel bacterium isolated from forest soil to produce different important hydrolyzing enzymes such as pectinase, xylanase, and cellulase. The bacterium degrades the agro-wastes producing simple sugars as byproducts. Since the simple sugars produced after fermentation by the bacterium can be used as the source for producing other value-added products, this novel bacterium can be a potential candidate for waste management and various industrial applications.

2. Materials and methods

2.1. Biomass collection

The varieties of agro wastes such as barley straw, orange peel, maple leaf, pomegranate peel, banana peels, canola straw, and pumpkin pulp and seeds were locally collected in Thunder Bay, Ontario, Canada. The brewer's spent grains were obtained from the local brewing industry (Sleeping Giant Brewing Co.). Simple treatments like cutting, grinding, and washing of the biomass samples were performed. Except for the brewer's spent grains, all agro-wastes were cut manually into small pieces and dried in a hot air oven at 50°C for 2 days till they were completely dried. Dried samples were powdered by a coffee grinder and were washed with warm water several times till the reducing sugar was detected by dinitrosalicylic acid method (Miller,

1959). The washed biomass samples were dried, as mentioned before. Once dried, all the samples were stored at room temperature in an airtight container.

2.2. Bacterium isolation and identification

The bacterium was isolated in a nutrient agar (NA) plate after serial dilution of forest soil sample and spread plate method. The isolate was sub-cultured several times till the pure isolated colonies were observed, and the pure culture of the isolate was stored at 4°C for further studies. DNA was extracted by the freeze-thaw cycles method as described by Chen et al. (Chen et al., 2020). PCR was performed using Taq DNA polymerase with a universal eubacterial primer set, (Forward Primer) 27F-5'-AGAGTTTGATCCTGGCTCAG-3' and (Reverse Primer) 1492R-5'-GGTTACCTTGTTACGACTT-3'. Then 1% (w/v) agarose gel electrophoresis was performed to determine the PCR products. The band of target fragments from the gel was excised, and a gel extraction minipreps kit (Bio Basic Inc.) was used to extract 16S rDNA from the gel. Then the extracted 16S rDNA was sent for DNA sequencing.

2.3. Phylogenetic analysis

After getting the 16S rDNA sequence result, the sequence was compared with the possible sequences found in the database of the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) (Https://blast.ncbi.nlm.nih.gov/Blast.cgi?, 2022) to identify possible genus. The phylogenetic tree was constructed using a Neighbor-Joining (NJ) tree. Also, uncorrected genetic divergence between the bacteria was calculated using MEGA 7 to distinguish the possible species (Kumar et al., 2016).

2.4. Screening biomass degradation abilities of *S. thermocarboxydus*

The different agro-wastes, including barley straw, banana peel, brewer's spent grains, orange peel, pomegranate peel, pumpkin pulp+seeds, canola straw, and maple leaf, were used as carbon

sources. The biomass degradation abilities of the bacterium were performed by flooding iodine solution over the colony grown on modified minimal salt (MMS) agar plate. MMS media containing 0.1% $NaNO_3$, 0.1% KCl, 0.1% K_2HPO_4, 0.05% yeast extract, 0.05% $MgSO_4$, and 0.3% peptone was supplemented with 0.5% different biomasses or pectin or xylan or CMC, and incubated at 37 °C for 48 hours (Guo et al., 2017). Then the iodine solution was flooded over the colony and observed the clear area around. The biomass degrading ability of the bacteria is based on the size of the clear or halo area produced on the plate by the bacterial strain after staining with the iodine solution. The larger the size of the clear zone, the higher the degrading capacity (Kasana et al., 2008; Wu et al., 2021). Iodine solution produces brownish color, but a clear area around the colony after iodine addition indicated that the organism was able to hydrolyze agro-wastes (Kasana et al., 2008).

2.5. Enzymatic activities by *S. thermocarboxydus* using different biomasses

The overnight cultured bacterium was inoculated in the basal broth medium (containing 0.2% KNO_3, 0.2% K_2HPO_4, 0.2% KH_2PO_4, and 0.3% yeast extract) supplemented with 0.1% (w/v) biomass. Then was incubated in a rotatory shaker incubator at 35℃, 200 rpm for 96 hours. Every 24 hours, the cultured broth was taken out aseptically, centrifuged, and the cell-free supernatant (enzyme extract) was used for enzyme activity analysis. The enzyme activities (pectinase, cellulase, and xylanase) were determined by using the standard graph prepared from different concentrations of substrates (citrus pectin, glucose, and beechwood xylan) as described in a previous study (Shrestha et al., 2021b) and measuring the reducing sugar content released from the different substrates used following the dinitrosalicylic acid (DNS) method (Miller, 1959). The substrates used for pectinase, cellulase, and xylanase activities were citrus pectin, CMC, and corn cob xylan, respectively. In brief, 10 µL of crude enzyme extract was added to 20 µL of

substrate solution in the wells of a microplate, incubated in a 50℃ water bath for 10 min, cooled, and 60 µL of DNS reagent was added. Then the microplate was covered and heated in boiling water for 5 mins. After it was brought down to room temperature, 200 µL of distilled water was added, and the absorbance at 540 nm was recorded to calculate the amount of reducing sugar released. Each enzymatic activity (pectinase, cellulase, or xylanase) was expressed as the amount of enzyme that releases 1 µmol of simple sugar unit (galacturonic acid, glucose, or xylose, respectively) in 1 min under assay conditions. Similarly, biomasses of different concentrations were added to the basal media to study the ability of *S. thermocarboxydus* to produce different hydrolyzing enzymes (pectinase, xylanase, and cellulase).

2.6. Determination of substrate degradation ratio

The washed and dried biomasses were used as carbon sources. Both biomasses and broth medium were autoclaved at 121°C for 30 mins separately, and they were mixed aseptically. An overnight culture of *S. thermocarboxydus* (1% v/v) was inoculated in a flask containing medium. Similarly, another set was taken but without bacterial inoculation as a control, and all were incubated at 37°C, 200 rpm, and for 96 hours. After every 24 hours of incubation, the cultures were centrifuged at 5000 rpm for 5 mins, and the supernatant was accumulated to determine enzyme (pectinase, cellulase, and xylanase) activity. The residue was treated with an acetic-nitric acid reagent (1M) and washed with distilled water until a neutral pH was obtained. Then the unused substrates were separated by centrifuging at 10000 rpm for 5 mins, air-dried at 50°C until constant weight, and used to determine the weight loss. The percent (%) substrate degradation was defined as the ratio of the final weight of substrates to the initial weight of substrates.

2.7. Scanning electron microscopy

The microstructures of all the different agro-wastes (bacterial treated and untreated) were investigated using a scanning electron microscope (SEM) and compared to verify the degradation of the agro-wastes. For SEM, both dried samples (bacterial treated and not treated) were freeze-dried and mounted on a stub using double-sided carbon tape. Then the sample was coated with gold and examined in Hitachi SU 70 SEM operating at 5 kV accelerating voltage.

2.8. Statistical analysis

In our study, all the experiments were performed in triplicates, data were recorded in excel, and the statistical analysis of the data was carried out by one-way analysis of variance followed by Tukey's comparison. The experimental data were considered statistically significant if the P-value equals or less than 0.05. All the values are presented as the mean of triplicate value ± standard deviation.

3. Results

The forest soil isolate was identified as *Streptomyces thermocarboxydus* on16S rDNA sequence analysis (Fig. 1).

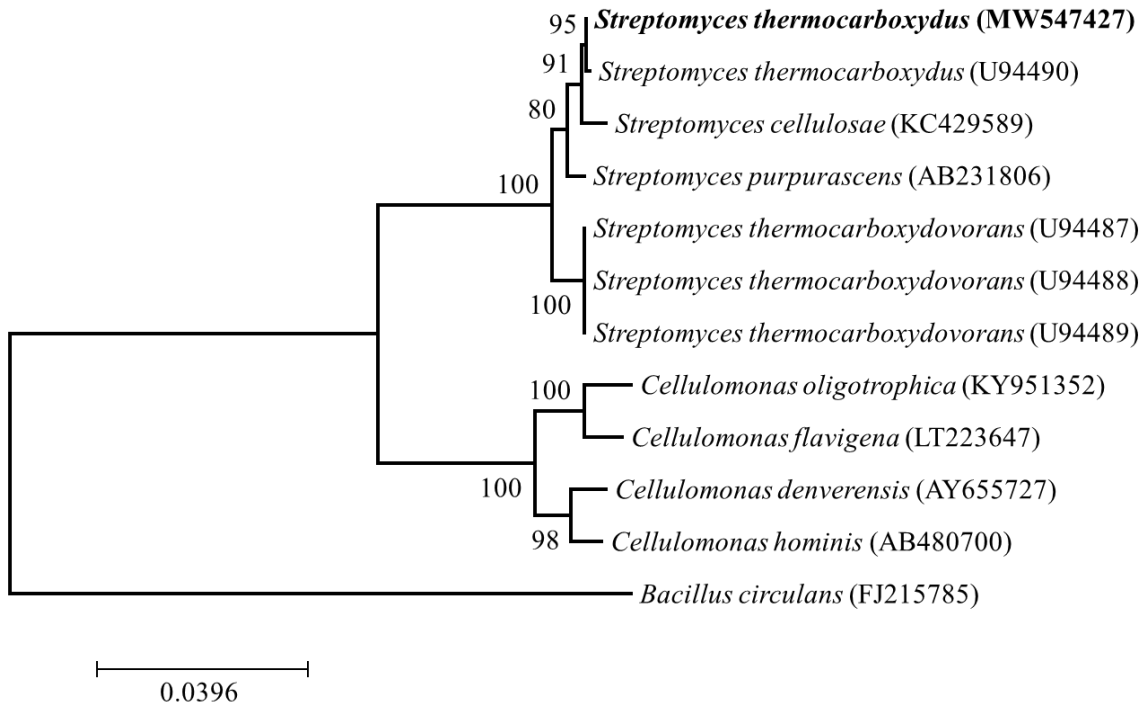


Fig. 1 Neighbor-joining (NJ) tree showing the phylogenetic relationships constructed based on the 16S rDNA sequence and those of other strains obtained from GenBank database. Numbers present on branches of the NJ tree are bootstrap support values.

The uncorrected genetic divergence between the bacterium and topotype *S. thermocarboxydus* (Kim et al., 1998) was 0.1% (Table 1).

Table 1 Uncorrected genetic distance between the species

S. N	Name of species	1	2	3	4	5	6	7	8	9	10	11	12
1	*S. thermocarboxydus* (MW547427)	0.000											
2	*S. thermocarboxydovorans* (U94487)	0.014											
3	*S. thermocarboxydovorans* (U94488)	0.014	0.000										

4	*S. thermocarboxydovorans* (U94489)	0.014	0.000	0.000									
5	*S. thermocarboxydus* (U94490)	0.001	0.015	0.015	0.015								
6	*C. oligotrophica* (KY951352)	0.087	0.086	0.086	0.086	0.088							
7	*C. flavigena* (LT223647)	0.082	0.085	0.085	0.085	0.083	0.016						
8	*C. denverensis* (AY655727)	0.081	0.083	0.083	0.083	0.082	0.032	0.031					
9	*C. hominis* (AB480700)	0.083	0.079	0.079	0.079	0.084	0.029	0.029	0.012				
10	*S. purpurascens* (AB231806)	0.006	0.011	0.011	0.011	0.007	0.089	0.084	0.083	0.085			
11	*S. cellulosae* (KC429589)	0.005	0.015	0.015	0.015	0.006	0.090	0.086	0.084	0.086	0.011		
12	*B. circulans* (FJ215785)	0.225	0.224	0.224	0.224	0.225	0.234	0.237	0.226	0.224	0.226	0.227	0.000

3.1. Screening for biomass degradation abilities by *S. thermocarboxydus*

The bacterium (*S. thermocarboxydus*) illustrated the ability to degrade different agro-wastes differently, as shown in Fig. 2a. Biomass containing agar plate gave a clear hydrolyzed zone after flooding the iodine solution over the bacterial colony after 24 hours. Iodine solution produces brownish color when combined with polysaccharides, but a clear area around the colony on iodine addition indicated that the organism was able to hydrolyze agro-wastes into simple sugars (Kasana et al., 2008).

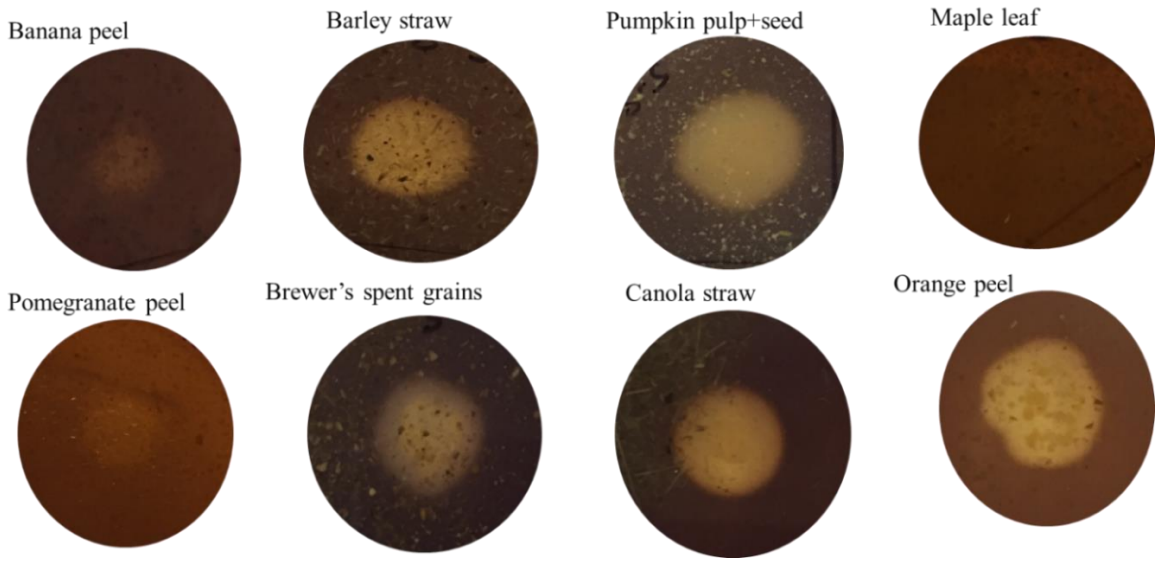


Fig. 2a Screening biomass degradation abilities of *S. thermocarboxydus* (flooding iodine solution over the colony after 24 hours of incubation)

Then on screening tests for pectinase, cellulase, and xylanase, the bacterium showed a clear zone around the colony on three agar plates indicating the bacterium produced all the tested hydrolyzing enzymes (pectinase, cellulase and xylanase) as shown in Fig. 2b.

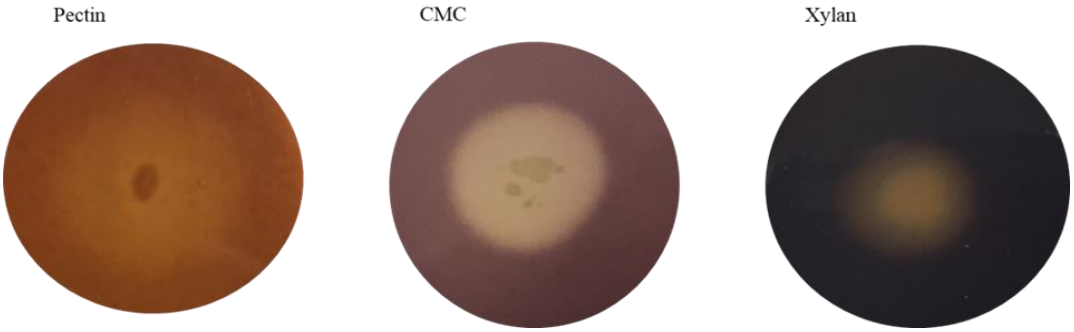


Fig. 2b Screening for pectinase, cellulase, and xylanase activity of *S. thermocarboxydus* (after flooding potassium iodine solution, Congo red, and iodine solution, respectively over 24 hours incubated colony)

3.2. Enzyme activity produced by *S. thermocarboxydus* using different agro-wastes/biomasses

3.2.1. Pectinase activity produced by *S. thermocarboxydus* using different biomasses

The bacterium significantly showed the highest pectinase activity when pomegranate peel was used as the carbon source. In contrast, the bacterium illustrated the least pectinase activity, almost negligible when no carbon source was added to the basal medium (shown in Fig. 3a).

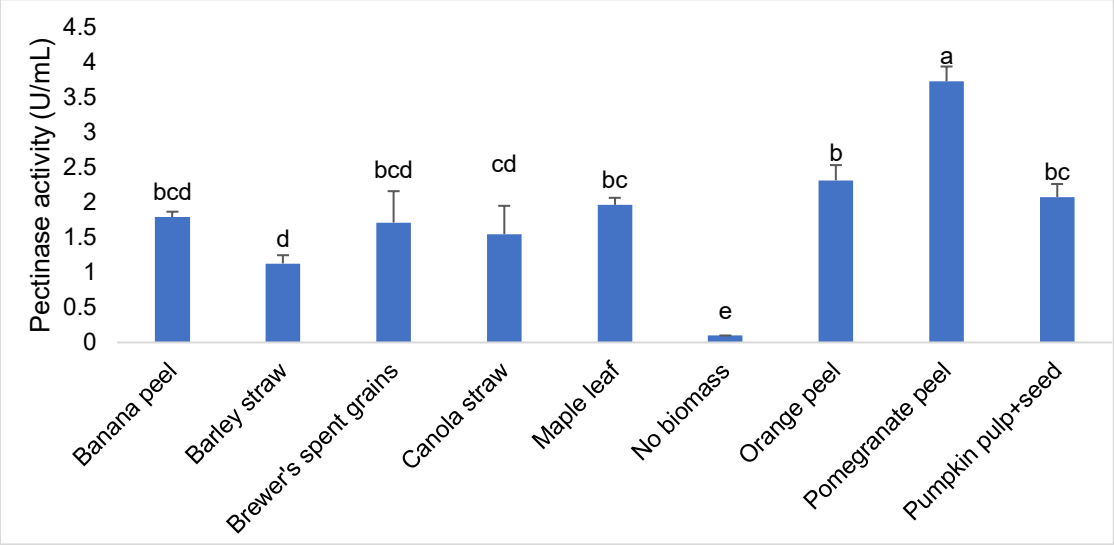


Fig. 3a Pectinase activity by *S. thermocarboxydus* using different agro-wastes/biomasses (Different lowercase letters above the bars represent the data is statistically significant)

In addition, the presence of pomegranate peel gradually increased pectinase activity until 72 hours, then it decreased. A similar trend was observed for pumpkin pulp+seed, but the pectinase activity did not decrease sharply; instead, the increase in pectinase activity was observed up to 120 hours. Banana peel exhibited the higher pectinase activity at 48 hours of incubation and after continuous decrease to 120 hours. Brewer's spent grains showed almost the same pectinase

activity from 24 to 120 hours. Canola straw and barley straw had a similar trend as brewer's spent grains but with less pectinase activity (Supplementary Fig. 1).

3.2.2. Cellulase activity produced by *S. thermocarboxydus* using different biomasses

The cellulase activity was maximum when pomegranate peel was used by *S. thermocarboxydus,* followed by orange peel, and the activities were statistically significant. Except for pomegranate and orange peel, the other agro-wastes did not exhibit cellulase activity (Fig. 3b).

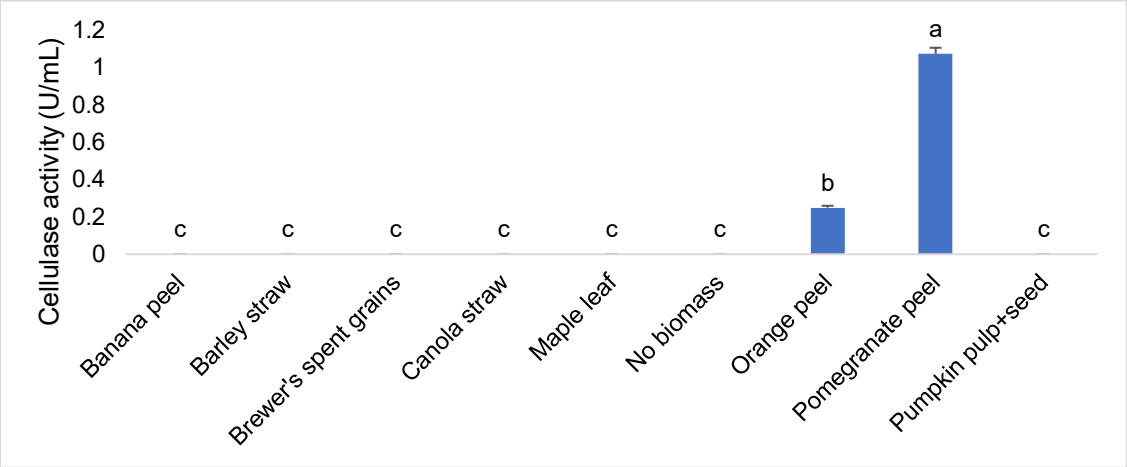


Fig. 3b Cellulase activity by *S. thermocarboxydus* using different agro-wastes (Different lowercase letters above the bars represent the data is statistically significant)

Cellulase activity was observed to be maximum (5.53 U/mL) at 72 hours for pomegranate peel and decreased sharply after 72 hours. While the orange peel only exhibited minimum cellulase activity (1.24 U/mL) in 96 hours (Supplementary Fig. 2).

3.2.3. Xylanase activity produced by *S. thermocarboxydus* using different biomasses

The xylanase activity was observed maximum when barley straw (1%) was used by the bacterium, whereas the least activity was observed when orange peel (0.5%) was used as a carbon source (Fig. 3c).

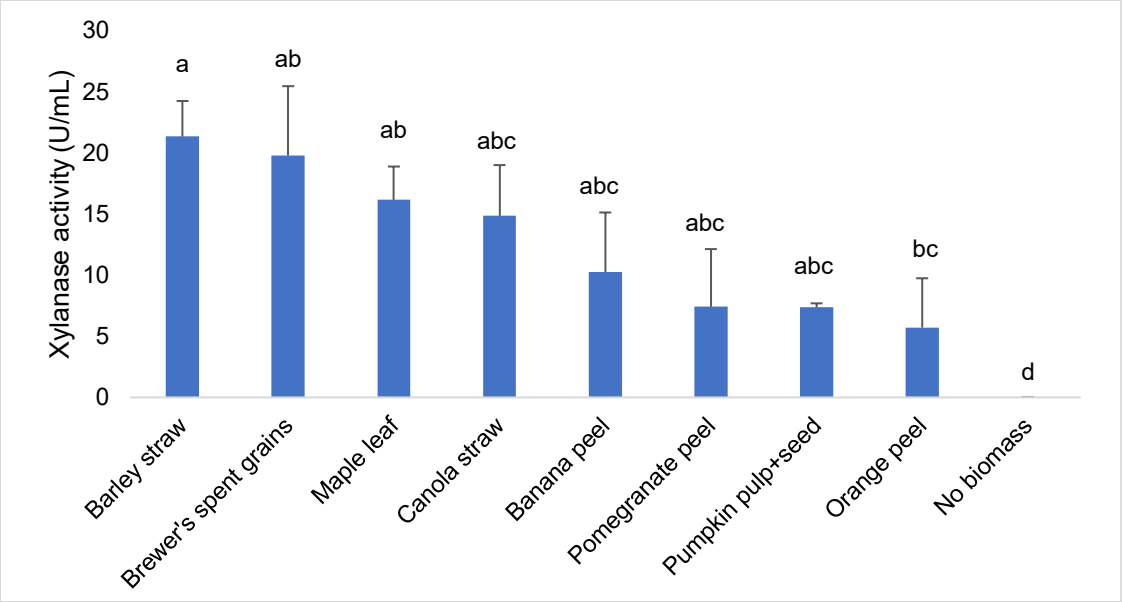


Fig. 3c Xylanase activity by *S. thermocarboxydus* using different agro-wastes (Different lowercase letters above the bars represent the data is statistically significant)

Fig. 3c also depicts that no xylanase was produced by *S. thermocarboxydus* when no carbon source was supplemented in the basal media. The xylanase activity for barley straw, brewer's spent grains, canola straw, orange peel, pumpkin pulp+seeds continuously increased till 96 hours and then decreased. Banana peel exhibited a continuous increase of xylanase activity up to 120 hours. Overall, maple leaf showed a rapid increase in xylanase activity from 72 hours onwards and exhibited the highest xylanase activity (42.06 U/mL) at 120 hours among different agro-wastes studied (Supplementary Fig. 3).

3.3. Enzymatic activities by *S. thermocarboxydus* at different concentrations of agro-wastes added

S. thermocarboxydus demonstrated the ability to produce different hydrolyzing enzymes such as pectinase, cellulase, and xylanase. However, all biomasses used did not induce these three hydrolyzing enzymes at 1% w/v of biomasses in the basal media.

3.3.1. Pectinase activity produced by *S. thermocarboxydus* using different concentrations of biomasses

The different agro-wastes at different concentrations (0.5%, 1%, 1.5%, and 2%) were used to study the enzyme activity produced by *S. thermocarboxydus.* The highest pectinase activity (2.95 U/mL) was observed at 1% pomegranate peel and 2% of brewer's spent grains, followed by 2% barley straw (2.67 U/mL) among the different agro-wastes used in this study. In contrast, the least pectinase activity (0.56 U/mL) was exhibited by 0.5% orange peel (Fig. 4a).

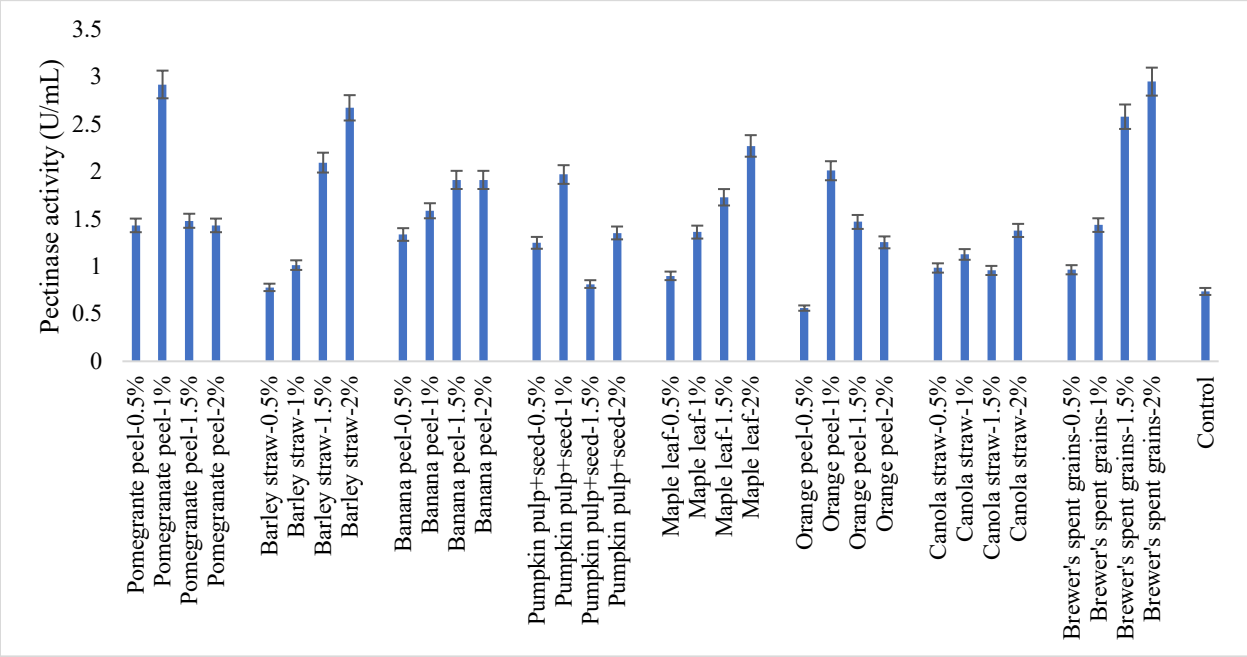


Fig. 4a Pectinase activity produced by *S. thermocarboxydus* at different concentrations of agro-wastes

The study illustrated the pectinase activity continuously increased with the increase in the concentration of agro-wastes such as barley straw, banana peel, maple leaf, canola straw and brewer's spent grains and showed the highest pectinase activity at 2%. However, pomegranate peel, pumpkin pulp+seeds and orange peel showed the highest pectinase activity at 1% (Fig. 4a).

3.3.2. Xylanase activity produced by *S. thermocarboxydus* using different concentrations of biomasses

The trends of xylanase activity exhibited by *S. thermocarboxydus* using different concentrations of agro-wastes were different. The highest xylanase activity was found in 1.5% barley straw and the least in 0.5% pumpkin pulp and seeds (Fig. 4b).

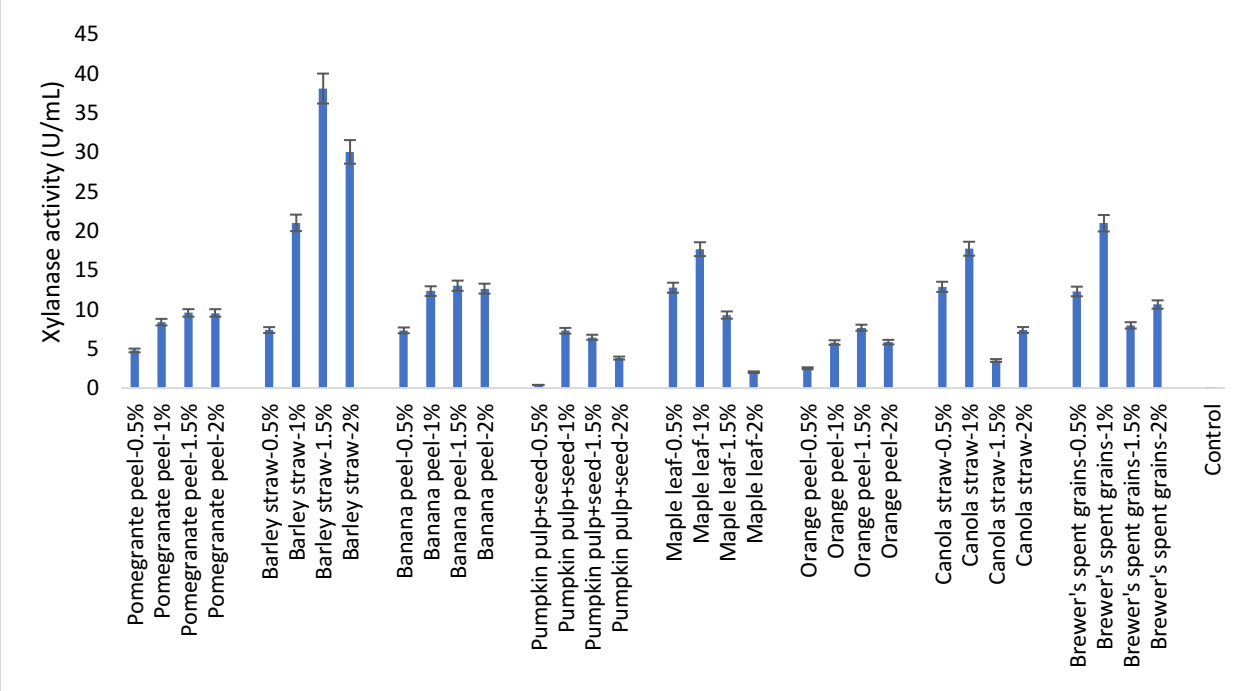


Fig. 4b Xylanase activity produced by *S. thermocarboxydus* at different concentrations of agro-wastes

In addition, the present study depicts the maximum xylanase activity when 1% of maple leaf, canola leaf, brewer's spent grains, and pumpkin pulp and seeds were used. The xylanase activity increased continuously with the concentration of pomegranate peel (0.5% to 2% w/v) while xylanase production did not change with changes in concentration of banana peel at 1%, 1.5%, and 2% (Fig. 4b).

3.4. Determination of substrate degradation ratio

The different agro-wastes that were examined showed different rates of degradation when treated with *S. thermocarboxydus*. The substrate degradation ratio is depicted in Fig. 5a, indicating the biomasses have been degraded. The highest substrate degradation ratio by *S. thermocarboxydus* was 80% for barley straw, followed by banana peel (77.2%), pomegranate peel (68.6%), and so on, and the least substrate degradation was observed in pumpkin pulp+seeds (Fig. 5a).

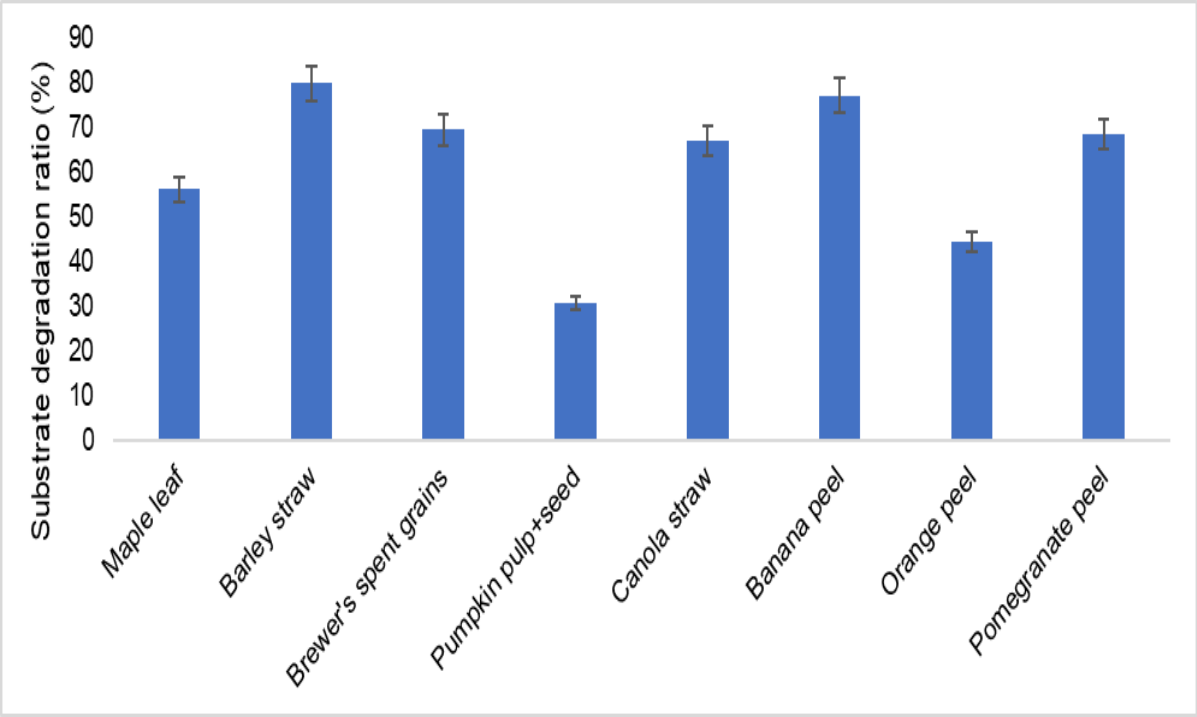


Fig. 5a Substrate degradation ratios exhibited by using *S. thermocarboxydus*

3.5. Scanning electron microscopy

The SEM images of all the agro-wastes before and after bacterial treatment were taken and are depicted in Fig. 5b. The changes in the morphological structure of agro-wastes such as rough surfaces of biomass after bacterial treatment observed in SEM images, show and corroborate the degrading capabilities of the bacterium.

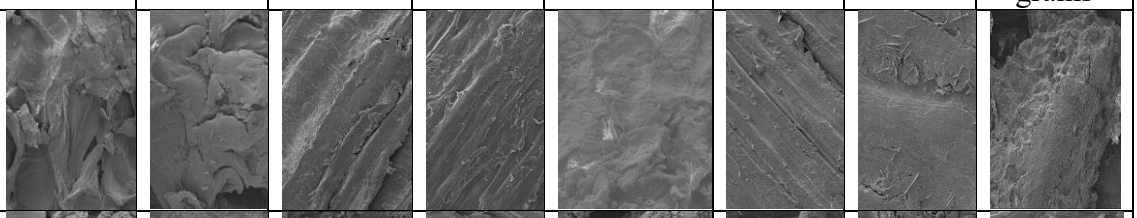
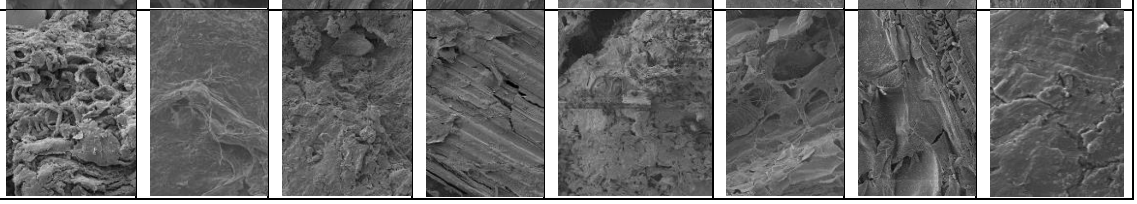
Biomass	Pomegranate peel	Orange peel	Banana peel	Maple leaf	Pumpkin pulp+seed	Barley straw	Canola straw	Brewer's spent grains
No bacterial treatment								
Bacterial treatment								

Fig. 5b Scanning electron micrograph (SEM) of different agro-wastes before and after bacterial treatments

4. Discussions

Waste production is increasing continuously, and efficient management of these wastes is crucial to reduce pollution. We isolated a native forest soil bacterium and examined its ability to degrade agro-wastes and hydrolyze some polysaccharides. Analysis of 16S rDNA sequence of the bacterium isolate showed that it clustered with *S. thermocarboxydus* (GenBank accession: U94490) (Fig. 1). In addition, when the uncorrected genetic distance between bacteria having similar sequences was calculated, their distance was found to be very minimum i.e 0.1% (Table 1). Therefore, the bacterial isolate was confirmed to be *S. thermocarboxydus.*

The various agro-wastes are the alternative feedstock for the sustainable production of value-added products, and several microorganisms can produce various enzymes and other products by utilizing agro-wastes (Thite and Nerurkar, 2018; Shrestha et al., 2020). Hence, *S. thermocarboxydus* was exploited to study its biodegrading capacity by culturing it on agar plates containing different agro-wastes (0.5% w/v) at 35℃ for 24 hours.

Different agro-wastes contain various components such as cellulose, hemicellulose, pectin, etc., and for the biomass degradation, different hydrolyzing enzymes are necessary to hydrolyze those

agro-waste components (Shrestha et al., 2020). Therefore the bacterium was screened for different hydrolyzing enzymes (pectinase, cellulase, and xylanase). The bacterium had the potential to degrade different agro-wastes as it produced different hydrolyzing enzymes and showed a clear zone around the colony.

Previous studies have shown that xylanase, cellulase, and pectinase are essential enzymes for biomass degradation and saccharification (Thite and Nerurkar, 2018; Shrestha et al., 2020). *S. thermocarboxydus* can produce different enzymes and has been exploited in various applications (Beg et al., 2000a; de Lima Procópio et al., 2012; Ramírez-Tapias et al., 2015; Mihajlovski et al., 2020; Shrestha et al., 2021b) and thus has economic values. In the present study, the pectinase activity was observed in all agro-wastes containing media which served as carbon sources. In addition, pectin in different agro-wastes acts as an inducer for pectinase production (Ghazala et al., 2015).

Table 2 Previous studies on enzyme production by using different agro-wastes

Organism used	**Biomass used**	**Conditions**	**Maximum enzyme activity**	**References**

Aspergillus terreus		Black gram peel, mustard oil cake, green gram peels, neem oil cake, wheat bran, groundnut oil cake, orange peels, finger millet waste, banana peels, broken rice, apple pomace	pH 5, 30℃ and 96 hours of incubation, Liquid static surface fermentation (LSSF) and Solid state fermentation (SSF)	Banana peel, pectinase LSSF:400 ± 21.45 U/ml; SSF:6500 ± 1116.21 U/ g	(Sethi et al., 2016a)
Bacillus	*licheniformis*	Citrus peel, apple pomace, carrot pomace, wheat bran, peanut husk, gram bran	pH 9.5, 37℃, 120 hours, Submerged fermentation	Orange peel, Pectinase 219 U/mL	(Bibi et al., 2016)
Bacillus	*amyloliquefaciens*	Banana peel, rice bran, orange peel, spent coffee grounds, wheat bran	37℃, 96 hours, Submerged fermentation	Wheat bran, pectinase 0.75 U/mL	(Doan et al., 2021)
B. mojavensis		Carrot peel, potato peel, wheat bran, prickly pear powder, barley bran, grapefruit bark powder, hay, grenade bark powder, carob powder	60℃, pH 8, 32 hours, submerged fermentation	Carrot peel; pectinase 64.8 U/mL	(Ghazala et al., 2015)

Aspergillus niger	Sugarcane bagasse, cassava pulp, banana peel	30°C, 200 rpm	Banana peel; pectinase 865.67 U/mL, sugarcane bagasse; xylanase 3910.06 U/mL and CMCase 51.28 U/mL	(Laothanachareon et al., 2022)

Table 2 depicts that pectinase production varied with the type of agro-waste used. Also, the table shows it is crucial to select suitable agro-industrial residues as the substrate, fermentation conditions, and microorganisms for enzyme production (Table 2).

The different enzyme activities observed in this study indicated that *S. thermocarboxydus* could utilize various biomasses differently and exhibited distinct trends of enzyme activity within 120 hours of the incubation period. The bacterium produced pectinase and xylanase by using almost all agro-wastes as a carbon source but cellulase was produced only when pomegranate peel and orange peel were used as carbon sources. This result reveals that not all biomass used in the present study was suitable for cellulase production by *S. thermocarboxydus,* or some other pretreatments of biomasses need to be followed. The pretreatment method is essential and critical in biomass hydrolysis into different fermentable sugars (Banerjee et al., 2011). A study has concluded that the variety of chemical compositions of varying agro-waste and the inherent capability of microorganisms play a vital role in the production of enzymes. Agro-waste-induced enzymes according to the availability or exposure of carbohydrates or substrate on the waste (Laothanachareon et al., 2022).

The cellulase-free pectinase-xylanase enzyme extract can be utilized in pulp and paper bio-bleaching. The enzymes can reduce biological oxygen demand, chemical oxygen demand, and

chlorine consumption, maintaining the same optical properties of pulp and paper and increasing the paper quality (Sharma et al., 2021). The crude xylanase-pectinase is applicable in the eco-friendly scouring of ramie fibers by increasing whiteness, fiber brightness, and reducing the harmful chemicals consumption by related industries (Singh et al., 2020).

Similarly, in another study, cellulase and xylanase producing *S. thermocarboxydus* was isolated from various sediments and used for agro-wastes management and wastewater treatment (Limaye et al., 2017).

The carbon source concentration plays a vital role in inducing the increase in enzyme yield, indicating that the carbon source regulates pectinase production (Ghazala et al., 2015). The result of the current study illustrated the bacterium utilized different agro-wastes and exhibited multi-enzyme activity. However, the bacterium displayed different trends for different concentrations and different agro-wastes. Barley straw, banana peel, maple leaf, and brewer's spent grains showed increasing pectinase activity with an increase in the concentrations.

The cellulase activity produced by *S. thermocarboxydus* using different concentrations of agro-wastes is no further described because the bacterium exhibited negligible cellulase activity only when pomegranate peel and orange peel were used.

In the present study, agro-wastes were treated with bacterium for 96 hours and compared the weight loss with the agro-wastes without bacterial treatment. The study showed a loss in agro-wastes weight when they were treated with the bacterium. The weight loss of the biomasses after bacterial treatment supports the bacterium is helpful in degrading the biomasses. Furthermore, the data reveals *S. thermocarboxydus* can degrade the agro-wastes, but the ability varies with the agro-waste types. Other studies also illustrated that the decomposition of agro-waste depends on the strains of microorganisms used (Saha et al., 2016) and the cultural conditions (Maki et al.,

2012). For examples; a study illustrated that the corn stover degradation by 26 different fungi was different, and the ability to degrade the corn stover varied with the strains of fungi used (Saha et al., 2016). In another study, the by-products formed in the cultured solution, changes in pH, structural changes of the substrate, and enzymes were the essential factors affecting the decomposition of substrates/agro-wastes, and each bacterium has a unique bacterial system for decomposition (Maki et al., 2012).

As observed in SEM images before bacterial treatment, the surfaces of all agro-wastes were somewhat smooth; however, in the bacterial treated agro-wastes, the surfaces were observed to be rough. Other studies also revealed the morphological changes of the substrate under SEM analysis are due to enzymatic activities (Kocabaş et al., 2015; Poondla et al., 2015; Liu et al., 2017). Thus, the change in morphology of agro-wastes might be due to the use of agro-wastes as a carbon source by *S. thermocarboxydus* and the degradation of polysaccharides present in agro-wastes. Hence, the results showed that *S. thermocarboxydus* degraded different agro-wastes and produced pectinase and xylanase like hydrolyzing enzymes differently in various concentrations.

5. Conclusions

The present study illustrated that the bacterium isolated from forest soil was identified as *S. thermocarboxydus* and produced pectinase, cellulase, and xylanase enzymes; thus it is a good candidate for lignocellulosic biomass degradation. This strain has potential to degrade the different biomasses exhibiting different enzyme activities. The different agro-wastes have different trends for enzyme production, and *S. thermocarboxydus* produced xylanase and pectinase by utilizing all the agro-wastes studied but little cellulase only from pomegranate peel and orange peel. Moreover, enzyme production also depends on the concentration and types of biomasses used. Since the bacterium uses different agro-wastes as low-cost substrates/carbon

sources and produces different enzymes, the bacterium is of great potential in reducing waste disposal problems and production costs. Thus, this bacterium is helpful in waste management and the economic growth of industries. A further detailed study in optimizing enzyme production using biomasses as carbon sources and rapid degradation of biomasses is recommended. Also, other organic wastes and their comparisons based on the different seasons and degradation by using other microorganisms need to be studied as this study included only eight different locally available agro-wastes and *S. thermocarboxydus.* It is also urged to explore and improve the eco-friendly utilization of agro-wastes in various avenues.

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5B Formulation of the Agro-waste Mixture for Multi-enzyme (Pectinase, Xylanase, and Cellulase) Production by Mixture Design Method Exploiting *Streptomyces* sp.

Abstract: The demand for enzymes in different industries is continuously increasing, and the use of a pure form of carbon and nitrogen source for enzyme production is expensive. Thus, organic waste as a low-cost substrate is used to produce enzymes, mitigate the market demand, and reduce the production cost. Besides, this action helps in decreasing organic waste and finally pollution. This study aims to utilize agro-waste for maximum multi-enzyme production by *Streptomyces* sp. and to formulate the three agro-waste mixtures using a statistical approach; extreme vertices mixture design (EVMD). The study illustrated the bacterium produced a maximum of 4.24±0.05 U/mL pectinase, 2.88±0.05 U/mL polygalacturonase (PGase), 9.43±0.16 U/mL xylanase, and 1.62±0.09 U/mL cellulase by formulating the agro-waste mixture of orange peel, pomegranate peel, and pumpkin pulp+seeds. Also, the mixture of agro-waste enhanced enzyme production rather than the single feedstock, and the enzyme production depends on the agro-waste mixture.

Keywords: *Streptomyces* sp., multi-enzymes (pectinase, xylanase, cellulase), agro-waste, mixture design

1. Introduction

The increase in population and awareness of fruit and vegetable consumption directly or indirectly increases organic waste production. Organic agro-wastes are rich in carbohydrate polymers such as cellulose, hemicellulose, xylan, and pectin. Thus the organic wastes are suitable as a low-cost resource to produce value-adding products such as enzymes, biofuels, chemicals, and substrates for different microbial growth and enzymes production (Panda et al., 2018; Shrestha et al., 2020; Laothanachareon et al., 2022; Ozzeybek and Cekmecelioglu, 2022).

Although the waste management program emphasizes reducing, reusing, and recycling waste, not all countries can manage the waste produced adequately (https://datatopics.worldbank.org/what-a-waste/trends_in_solid_waste_management.html). In recent years the interest in converting agro-waste into different valuable commodities is increasing because the agro-waste is renewable, eco-friendly, cheap, and abundantly available resources (Limkar et al., 2019; Shrestha et al., 2020). For example, pectinase, xylanase, and cellulase are the three most common industrial enzymes with broad biotechnological applications. Moreover, these enzymes play an essential role in the degrading plant biomass (Bharathiraja et al., 2017; Toushik et al., 2017; Ravindran et al., 2018) and help protect the environment by degrading different agro-waste (Amadi et al., 2022).

Additionally, the production of the multiple enzymes or enzyme cocktails simultaneously by using a single microorganism and degrading multiple carbohydrate polymers of low-cost substrates is also earning more interest (Amadi et al., 2022). Furthermore, the mixture of enzymes can act as a co-catalyst in different industries and substitute expensive feedstocks with low-cost agro-waste for multi-enzyme production (Ravindran et al., 2018; Limkar et al., 2019; Laothanachareon et al., 2022; Ozzeybek and Cekmecelioglu, 2022). The utilization of organic waste not only decreases environmental pollution, CO_2 emissions, global warming, and disposal problems but also helps in reducing production costs (Sadh et al., 2018; Shrestha et al., 2020, 2021a). Due to the different chemical compositions (pectin, hemicellulose, cellulose, carbon, nitrogen, moisture, vitamin, etc.) in agro-waste, the agro-waste are utilized for enzymes production; otherwise, they can pollute the environment when thrown as garbage (Sadh et al., 2018). Furthermore, enzyme production also depends on microorganisms (Laothanachareon et al., 2022; Ozzeybek and Cekmecelioglu, 2022).

Many studies have been performed regarding the optimization of growth conditions to produce a single enzyme. Still, very little (countable) research has been reported on the concurrent production of more than one enzyme in one medium. For instance, Ozzeybek and Cekmecelioglu, (Ozzeybek and Cekmecelioglu, 2022) formulated the apple pomace, hazelnut shell, and orange peel mix for the production of pectinase and cellulase enzyme from *Bacillus subtilis*. The co-production of xylanase and alkaline protease was explored by using *Bacillus* sp. as well (Limkar et al., 2019). Similarly, tannase and pectinase were co-produced by *Rhodotorula glutinis* from tannin-pectin containing media (Taskin, 2013). Also, the study has illustrated pectinase to enhance agro-waste saccharification in combination with xylanase and cellulase (Thite and Nerurkar, 2018).

There is a need to explore synchronous production of industrially important enzymes cocktail using the same media and microorganism because it is an economic concern with energy, time, and production cost reduction. Therefore, this study concentrates on producing multi-enzymes by exploiting locally isolated forest soil bacterium (*Streptomyces* sp.) and utilizing various locally available organic waste such as orange peel, pomegranate peel, pumpkin pulp+seeds, canola straw, and brewer's spent grains. The response surface methodology (RSM) has been applied in many studies to optimize cultural conditions, and it is an effective statistical method. However, another statistical technique known as extreme vertices mixtures design (EVMD) is applied in this study. As the name indicates, it is handy to formulate the agro-waste mixture with fewer experimental runs. The best formulation of different agro-waste mixtures was determined through EVMD. In addition, the multi-enzyme produced were compared with single agro-waste feedstock and between two sets of agro-waste mixture formulations.

2. Materials and methods

2.1. Materials

In this study, different locally available agro-wastes such as orange peel, pomegranate peel, pumpkin pulp+seeds, and canola straw were used. Brewer's spent grains was also obtained from a local brewing company, Sleeping Giant Brewing Co. (Thunder Bay, Ontario). All those agro-wastes were dried in shade for 3-4 days. Once they were dried, they were ground with a coffee grinder. The powered agro-wastes were washed with warm water to remove impurities present such as simple sugar. The presence of simple (reducing) sugar was tested by dinitrosalicylic acid (DNS) method. Each agro-waste was washed with warm water till reducing sugar was observed. Then they were dried in a 50℃ oven for 48 hours (consistent weight was noticed) and kept in airtight containers for further use.

2.2. Microbial strain and inoculum preparation

This study used a novel *Streptomyces* sp. isolated from the boreal forest soil in Thunder Bay, Ontario, Canada and preserved at -80℃. The bacterium was identified as *Streptomyces* sp. based on colony morphology, biochemical characteristics, microscopic characterization, and 16S rDNA sequencing (Shrestha et al., 2021b). The stored bacterium was activated by sub-culturing in LB at 35℃ for 24 hours and used as the seed culture.

2.3. Qualitative or plate screening tests

The bacterium was screened for the capabilities of producing pectinase, xylanase, and cellulase by culturing the bacterium on the nutrient agar plates containing pectin, xylan, carboxymethyl cellulose (CMC) as the only carbon source. When the isolated colonies on the plates were observed, the colonies were flooded with potassium iodide solution for pectinase and Congo red

solution for xylanase and cellulase activity. A clear zone around the bacterial colony indicates the bacterium has enzyme activity.

2.4. Growth medium and enzyme production

The simple growth medium was prepared by mixing 3% (w/v) yeast extract, 2% (w/v) K_2HPO_4, 2% (w/v) KH_2PO_4, and 2% (w/v) KNO_3 in distilled water. The pH of the mixture was maintained at 7 by adding potassium hydroxide or HCl and autoclaved at 121℃ for 15 mins. The 1% (w/v) pectin or agro-waste was separately sterilized and added aseptically to the mixture after. The 24 hours grown seed culture (1% v/v) was inoculated in the growth media after cooling to room temperature and incubated at 35℃, 200 rpm for 96 hours. The samples were collected every 24 hours aseptically, centrifuged, and cell-free supernatant was used for enzyme activity assays. The different enzyme activities: pectinase, polygalacturonase (PGase), xylanase, and cellulase were measured by the dinitrosalicylic acid (DNS) method using 1% w/v pectin, polygalacturonic acid, xylan, and CMC as substrate. One unit of enzyme activity is expressed as the amount of enzyme that releases 1 μmol of simple sugar (galacturonic acid, xylose, and glucose from pectin/polygalacturonic acid, xylan, and CMC, respectively) per minute under assay conditions (Miller, 1959).

2.5. Experimental design and analysis

Two sets of experimental designs were formulated by the extreme vertices mixture design (EVMD) method. The first set (Set-I) included the proportion of orange peel, canola straw, and brewer's spent grains. Another set (Set-II) integrated orange peel, pomegranate peel, and pumpkin pulp+seeds. In both sets, all feedstock was used in a range of 20 to 100%, which resulted in 7 runs in each set.

2.6. Statistical analysis

In this study, the experiments were performed in triplicates and the results of the experiments were presented as mean values with standard deviations. The differences in the results values were identified by One-way analysis of variance (ANOVA) and compared using Tukey's range test. The values were considered statistically significant when the p-value was less than 0.05.

3. Results and discussion

3.1. Microbial strain and screening test

The study exploited *Streptomyces* sp. that was isolated from the boreal forest soil in Thunder Bay, Ontario, Canada and preserved at -80°C (Shrestha et al., 2021b). The bacterium was revived by culturing in LB broth and incubated at 35°C, 200 rpm for 24 hours. It is worth having a bacterium capable of producing multi-enzyme simultaneously. Thus, the bacterium was screened for pectinase, cellulase, and xylanase by culturing in nutrient agar plates having pectin, CMC, and xylan as a carbon source. The bacterium illustrated the clear area around the colony after flooding of potassium iodide solution and Congo red, indicating the bacterium's capability to produce pectinase, cellulase, and xylanase (shown in Fig.1).

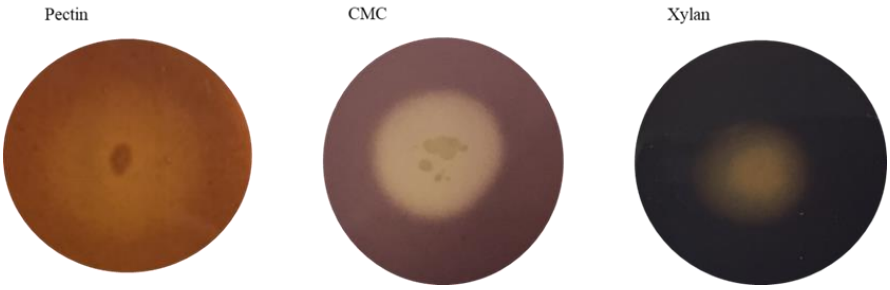

Fig. 1 Screening (qualitative test) *Streptomyces* sp. for different enzyme activity

The clear zone around the bacterial colony is the result of the bacterium's ability to utilize or hydrolyze pectin, CMC, and xylan when used as the carbon source by producing relative enzymes; pectinase, cellulase, and xylanase, respectively. The iodine solution gives brown or

brown-black coloration when bound with unutilized pectin. In contrast, Congo red gives reddish to brown coloration when CMC and xylan are present in the agar plates.

3.2. Enzyme production

Since the bacterium was screened for multi-enzyme and illustrated its ability to produce different enzymes, it was subjected to the degradation of varying agro-waste. Here, agro-waste such as pomegranate peel, orange peel, canola straw, brewer's spent grains, and pumpkin pulp+seeds was used as the substrate (carbon source). The bacterium was cultured in the broth containing fundamental constituents (K_2HPO_4, KH_2PO_4, KNO_3, yeast extract, and agro-waste) at 35℃, 200 rpm for 96 hours. The sample was collected every 24 hours and centrifuged at 5000 rpm for 8 mins to get the cell-free supernatant. The hydrolysates produced after centrifugation were analyzed for different enzyme production and elucidated in Table 1. The result showed that cellulase was not produced by *Streptomyces* sp. when it was grown separately in the media containing 1%w/v of brewer's spent grains, canola straw and pumpkin pulp+seeds. However, the bacterium can produce pectinase, xylanase and cellulase using orange peel and pomegranate peel. This may be due to high cellulose and hemicellulose content in pomegranate and orange peel which might induce the bacterium to produce cellulase. This result shows that a single organism can produce multi-enzymes simultaneously but vary with agro-waste. Here, *Streptomyces* sp. had the higher pectinase using pomegranate peel (3.73±1.33), followed by orange peel (2.31±0.32). Similarly, the highest PGase was produced by using canola straw (1.72±0.44) followed by orange peel (1.67±0.24), and xylanase from orange peel (9.11±3.81) followed by canola straw (8.24±0.75).

Table 1 Enzyme activities performed by *Streptomyces* sp. using different agro-waste (mean ± standard deviation)

Agro-waste	Pectinase (U/mL)	PGase (U/mL)	Xylanase (U/mL)	Cellulase (U/mL)
Pomegranate peel	3.73±1.33	1.12±0.25	2.08±0.59	1.30±0.77
Orange peel	2.31±0.32	1.67±0.24	9.11±3.81	0.25±0.36
Canola straw	1.55±0.27	1.72±0.44	8.24±0.75	-
Brewer's spent grains	1.71±1.07	1.37±0.47	5.64±0.65	-
Pumpkin pulp+seeds	2.07±0.86	0.91±0.13	7.36±0.34	-

*'-' represents no production

The differences in the enzyme production by the same bacterium using different agro-waste is due to the variation in compositions and the carbon/nitrogen content of agro-waste. The previous study illustrated the profound effect of *Aspergillus niger* on types and enzyme production by using different agricultural waste. The study also demonstrated the capabilities of producing enzymes depend on the inheritance of microbial strain (Laothanachareon et al., 2022). Another study also illustrated that higher pectinase and xylanase were produced from an optimized production medium containing wheat bran and citrus peel from two *Bacillus* sp. (*B. safensis* and *B. altitudinis*) (Thite et al., 2020).

From different pieces of literature, it is found that the fruits and vegetable pomace contains pectin (1.50–13.40%), cellulose (7.20–43.60%), hemicelluloses (4.26–33.50%), and lignin (15.30–69.40%) as the main constituents (Szymanska-Chargot et al., 2017) and also can be observed in Table 2.

Table 2 Main components of the different agro-waste used in the study (collected from different pieces of literatures)

Agro-waste	Pectin (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Orange peel	20.0-42.5	9.21-37.0	10.5-16.0	5.0-7.5	(Rezzadori et al., 2012; Tsouko et al., 2020)
Pomegranate peel	6.8-27.9	10.8-27.6	26.2-32.8	5.6-28.5	(Barathikannan et al., 2016; Pocan et al., 2018)
Canola straw	2.3-2.5	22.4-40.6	16.9-23.2	19.6-21.3	(Adapa et al., 2011; Müller-Maatsch et al., 2016; Wang et al., 2020)
Brewer's spent grains	-	16.0-22.4	15.0-29.0	19-30.5	(Dos Santos et al., 2015)
Pumpkin pulp	5.2-5.6	18.3-45.7	3.4-16.9	18.7-19.9	(Derkanosova et al., 2018; Ma et al., 2018)

Thus, the differences in chemical compositions of the agro-waste and fermentation conditions play crucial roles in the types and levels of enzyme production (Amadi et al., 2022; Ozzeybek and Cekmecelioglu, 2022). From the literature and as mentioned above in the Table 2, it is observed that the orange peel and pomegranate peel contain higher pectin, which may be the factors for higher pectinase production compared to canola straw and pumpkin pulp+seeds. However, xylanase is higher in agro-waste containing high hemicellulose. Besides, the production of enzymes is also affected by the microorganisms used and the agro-waste types (Ozzeybek and Cekmecelioglu, 2022).

3.3. Formulation of agro-waste mixture

Due to the different compositions present in agro-waste that play an influencing role in enzyme production, this study attempted to produce higher multi-enzyme production simultaneously by formulating the agro-waste mixture. The optimal proportions of the agro-waste mixture were determined by EVMD. In the current study, two sets of mixture were formulated. One used orange peel, canola straw, and brewer's spent grains (Set-I, shown in Table 3) and another set contained orange peel, pomegranate peel, and pumpkin pulp+seeds (Set-II, shown in Table 4). Both sets included 40-100% orange peel and another agro-waste was 20-100%.

In Set I (Table 3), the significantly highest pectinase was observed in run 6 followed by runs 2 and 4, PGase was maximum in run 2 followed by run 6, xylanase was highest in 1st run followed by 2nd and 5th run, and cellulase was only negligibly observed (thus not shown in Table 3). As observed in Table 3, the runs with higher orange peel concentration gave higher pectinase and PGase. Both runs 6 and 2 constituted an equal proportion of canola straw and brewer's spent grains, whereas orange peel is more than double canola straw or brewer's spent grains.

Table 3 The experimental results of enzyme productions by using orange peel (OP), canola straw (CS), brewer's spent grains (BS) under varying mixture formulation (Set I)

Run	OP (%w/v)	CS (%w/v)	BS (%w/v)	Pectinase (U/mL)	PGase (U/mL)	Xylanase (U/mL)
1	40	20	40	0.53±0.02^{e}	0.98±0.00^{de}	17.35±0.38^{a}
2	60	20	20	1.56±0.05^{b}	2.45±0.03^{a}	12.38±0.36^{b}
3	40	40	20	0.78±0.03^{d}	0.98±0.03^{de}	11.43±0.43^{c}
4	46.67	26.67	26.67	1.55±0.07^{b}	1.67±0.08^{c}	9.61±0.25^{d}
5	43.33	23.33	33.33	0.76±0.06^{d}	0.90±0.08^{e}	12.71±0.26^{b}
6	53.33	23.33	23.33	1.79±0.03^{a}	1.94±0.07^{b}	7.62±0.16^{e}
7	43.33	33.33	23.33	1.08±0.03^{c}	1.08±0.04^{d}	12.08±0.17^{bc}

Data are expressed as mean ± standard deviation, and the different lower-case alphabets denote the values are significantly different.

In Set II (Table 4), pectinase production was observed the highest in run 3 followed by run 2 which has a higher proportion of orange peel. The xylanase production was maximum in run 6 followed by run 1, whereas cellulase production was higher in run 2 and 6 followed by run 1. From this observation, it is also obvious that orange peel has a vital role in different enzymes production. Compared to the formulation of Set I, Set II is better if pectinase, PGase, and cellulase are to be considered. However, if xylanase is the main enzyme to consider, Set II formulation is better than Set I.

Table 4 The experimental results of enzymes production by using orange peel (OP), pomegranate peel (PP), and pumpkin pulp+seeds (PPS) under varying mixture formulation (Set II)

Run	OP (%w/v)	PPS (%w/v)	PP (%w/v)	Pectinase (U/mL)	PGase (U/mL)	Xylanase (U/mL)	Cellulase (U/mL)
1	40	20	40	2.88 ± 0.08^{cd}	2.67 ± 0.11^{ab}	6.41 ± 0.19^{b}	1.33 ± 0.13^{ab}
2	60	20	20	3.81 ± 0.09^{b}	2.88 ± 0.05^{a}	4.43 ± 0.25^{c}	1.58 ± 0.04^{a}
3	40	40	20	4.24 ± 0.05^{a}	2.31 ± 0.02^{c}	4.49 ± 0.21^{c}	1.19 ± 0.09^{bc}
4	46.67	26.67	26.67	1.36 ± 0.08^{e}	0.49 ± 0.08^{d}	3.59 ± 0.16^{d}	0.00 ± 0.00^{d}
5	43.33	23.33	33.33	3.09 ± 0.08^{c}	2.58 ± 0.15^{b}	4.92 ± 0.16^{c}	0.08 ± 0.03^{d}
6	53.33	23.33	23.33	2.73 ± 0.05^{d}	2.19 ± 0.06^{c}	9.43 ± 0.16^{a}	1.62 ± 0.09^{a}
7	43.33	33.33	23.33	3.91 ± 0.17^{b}	2.75 ± 0.06^{ab}	2.86 ± 0.08^{e}	1.01 ± 0.19^{c}

The values are expressed as mean ± standard deviation, and the different lower-case alphabets denote the values are significantly different.

There are many optimization studies for enzyme production, however, there is little regarding the agro-waste mixture formulation study for enzyme production. Also, the availability of different agro-waste in different places varies, and bacteria too which could be the main reason for not finding exactly similar research. Due to the lack of similar studies using mixture design for the formulation of orange peel, pomegranate peel, pumpkin pulp+seeds, canola straw, and brewer's spent grains, the current study results could not be compared directly. However, one study was found in which the agro-waste mixture was formulated using apple pomace, hazelnut shell, and orange peel to co-produce bacterial pectinase and cellulase by the mixture design method (Ozzeybek and Cekmecelioglu, 2022). In their study, *Bacillus subtilis* and acid-pretreated biomass were used. They illustrated the mixture composition of 20% apple pomace, 50% hazelnut, and 30% orange peel, producing a maximum of 8.27 U/mL pectinase and 0.5 U/mL cellulase (Ozzeybek and Cekmecelioglu, 2022). In that study, the pectinase production was higher; however, cellulase was lower than in our study. In addition, our study explored the production of PGase and xylanase. The concurrent production of xylanase, cellulase and pectinase was analyzed using citrus peel from *Bacillus* sp. under solid state fermentation (Amadi et al., 2022). A few studies have shown that citrus peel, including orange peel, is a good source of enzyme production (Ahmed et al., 2016a; Bibi et al., 2016; Pili et al., 2018). Pili et al. (Pili et al., 2018) demonstrated that pectin lyase production by *Aspergillus brasiliensis* was higher in the agro-waste residue containing media (orange peel, corn steep liquor, and parboiled rice water) compared to the synthetic media. The reason behind this is that agro-waste contains useful

polysaccharides inducers; for example, pectin present in agro-waste may act as the inducer for pectinase.

The 3D response surface plots of EVMD can be used to determine the interaction between the proportions of each feedstock. From Fig. 2, it is evident the proportions of the feedstocks play an important role in enzymes production. It is noted that pectinase production increased with high orange peel and pumpkin pulp+seeds (Fig. 2a), PGase is higher with low pumpkin pulp+seeds and pomegranate peel (Fig. 2b). The xylanase and cellulase production increased when the orange peel was more than double that of pomegranate peel and pumpkin pulp+seeds (Fig. 2c and 2d).

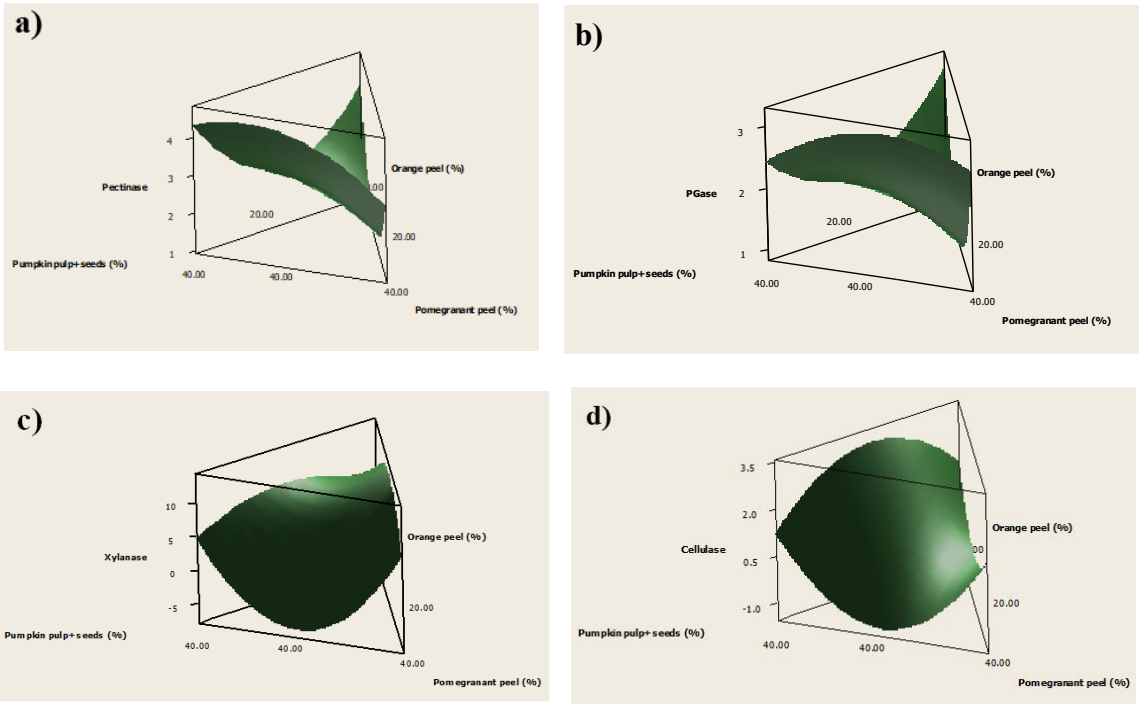

Fig. 2 Mixture surface plot of a) pectinase, b) PGase, c) xylanase, and d) cellulase from orange peel, pomegranate peel and pumpkin pulp+seeds

The maximum pectinase (1.79±0.03) in Set I was observed in run 6, having a mixture of 53.33% orange peel, 23.33% canola straw and 23.33% brewer's spent grains. Similarly, in Set II,

maximum pectinase (4.24±0.05) was observed in run 3, constituting 40% orange peel, 40% pumpkin pulp+seeds, and 20% pomegranate peel. Thus, additional experiments were performed for the verification of this agro-waste mixture formulation, and approximately similar values of enzyme productions were obtained, as depicted in Table 5.

Table 5 Enzyme production by agro-waste mixture for verification (mean ± standard deviation)

Agro-waste mixtures	**Pectinase (U/mL)**	**PGase (U/mL)**	**Xylanase (U/mL)**	**Cellulase (U/mL)**
Orange peel, canola straw, brewer's spent grains	2.42±0.15	2.17±0.10	11.83±0.97	0±0.18
Orange peel, Pomegranate peel, Pumpkin pulp +seeds	4.02±0.09	2.39±0.09	5.78±0.55	1.39±0.21

4. Conclusions

The current study demonstrated the forest soil bacterium (*Streptomyce*s sp.) is a potent candidate for different agro-waste biodegradation as it produces multi-enzymes. In addition, the study depicted that enzyme production depends on the compositions, their proportions, and constituents of the media. Here, the set of media containing mixture of orange peel, pomegranate peel and pumpkin pulp+seeds produced higher pectinase, PGase, and cellulase. However, xylanase was higher in the media containing orange peel, canola straw, and brewer's spent grains mixture. Further investigations for optimizing various parameters (temperature, pH, inoculum volume) are recommended for maximum agro-waste utilization, degradation, and enzyme production.

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5C A Sustainable Source for Phytochemicals and Potential Antibacterial Applications

Abstract

The present study aimed to screen various agro-wastes for the presence of phytochemicals. In addition, the study focused on determining phenolic content, total flavonoid, antioxidant activity and total antioxidant capacity of agro-wastes by using gallic acid, rutin, trolox, and ascorbic acid as standard, respectively. Further, pectin extraction from agro-wastes by traditional and microwave-assisted methods was compared. The agar well diffusion assay studied the antibacterial potency of those agro-wastes extracts using five bacteria.

Of the various agro-wastes studied, pomegranate peel and maple leaf illustrated higher flavonoid, total phenolic content, and antioxidant activity. However, different solvents used in extraction showed different potentials for evaluating total phenolic content, total flavonoid, and antioxidant capacity. The inhibition zone varied on the agro-wastes extract and the bacterial strains. Pomegranate peel extract exhibited the highest inhibition zone against *Cellulomonas* sp. (S-10) and *Bacillus* sp. (S-17), while the aqueous extract of pumpkin pulp+seeds did not show any inhibition. Besides, the study revealed higher pectin yield from pumpkin pulp+seeds followed by orange peel, banana peel, pomegranate peel, and others. The traditional and microwave-assisted methods showed almost similar pectin yield from most of the agro-wastes used.

This study supports different agro-wastes as potential low-cost resources for innovative and competitive production of phytochemicals, including total flavonoid, phenolic content, and pectin. In addition, those agro-wastes exhibited antibacterial potency and have the potential to be used in the pharmaceutical industries.

Keywords: agro-waste, phytochemical, antioxidant, antibacterial potency

1. Introduction

The world's population is continuously rising and is proportionate to waste production (Supangkat and Herdiansyah, 2020). These huge wastes, if not managed or disposed of properly, may cause other health and environmental issues (Supangkat and Herdiansyah, 2020; Yunis and Aliakbari, 2021). However, organic wastes, particularly horticultural and agricultural residues, can be used as feedstocks for producing many value-added products such as enzymes, bioactive compounds, pectin, dietary fiber, organic acids, biofuels, and others (Happi Emaga et al., 2008; Campos et al., 2020; Suleria et al., 2020; Shrestha et al., 2021a). Using those agro-wastes in various avenues can finally be helpful in the disposal and management of wastes (Mussatto et al., 2012; Suleria et al., 2020; Shrestha et al., 2021a).

Phytochemicals are secondary metabolites such as alkaloids, terpenes, pectin, and phenolic compound that has a health-promoting capacity and are mainly derived from fruits and vegetables (Azmir et al., 2013; Hilali et al., 2019; Bose et al., 2020; Shrestha et al., 2021a). Although they are not nutrients or do not have nutritional value to our health, they play an essential role in maintaining good health. Phenolic compounds and flavonoids have high antioxidant and antiproliferative activity (Saini et al., 2019). The interest in phytochemicals is spreading and widely applied in curing different diseases (Gul et al., 2017), including microbial infectious ailments, depression, anxiety, cancer, and wound healing with no or fewer side effects. Further, they significantly contribute to the cosmetics and perfume industries (Bose et al., 2020; Coman et al., 2020). The solid-liquid extraction method using organic solvents is commonly used for bioactive compound extraction. However, the extraction process through organic solvents might damage some important functional groups essential for healthcare benefits (Azmir et al., 2013).

Pectin is the most complex heteropolysaccharide and is mostly found in the cell wall of plants, including fruits and vegetables. Commercially pectin is produced from apple pomace or citrus fruits (Nasseri and Thibault, 2008; Hilali et al., 2019) and is used in various industries such as cosmetic, food, and pharmaceutical industries (Nasseri and Thibault, 2008; Shrestha et al., 2021c). Besides apple and citrus fruits, many other fruits and vegetable wastes can be used as low-cost resources for pectin production (Müller-Maatsch et al., 2016) to fulfill the increasing demand for pectin. Thus, this study screened different agro-wastes for phytochemicals presence and quantified total phenolic content, flavonoid, antioxidant capacity and pectin extraction from different readily available agro-waste. As a result, phytochemicals can be used in pharmaceutical and cosmetic industries because they have antibacterial activity (Rakholiya et al., 2014). The synthetic production of polyphenols and antibiotics like bioproducts do not have their original natural flavor and can be expensive. Thus, natural bioproducts with original flavor can be produced from agro-wastes reducing the production cost on the one hand, and the waste disposal problem can be solved in another hand. Furthermore, this study investigated the antibacterial effects of the agro-wastes extracts to know the new source of antibacterial. Also, the effects of different solvents in phenolic content, total flavonoid, and antioxidants from different agro-wastes were studied. This study explores the different prospects of various agro-wastes in a simple and easy way for green economic growth.

2. Materials and methods

2.1. Sample collection

The varieties of agro-wastes such as barley straw, orange peel, maple leaf, pomegranate peel, and banana peels available locally (Thunder Bay, Ontario, Canada) were collected. They were cut

manually into small pieces, dried in a 40°C hot air oven for 2 days, powdered by a coffee grinder and stored at room temperature until use.

2.2. Sample extract

The agro-wastes powders (1 g) were extracted with 10 mL of the different solvents, including water, ethanol, methanol, and acetone, at room temperature (35°C) for 24 hours and centrifuged to collect the supernatant. Then the clear supernatant (agro-waste extract) was stored at 4°C until use.

2.3. Screening tests for phytochemicals

Each agro-wastes extracts was used for screening different bioactive compounds: phenol and tannins, flavonoids, saponins, glycosides, steroids, and terpenoids according to the standard procedure described (Ishikawa et al., 2006; Ayoola et al., 2008; Gul et al., 2017; Singh et al., 2018).

2.3.1. Test for tannins

An equal volume of biomass extract and 2% $FeCl_3$ (1 mL each) was mixed in a clean test tube, and the color formation was noticed. The presence of tannins was indicated by the green, blue, or black color.

2.3.2. Test for flavonoids

One milliliter of extract was taken in a clean test tube, and a few drops of dilute NaOH was added, which was then neutralized by adding a few drops of HCl. The color developed before adding a few drops of HCl will disappear after indicating the presence of flavonoids.

2.3.3. Test for saponins

Two milliliters of distilled water were added to the extract in the test tube and mixed vigorously in a vortex. The formation of frothy foam in the test tube indicated the presence of saponins.

2.3.4. Tests for glycosides

Liebermann's Test: Two milliliters of acetic acid and chloroform were added to the extract in the test tube. Then a few drops of concentrated H_2SO_4 were added to the mixture. The blue or bluish-green color formation indicated the presence of a steroidal ring i.e., aglycone portion of cardiac glycoside.

Keller-Kiliani Test: Two milliliters of glacial acetic acid were mixed with one drop of 2% $FeCl_3$. The mixture was added to the 5 ml aqueous extract, followed by 1 mL concentrated H_2SO_4. A brown ring at the interface layers indicated the entity of cardiac steroidal glycosides. A green layer of acetic acid may form just above the brown ring that can spread throughout the layer.

2.3.5. Test for terpenoid

One milliliter of extract was mixed with 1mL of conc. H_2SO_4 and incubated in a water bath for 4 minutes; the formation of greyish color indicated the presence of terpenoids.

2.4. Total flavonoid content

The spectrophotometric method (Aluminium chloride colorimetric technique) was used to determine the total flavonoid content (TFC) (Suleria et al., 2020). The principle of this method is as follows: under alkaline conditions, flavonoids dissolved in ethanol and combined with trivalent aluminium ions to form a red complex with maximum absorption at 510 nm wavelength. In a specific range of concentration, the concentration and absorbance follow Beer–Lambert's law. Rutin was used as the standard for flavonoid study. The sample or standard solution (or water for blank control) 275 µL was mixed with 100 µL sodium nitrite solution ($NaNO_2$, 50 g/L), 150 µL aluminium nitrate solution ($Al(NO_3)_3$, 100 g/L), and 350 µL sodium hydroxide solution (NaOH, 200 g/L). After 15 min of incubation, 200 µL of the mixture was transferred to a 96-well microplate and absorbance at 510 nm was determined with an Epoch microplate spectrophotometer (BioTek,

USA). The TFC was determined using the standard calibration curve of rutin, and the result was expressed as mg rutin equivalent to g of dry agro-waste (mg RE/g).

2.5. Total phenolic content

The total phenolic content (TPC) was determined by the spectrophotometric method using the Folin–Ciocalteau reagent. In brief, 0.125 mL of sample (1 mg/mL) was added to the glass test tube, followed by 0.5 mL of Folin-Ciocalteu's phenol reagent and 0.125 mL of distilled water. After 5 min, 1.250 mL of 7% Na_2CO_3 solution was added to the mixture, followed by addition of 3 mL of distilled water, thorough mixing, keeping the mixture at room temperature in the dark for 90 min, and reading the absorbance at 750 nm. The TPC was determined using the standard calibration curve made by gallic acid solution. The estimation of TPC was carried out in triplicate and expressed as milligrams of gallic acid equivalents (GAE) per gram of dried weight sample.

2.6. Antioxidant activity by ABTS radical-scavenging assay

Free radical scavenging activity of the extracts was determined by ABTS radical cation decolorization assay as mentioned by Ali et al. (2018) and Suleria et al. (2020) with slight modification (Ali et al., 2018; Suleria et al., 2020). The 7 mM stock solution of ABTS was reacted with 2.45 mM potassium persulfate to produce ABTS radical cation ($ABTS*^+$) and allowed the mixture to stand in the dark at room temperature (25±2°C) for 12 h before use. The obtained $ABTS*^+$ solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm. 150 µL of $ABTS*^+$ solution prepared in this way were added to each well containing 50 µL of sample. The mixture was shaken gently and left to stand in dark for 15 min at room temperature. The absorbance was measured at 734 nm spectrophotometrically. The $ABTS*^+$ scavenging capacity of the extract was calculated as:

ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})] \times 100 / (Abs_{control})$

2.7. Total antioxidant capacity

The total antioxidant capacity of the different agro-wastes was determined by the phosphomolybdate method using ascorbic acid as a standard as in Suleria et al. (2020) (Suleria et al., 2020). In the process, 0.1 mL of sample solution and 1 mL of reagent solution were mixed in a test tube. The reagent solution was prepared from 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The tubes were capped, incubated in a water bath at 95°C for 90 min, and cooled. After the samples were cooled down to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. A blank contained 1 mL of the reagent solution and the appropriate volume of the solvent and was incubated under the same conditions. The antioxidant capacity was expressed as microgram of ascorbic acid equivalents (AAE) per gram dry weight of sample.

2.8. Antibacterial efficiency test

The antibacterial potency of the aqueous extract of agro-wastes was tested against bacteria (S-5: *Streptomyces* sp., S-10: *Cellulomonas* sp., S-14: *Streptomyces* sp., S-17: *Bacillus* sp., and C-19: *Bacillus* sp.). The bacteria were isolated, identified and preserved in -80℃ in our laboratory (Lakehead University, Thunder Bay, Ontario, Canada) (Shrestha et al., 2021b, 2022). To revive the bacteria, they were grown overnight in LB broth at 35℃ and 200 rpm. Then 100 µL of the cultured broth was spread on the Mueller-Hinton agar (MHA) plate, followed by making 6 mm wells on the agar plates, adding 50 µL of agro-wastes extracts to the wells and incubating the plates at 35℃. After 24 hours, the clear zone around the well was observed and measured the diameter.

2.9. Pectin extraction from agro-waste

Different agro-wastes such as orange peel, pomegranate peel, barley straw, maple leaf, pumpkin pulp+seeds, canola straw, and barley spent grains were used in this study for pectin extraction. Pectin from different agro-wastes was extracted by the process mentioned by Liew et al. and Yang et al. with some modifications (Liew et al., 2014; Yang et al., 2018). In brief, 4 grams of agro-waste powder was mixed with 100 mL distilled water maintaining the mixture pH of 3 with 0.1N citric acid. The mixture was stirred till the agro-waste powder was evenly wetted with acidic water. Once the agro-waste was mixed with acidic water, it was incubated in a 70℃ water bath for 90 mins. Then the mixture was kept at room temperature for 24 hours. After, the precipitated pectin was recovered by centrifugation at 13,300 g for 10 mins. The obtained supernatant was ethanol precipitated by 95% ethanol in a 1:2 v/v ratio and kept in the dark at room temperature for 24 hours. The floated pectin was then filtered, washed with acetone, and dried in a hot air oven at 50℃ for 48 hours. The pectin yield was determined using the following formula:

$$\text{Pectin yield (\%)} = \frac{\text{Product obtained (g)}}{\text{4 g agro-waste}} \text{ X } 100$$

A similar process was followed for microwave-assisted pectin extraction, except the mixture was microwaved for 3 mins after the complete homogenization of agro-wastes with acidic water.

2.10. Statistical analysis

In this study, all the experiments were conducted in triplicate and the results are expressed as mean ± standard deviation.

3. Results and discussions

3.1. Screening for phytochemicals

The different agro-wastes: corncob, agave leaf, banana peel, orange peel, pumpkin pulp+seeds, pomegranate peel, barley straw, and ginkgo leaf were screened for different phytochemicals, and the screening result is presented in Table 1. The screening result showed that agro-waste has at least two phytochemicals indicating that agro-wastes might have some significant potential. The ginkgo leaf has all phytochemicals tested, whereas barley straw has only steroidal glycosides and terpenoids. Similarly, pumpkin pulp+seeds have saponins and terpenoids, and corncob has saponins, steroidal glycosides and terpenoids. Table 1 depicts different agro-wastes possessing various phytochemicals. This screening test helped in knowing the general idea of different kinds of phytochemicals the biomass contains and provide the possibility for medicinal use.

Table 1 Screening test for different phytochemicals in agro-wastes

Phyto chemicals	CC	AL	BP	OP	PP	SS	PoP	BS	GL
Phenol and tannin	-	+	-	+	-	-	+	-	+
Flavonoid	-	-	-	-	-	-	+	-	+
Saponins	+	+	+	-	+	+	-	-	+
Glycosides	-	-	-	+	-	-	-	-	+
Steroidal glycosides	+	+	+	+	-	+	+	+	+
Steroid	-	-	-	+	-	+	+	-	+
Terpenoids	+	+	+	+	+	+	+	+	+

Note: CC: Corncob, AL: Agave leaf, BP: Banana peel, OP: Orange peel, PP: Pumpkin pulp+seeds, SS: Sesame seed, PoP: Pomegranate peel, BS: Barley straw, GL: Gingko leaf; '+' denotes present, and '-' denotes absent

3.2. Total phenolic content (TPC)

The TPC was higher in pomegranate peel (except acetone extract) followed by maple leaf, and the minor phenolic content was illustrated by barley straw in all solvent extract. Methanol increased the phenolic content of orange peel, banana peel, maple leaf and pomegranate peel. While acetone extracted higher phenolic content from barley straw, sesame seeds, pumpkin pulp+seeds, and water was observed as a suitable solvent for agave leaf. Gingko leaf illustrated a slight difference when different solvents were used for phenolic content. The study performed by Iloki-Assanga et al. (Iloki-Assanga et al., 2015) demonstrated methanol to be the most effective solvent and acetone the least effective solvent for phenolic content extraction, which is in parallel with our study. The phenolic content exhibited by orange peel and banana peel in our study is lower than the values determined by Kuppusamy et al. (Kuppusamy et al., 2020) and Suleria et al. (Suleria et al., 2020). The differences in the values demonstrated by different studies could be due to the variations in geographic locations, growth conditions, varieties of agricultural products, and harvesting time of the tested samples. All these parameters are important factors for plant phenolic compound accumulation (Iloki-Assanga et al., 2015; Kuppusamy et al., 2020).

Table 2 Total phenolic content of different agro-wastes in different extract solution

Different agro-wastes	Total phenolic content (mg GAE/100g)			
	Water extract	Ethanol extract	Acetone extract	Methanol extract
Barley straw	5.53±0.54	3.15±0.19	9.43±0.23	4.46±0.16

Pumpkin pulp+seeds	5.28±0.63	8.05±0.44	13.82±0.16	9.81±0.64
Pomegranate peel	47.60±0.28	47.33±2.22	28.05±1.12	48.36±2.33
Orange peel	11.02±1.37	13.77±0.69	17.68±1.19	21.93±0.58
Maple leaf	46.81±0.84	46.99±1.94	46.84±1.04	47.96±1.67
Banana peel	13.51±1.01	10.15±0.57	10.71±0.20	15.94±1.13
Sesame seeds	Nd	7.54±0.52	14.55±0.27	9.09±0.51
Gingko leaf	5.96±0.16	14.25±0.41	13.13±0.66	14.01±0.81
Agave leaf	14.62±0.36	4.26±0.28	10.68±0.15	6.66±0.41
Corncob	21.96±0.42	6.44±0.28	8.54±0.31	6.04±0.15

Data are expressed as mean ± standard deviation, Nd: not determined

3.3. Total flavonoid content (TFC)

The TFC presented in the agro-waste is revealed in Table 3. Table 3 depicted the absence of phenolic content in agave leaf, corncob, and barley straw in all different solvent extracts. The pomegranate peel and maple leaf only gave the phenolic content in the water extract. Interestingly, a higher flavonoid content was observed in pumpkin pulp+seeds and sesame seeds in acetone extract while significantly less in ethanol extract. Orange peel exhibited low flavonoid content only in ethanol extract. The differences in flavonoid content in various solvents may be due to the different solubility of the flavonoid in the solvent used. The nature of the agro-wastes, like pH, solidity and the extraction temperature, might also be the affecting factors for the solubility of compounds in the solvent used (Kuppusamy et al., 2020). The TFC of orange peel and banana peel calculated in this study was lower than the value demonstrated by Suleria et al. (Suleria et al., 2020), however, pomegranate peel exhibited almost similar value.

Table 3 Total flavonoid content of various agro-wastes in different extract solution

	Total flavonoid (mg RE/100g)

Different agro-wastes	Water extract	Ethanol extract	Acetone extract	Methanol extract
Barley straw	0±4.74	0±2.01	0±7.59	0±6.22
Pumpkin pulp+seed	0±0.76	56.40±4.23	435.35±3.79	0±2.63
Pomegranate peel	230.57±4.69	245.44±2.01	37.98±0.76	314.25±3.30
Orange peel	0±4.23	3.33±0.76	0±11.35	0±3.79
Maple leaf	336.69±3.27	234.04±3.79	182.72±2.01	350.26±3.48
Banana peel	0±0.76	0±2.27	0±3.48	0±2.01
Sesame seed	Nd	30.96±2.01	793.68±6.58	0±3.94
Gingko leaf	0±1.32	125.70±3.31	203.59±5.17	102.02±2.74
Agave leaf	0±3.48	0±7.44	0±4.11	0±3.95
Corncob	0±5.73	0±2.11	0±0.00	0±2.01

Data are expressed as mean ± standard deviation, Nd: not determined

TPC and TFC in different extraction solvents indicated that the solvent used for extraction has a vital role in the extraction of flavonoid and phenolic content. This fact is supported by Kuppusamy et al. (Kuppusamy et al., 2020). They demonstrated the significant differences between the solvents used to extract phenolic and flavonoid content from various tested food waste (Kuppusamy et al., 2020).

3.4. Antioxidant activity and total antioxidant capacity (TAC)

Among different agro-waste in water extract, pomegranate peel illustrated the highest antioxidant activity by scavenging ABTS free radical of 70.86% followed by gingko leaf, barley straw, orange peel, agave leaf, sesame seeds, banana peel, maple leaf, and the least by corncob. Similarly in the methanol extract, gingko leaf exhibited the maximum antioxidant activity (78.47%) and least by sesame seeds. However, the ethanol extract of pomegranate peel demonstrated the highest

scavenging capacity (71.69%) followed by maple leaf, gingko leaf, banana peel, orange peel, and the least by sesame seeds (Table 4).

Table 4 Antioxidant activity exhibited by different agro-waste in different extract solutions

Different biomasses	Scavenging activity (%)		
	Water extract	Methanol extract	Ethanol extract
Banana peel	55.32	77.23	67.96
Barley straw	69.83	55.52	13.05
Pumpkin pulp+seeds	30.66	53.59	19.27
Orange peel	67.96	77.45	64.03
Sesame seeds	58.22	39.80	7.67
Gingko leaf	69.83	78.47	68.17
Agave leaf	66.93	77.79	40.61
Pomegranate peel	70.86	78.36	71.69
Maple leaf	32.53	76.55	71.28
Corncob	1.86	67.50	36.67

In this study, the antioxidant activity of agro-wastes was determined by scavenging free ABTS radical which is one of the widely used methods. This method is based on the tendency of phenolic compounds to donate hydrogen atom. The antioxidants present in sample reduce the free radical of scavenged ABTS and forms stable free radical which is measured calorimetrically (Katalinic et al., 2006; Suleria et al., 2020). The highest ABTS radical scavenging ability in this study was found in water, methanol, and ethanol extract of pomegranate peel referring the best natural antioxidant.

Moreover, the TAC of the agro-waste is determined by using an electron transfer mechanism. In this assay, molybdenum (VI) is reduced by any phenolic or antioxidant compounds to molybdenum (V) (Suleria et al., 2020). The different agro-wastes illustrated the different TAC in different extract solutions. As presented in Table 5, pomegranate peel extracted in water showed the highest TAC (55.03 ± 2.56 µg AAE/gDW) compared to all other agro-waste, whereas barley straw in ethanol extract exhibited the least (0.61 ± 0.09). Further, the table illustrated water as the best solvent for pomegranate peel, maple leaf, and banana peel to show TAC. Similarly, methanol was the best solvent for extracting antioxidants from pomegranate peel, banana peel, maple leaf, corn cob, ginkgo leaf, and orange peel.

Table 5 Total antioxidant capacity exhibited by different agro-waste in different extract solutions

Different biomass	**Total antioxidant capacity (µg AAE/gDW)**			
	Water extract	**Ethanol extract**	**Acetone extract**	**Methanol extract**
Barley straw	6.52 ± 0.11	0.61 ± 0.09	1.02 ± 0.01	1.94 ± 0.09
Banana peel	41.82 ± 0.94	6.27 ± 0.28	3.23 ± 0.15	22.23 ± 0.27
Maple leaf	50.45 ± 1.02	19.36 ± 0.66	10.74 ± 0.46	22.26 ± 0.44
Pumpkin pulp+seeds	5.18 ± 0.16	2.59 ± 0.21	1.83 ± 0.31	5.57 ± 0.05
Pomegranate peel	55.03 ± 2.56	23.99 ± 0.69	3.23 ± 0.1	27.31 ± 0.21
Corn cob	12.58 ± 0.08	6.13 ± 0.06	1.75 ± 0.05	22.02 ± 0.38
Gingko leaf	7.45 ± 0.14	6.7 ± 0.11	5.09 ± 0.09	13.71 ± 1.3
Sesame seeds	2.44 ± 0.05	2.35 ± 0.04	2.23 ± 0.09	1.41 ± 0.26
Agave leaf	5.8 ± 0.16	1.1 ± 0.09	1.85 ± 0.06	7.07 ± 0.13

Orange peel	8.61 ± 0.24	3.51 ± 0.12	2.7 ± 0.08	10.32 ± 0.46

Data are expressed as mean ± standard deviation

There are different assays or reactions or methods for determining antioxidant potential of plant materials because different phytochemicals have different complex nature and not a single method can determine the exact antioxidant potential (Suleria et al., 2020). From Tables 2 and 5, the values of TPC and antioxidant of pomegranate peel were observed to be higher, followed by maple leaf. The results might indicate the correlation between the total phenolic content and antioxidant of those agro-wastes. Pomegranate peel and maple leaf exhibiting higher TPC and total antioxidant can be used in cosmetic and dermatological applications as the phenolic compounds have been detected to have depigmentation properties, UV radiation absorbing capacity and inhibit UV irradiation-induced oxidative stress (Mapoung et al., 2021). The polyphenols from different samples depend on the types of solvent used, extraction procedures, plant parts used or origin of the sample, environment where the samples are grown (Iloki-Assanga et al., 2015), geographical location (Phuyal et al., 2020), age of the plant, and many other factors (Soto-Castro et al., 2021). The present study revealed that pomegranate peel and maple leaf having good flavonoid and phenolic content showed good antioxidant properties. This finding is supported by many other studies where literatures have mentioned that phenolic content having fruits and vegetables have good antioxidant activity (Jiao et al., 2019; Saini et al., 2019; Kuppusamy et al., 2020).

3.5. Antibacterial effect of agro-wastes extracts

The aqueous extract of agro-wastes illustrated antibacterial effects as represented in Fig. 1. Amoxicillin, a broad-spectrum antibiotic, was used as a positive control. The pumpkin pulp+seeds did not demonstrate any inhibition to the bacteria used. At the same time,

pomegranate peel, orange peel, maple leaf, banana peel, barley straw, barley spent grains, and canola straw showed antibacterial effects. The inhibition zone diameters of agro-wastes against bacteria were observed in the order of pomegranate peel against S-10 > S-17 > orange peel against C-19 > pomegranate peel against S-5 > orange peel against S-14 > barley straw against S-14 > maple leaf against S-5 and so on. Among those agro-wastes, orange peel illustrated an antibacterial effect against all bacteria used, while pomegranate peel extract did not inhibit C-19.

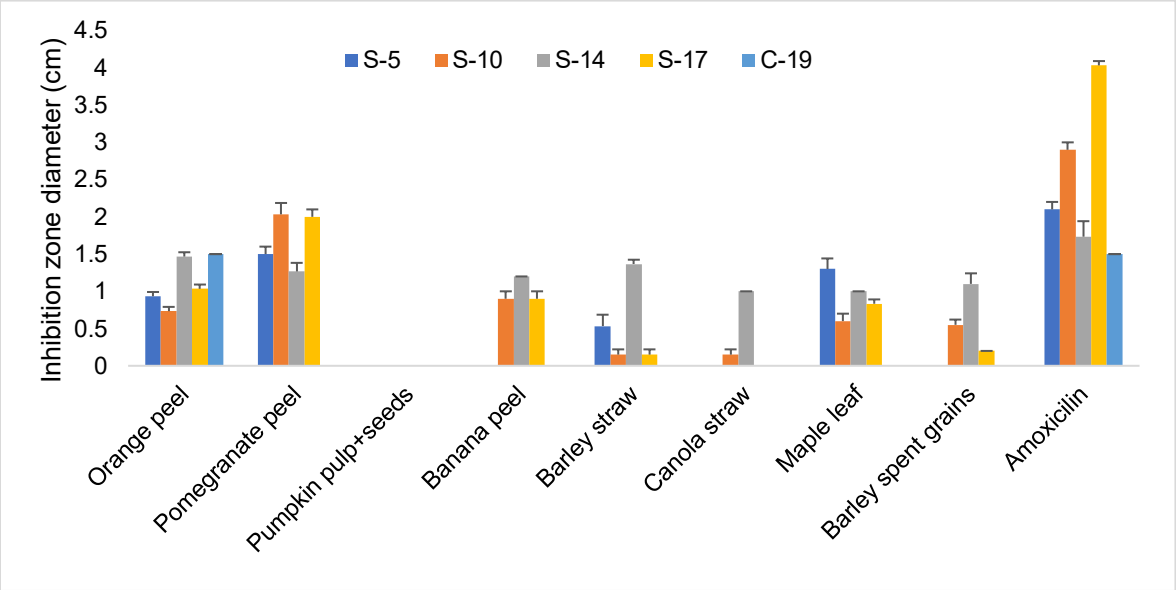

Fig. 1 Antibacterial effect of agro-wastes against bacteria (Bar represents the diameter of inhibition zone in cm ± standard deviation)

From Fig. 1, it is evident that C-19 was more resistant to agro-wastes extract. The antibacterial effect varies with the agro-wastes and bacteria used. This study is parallel to other studies, which illustrated that different seeds extracts and peels extracts exhibited different inhibition zone against various strains of bacteria (Abbas et al., 2019; Naqvi et al., 2020; Attia et al., 2021) . Naqvi et al. (Naqvi et al., 2020) illustrated ginger peel extracts to show the maximum inhibition zone against all bacterial strains, while onion peel did not show any inhibition zone among different agro-wastes peel studied. Similarly, Abbas et al. (Abbas et al., 2019) reported that the

presence of phytochemicals like terpenoids might be responsible for the antibacterial potency of agro-wastes seed extract and all seeds extracts were more susceptible to gram-positive bacteria compared to gram-negative bacteria. The bacterial cell wall component, peptidoglycan layer, plays a vital role in permeability of seeds extracts, resulting in the susceptibility of bacteria to the extracts (Abbas et al., 2019).

3.6. Pectin extraction from agro-waste

Besides total phenolic and flavonoid content, pectin can be extracted from those agro-wastes. Pectin has humongous applications in various industries, including the food and pharmaceutical industries. So, alternative innovative methods need to be studied for pectin production to comply with the demand for pectin. In the present study, pectin was extracted in traditional, and microwave assisted ways. From both methods, the maximum pectin was extracted from pumpkin pulp+seeds (11.24±0.21, 14.07±0.73), followed by orange peel (10.16±0.37, 9.96±0.11), banana peel (7.96±0.18, 9.46±0.24), pomegranate peel (7.91±0.21, 7.06±0.14), as depicted in Table 6. The microwave assisted method was found to be better for pectin extraction from pumpkin pulp+seeds and banana peel; however, the traditional method extracted slightly higher pectin from barley straw, pomegranate peel, and canola straw. There were negligible differences in pectin yield by both methods from maple leaf, orange peel, and barley spent grains (Table 6). The higher pectin yield from industrial pumpkin waste was 69.89 ± 2.90% under optimized conditions (89.98°C and pH 2.83 for 13 min) (Lalnunthari et al., 2020). Another study illustrated lower pectin yield by microwave heating (5.7%), while the increased pectin yield (10.5%) was observed with the microwave extraction after pumpkin powder was acid pretreated (Yoo et al., 2012). Our study showed that the pectin yield from orange peel (9.96±0.11%) was within the range to the pectin obtained by Benassi et al. (1-10%) (Benassi et al., 2021) and Patience et al.

(11%) (Patience et al., 2021). Similarly, the pectin yield from banana peel was within the range revealed by Oliveira et al. (Oliveira et al., 2016). The difference in pectin yield might be due to different extraction processes, extraction conditions, temperature, pH, pretreatment and types of agro-wastes, and more (Yoo et al., 2012; Oliveira et al., 2016; Lalnunthari et al., 2020; Patience et al., 2021). In the current study, the variation in heating procedures during extraction processes may be the reason for change in pectin yield. In the traditional technique, the mixture was incubated at 70℃ for 90 mins and microwave assisted technique the samples were microwaved for 3 mins, respectively.

Table 6 Pectin yield (%) from different agro-waste exploiting traditional and microwave assisted methods

Different biomass	Traditional Pectin extraction	Microwave assisted extraction
Barley straw	2.03±0.36	1.56±0.09
Pumpkin pulp+seeds	11.24±0.21	14.07±0.73
Pomegranate peel	7.91±0.21	7.06±0.14
Canola straw	3.65±0.37	1.97±0.17
Barley spent grains	6.37±0.34	6.01±0.18
Orange peel	10.16±0.37	9.96±0.11
Banana peel	7.96±0.18	9.46±0.24
Maple leaf	3.74±0.18	3.77±0.11

Data are represented as mean ± standard deviation

The traditional method is customarily used, simple, and conventional, but it is time consuming; thus, many alternatives are being studied. The microwave assisted extraction process is more

effective, accelerates energy transfer and is less time consuming (Raji et al., 2017). In this process, the heating occurs simultaneously and is fast. However, another study illustrated no conclusive differences in the microwave and conventional extraction of pectin from sugar beet pulp. The reason might be water use in microwave assisted extraction of pectin from sugar beet (Mao et al., 2019). A study illustrated acid used in pectin extraction is the main factor, and the citric acid enhanced the pectin extraction (Raji et al., 2017; Yang et al., 2018). Thus, our study exploited citric acid as an extraction solvent instead of hydrochloric acid as mineral acid. The yield of pectin also depends on different parameters like microwave time, pH, solid-liquid ratio, microwave power (Maran and Prakash, 2015), and extraction methods (Benassi et al., 2021). Benassi et al. extracted pectin from orange peel by exploiting various methods. They illustrated that the acidic hot water extraction method was superior, followed by the hot water microwave extraction method to all other methods (Benassi et al., 2021).

4. Conclusions

From this study, we can conclude that various agro-wastes contain different phytochemicals and exhibit antibacterial potency, which may potentially be used in diverse pharmaceutical industries. The study encourages using the cheap or low-cost biomass as a resource to decrease the production cost on one hand, and on the other hand pectin, polyphenols, and antibacterial can be extracted from the biomass. The extraction of products depends on the types of agro-wastes, methods and conditions of extraction, and solvents used. In addition, extracting value-added products such as polyphenols and pectin from different agro-wastes is a remarkable way to help to manage waste, decrease greenhouse gas emissions, and take economic advantages. Furthermore, the biopolymer from agro-wastes having antibacterial properties can be applied in bioplastic and food packaging material production. However, a detailed study of the extraction

process for optimal output of different health beneficial and economically important compounds from agro-wastes is recommended, and the process should be environmentally friendly, efficient, and cost-effective.

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5D Valorization of Agro-waste: Immobilization of Whole-cell for Pectinase Production, Adsorption of Crystal Violet for Color Remediation, and Composting

Abstract

An inexpensive, easily available, natural, and sustainable agro-wastes has the potential to be used as the immobilizing matrix for pectinase production, adsorbent for crystal violet, and forming compost. The pectinase production by immobilization depends on the concentration of substrate and inoculum volume, and the number of repeated runs. In addition, the study revealed orange peel as the best adsorbent and canola straw as the inferior adsorbent of crystal violet. The adsorption of crystal violet may be influenced by the agro-wastes, the concentration of crystal violet, and the time of exposure. Further, the study illustrated that laboratory-scale composts were formed within 90 days. During the composting process, there were changes in visual appearances of agro-wastes mixtures, temperature, and pH of composts. In the initial first week of composting, the temperature increased and declined. Similarly, the pH of the composts was low in the beginning and alkaline (around pH 9) at the end. The number of bacteria in all composts was 10^7 colony-forming units per gram of compost. Further, seven bacteria with different colony morphologies were observed. 100% moong seeds germinated in those composts; however, the weights of the seeds were different with the different composts. Thus, valorizing agro-wastes can finally help in waste management and pollution reduction.

Keywords: Agro-wastes; immobilization of bacteria; adsorption of crystal violet, composting

1. Introduction

Pectinase is one of the essential industrial enzymes which degrade the complex heteropolysaccharides pectin. The biotechnological application of pectinase in a wide range of industries is increasing. Pectinase is normally produced from fungi and bacteria for industrial

applications (Shrestha et al., 2021c). However, using pectinase in harsh conditions such as wastewater treatment and adding filamentous fungi in stirred tank bioreactors has technical challenges. Thus, to overcome such problems, immobilization methods can be applied (Zheng et al., 2022). Immobilizing the whole cell, such as bacteria and fungi onto solid substances limits cell mobility by physical means. Moreover, immobilization is an effective and economical method to boost some enzyme properties, including stability, activity, specificity, selectivity, and resistance to inhibitors. This process is effective for enzyme production, and improves enzyme properties, and is cost-effective as the immobilized cell can be recovered easily and used repeatedly (Bouabidi et al., 2019; Shrestha et al., 2021c).

Immobilization methods such as entrapment, covalent bonding, and adsorption are commonly used. However, the supporting materials can be organic or inorganic and should be selected wisely (Bouabidi et al., 2019). Low-cost agro-wastes can be an alternative for supporting cells (Kuhad et al., 2004; Ejaz et al., 2018) and reducing production costs (Ahmed et al., 2020). Further, the low-cost agro-wastes have the potential to be used in the adsorption of toxic metals or chemicals from different industrial effluent and research laboratories (Rezzadori et al., 2012). Due to the availability, cost-effectivity, recyclability of agro-wastes, and their applicability in different aspects, the low-cost natural adsorbents are widely used (Pagnanelli et al., 2005; Pérez-Marín et al., 2007; Li et al., 2008; Hameed and Ahmad, 2009). At the same time, the increasing demand of dyes in the textile industries is the main concern for the environmental threat because the dye containing wastewater is discharged into the water bodies. The chemicals present in water body is harmful for all living things, including animals, plants, and human health (Tahir et al., 2017) and it is opted to remove or reduce such harmful chemicals before being discharged into the water bodies.

Moreover, the low-cost wastes can be converted into soil additive via composting, a common and traditional method to manage and reduce waste. Further, waste management helps reduce pollution and eliminate disposal and other pollution-related problems (Yusuf, 2019). Composting is the simple, natural, and aerobic biological process of organic matter degradation into stable compost. This process is facilitated and accelerated by indigenous microorganisms (Limaye et al., 2017; Zainudin et al., 2022). Microorganisms like bacteria, fungi, and actinomycetes behave as composting agents (Zainudin et al., 2022) and degrade the organic materials present in organic wastes. The main compositions of the organic materials include cellulose, xylan, hemicellulose, and pectin (Edwards and Doran-Peterson, 2012; Saini et al., 2015; Shrestha et al., 2020) and their hydrolysis as well as degradation are possible only with the lignocellulolytic enzymes, including pectinases (Ravindran et al., 2018; Amadi et al., 2022; Shrestha et al., 2022). However, the variation in the agro-wastes components affects their degradation, subsequently influencing the composting process and the quality of compost products (Zainudin et al., 2022).

Different parameters for better compost formation include agro-wastes and microorganisms used, carbon-nitrogen ratio, moisture content, and aeration (Hariz et al., 2013; Külcü and Yaldiz, 2014; Zainudin et al., 2022). The presence of microorganisms during the process and after the maturation of compost is very important to determine the quality of compost (Zhang et al., 2018). In addition, the composting of different materials or agro-wastes requires different microorganisms (Zhang et al., 2018).

Thus, this study aims to valorize the agro-wastes by evaluating the potentiality of immobilizing bacterial cell for pectinase production and determining the best agro-waste among banana peel, canola straw, orange peel, and pomegranate peel for adsorption of crystal violet from aqueous

solution. Further, our study focuses on converting the agro-wastes mixture into compost by exploiting a single bacterium or bacterial mixture. The study explores the variation in temperature and pH during composting and the effect of composts in the germination of moong seeds. By utilizing agro-wastes in different aspects, the study helps in waste management, which finally aids in reducing pollution and disposal problems.

2. Materials and methods

2.1. Pretreatment of agro-waste

Agro-wastes such as orange peel, pomegranate peel, banana peel, brewer's spent grains, barley straw, and canola straw are locally available and were collected, dried, and ground in a coffee grinder. For adsorption and immobilization, they were alkali pretreated by placing those agro-wastes in 1% (w/v) sodium hydroxide solution at 50 mL/g and kept at room temperature overnight. The next day, the agro-wastes were washed with distilled water several times till neutral pH was observed and then dried in a hot air oven at 50°C for two days.

2.2. Microorganism and inoculum preparation for immobilization

The pectinase-producing bacteria (*Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Bacillus* sp. (S-17)) used in this study were isolated from forest soil near Lakehead University, Thunder Bay, Ontario, Canada and stored at -80°C. The bacterial identification was based on 16S rDNA sequence analysis (Shrestha et al., 2021b). For immobilization, *Streptomyces* sp. (S-5) was exploited, whereas for composting, *Streptomyces* sp. (S-5), *Cellulomonas* sp. (S-10), *Bacillus* sp. (S-17) and *Bacillus* sp. (C-19) isolated from the contaminated broth (Shrestha et al. 2022a) were used. The bacteria were revived by inoculating 1% v/v in 4 mL LB broth and incubated overnight at 35°C. Then 1 ml of overnight culture was inoculated onto fresh LB media till optical

density (OD_{600}) reached about 1. For pectinase production, 1% v/v was inoculated onto pectin containing media and incubated at 35℃, and 180 rpm.

2.3. Immobilization of the bacterium on canola straw

The pretreated canola straw (1% w/v) was taken in the flask with 50 mL of pectinase producing media, and 2% v/v of bacterial inoculum was added and incubated at 35℃ for 48 hours. Then the adsorbed bacterium was collected by centrifugation while the cell free supernatant (enzyme extract) was used to determine pectinase activity. The canola straw with bacterium was washed with distilled water three times, fresh pectinase production media added, and a further process was followed, as previously mentioned. Such process was repeated four times aseptically. The pectinase activity was estimated by determining the reducing sugar following the dinitrosalicylic acid (DNS) method (Miller, 1959) as described in previous study (Shrestha et al., 2021b).

2.4. Adsorption of crystal violet in agro-waste

For adsorption of crystal violet, four different agro-waste (canola straw, orange peel, pomegranate peel, and banana peel) were used. Three concentrations (0.5, 1, and 1.5% w/v) of crystal violet were added to the flasks containing 1% w/v agro-waste as test samples and without agro-waste as control. All the flasks were incubated at 32℃ and 180 rpm shaking incubator. At different time durations, the optical density at 660 nm (OD_{660}) was taken to calculate the adsorption percentage as follows.

$$\text{Adsorption (\%)} = \frac{(\text{OD at 0 hour} - \text{OD at time t})}{\text{OD at 0 hour}} \text{ X } 100$$

2.5. Composting units

Six big glass jars were cleaned, dried, and autoclaved. The mixture of barley straw (50 g), canola straw (50 g), brewer's spent grains (50 g), banana peel (40 g), orange peel (35g), and

pomegranate peel (10g) were added to the bottles. This proportion of the waste was determined based on the availability of the waste. Then 140 ml distilled water was added to the mixture of agro-wastes, mixed properly, and 20 mL of bacterial inoculum was added to each jar. Only distilled water (no bacteria) was added to 1st jar (N1), culture broth of S-5 to 2nd (N2), S-10 to 3rd (N3), S-17 to 4th (N4), a mixture of S-5, S-10, S-17 to 5th (N5), and a mixture of S-5, S-10, S-17, and C-19 to 6th (N6) jar. After, they were covered with lids and kept at room temperature for 90 days.

2.5.1. Maintaining the moisture level

In the initial few days, some amount of distilled water was added to maintain the moisture level. On the 1st day, 25 ml of distilled water was added, 10 mL on the 6th day, and 20 mL on the 10th day. After the 10th day, the mixture of agro-wastes appeared somewhat moist, so no distilled water was added.

2.5.2. pH and temperature of the composts

In certain time intervals, 1g of agro-wastes were collected in 50 mL centrifuge tubes, and 10 mL of distilled water was added, vortexed, and pH was measured. At the same time, the temperature was measured by inserting the thermometer in the middle of the agro-wastes mixture.

2.5.3. Germination test

Each compost sample (1 g) was collected, and 10 mL distilled water at a ratio of 1:10 (w/v) was added to the petri-dish. Ten moong seeds were placed on the petri-dish with compost and water and left for a few days to germinate and grow at room temperature. The number of germinated seeds was counted, and later the weight of the seedlings was taken and compared.

2.5.4. Number of bacteria

Five grams of each compost sample was taken and mixed with 100 mL of distilled water. Tenfold serial dilutions of each compost sample were performed and spread on NA plates for bacterial isolation. The plates were incubated at 30℃ for 2 days. The bacteria isolated were counted and expressed as colony-forming units per mL. The plate having colonies between 25 to 200 was counted.

2.6. Statistical analysis

All the experiments were performed in triplicates and all the results data were recorded and analyzed in excel.

3. Results and discussions

3.1. Immobilization of *Streptomyces* sp. (S-5) on canola straw

The alkali pretreated canola straw was used to immobilize *Streptomyces* sp. (S-5), for pectinase activity. It was observed that the bacterium was adsorbed on the surface of canola straw (Fig. 1). The immobilized bacterium demonstrated the pectinase activity for the continuous four runs. The scanning electron micrographs (SEM) depict that before immobilization of the bacterium, the surface of the canola straw was smoother (Fig. 1a) than after immobilization (Fig. 1b). The bulging surface observed in Fig. 1 b indicated the bacterium attached or restricted on the canola straw surface.

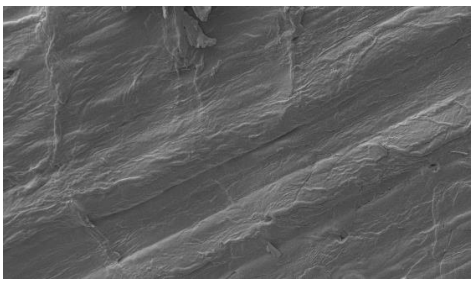
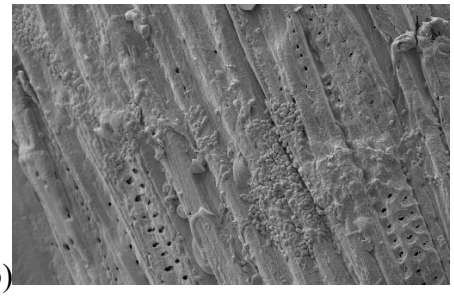
a)  **b)** 

Fig. 1 Scanning electron micrographs of canola straw **a**) before immobilization and **b**) after immobilization of *Streptomyces* sp. (S-5)

In general, immobilization of a whole cell or enzyme are conducted by entrapping or attaching to organic or inorganic carriers; organic includes agar, chitin, alginate, polymers, and inorganic includes porous glass, activated charcoal, and ceramics (Bouabidi et al., 2019). In our study, we used a readily available local natural organic carrier, canola straw, for immobilizing *Streptomyces* sp. (S-5) because it is readily available, non-toxic, insoluble, inexpensive, and provides an inert environment to bacterial cells. The bacterial cell immobilization to canola straw is via adsorption, which is a physical binding of a cell to water insoluble surface of canola straw (Bouabidi et al., 2019). Another study has illustrated the increased co-production of tannase and pectinase by immobilized yeast than the free cell (Taskin, 2013).

3.2. Pectinase activity by immobilized bacterium

The present study demonstrated that pectinase activity was observed to be significantly lower in 1st and 2nd runs; however higher in the 3rd run and again decreased in the 4th run. The pectinase activity was significantly higher in the 3rd run but not significantly different between the free bacterium and the 4th run of immobilized bacterium (Fig. 2).

The significant decrease in pectinase activity illustrated by the immobilized bacterium in the 1st and 2nd runs might be due to some time required by the bacterium to adjust in a new environment or might be due to washed out of the adsorbed bacteria while washing and not sufficient for enzyme activity.

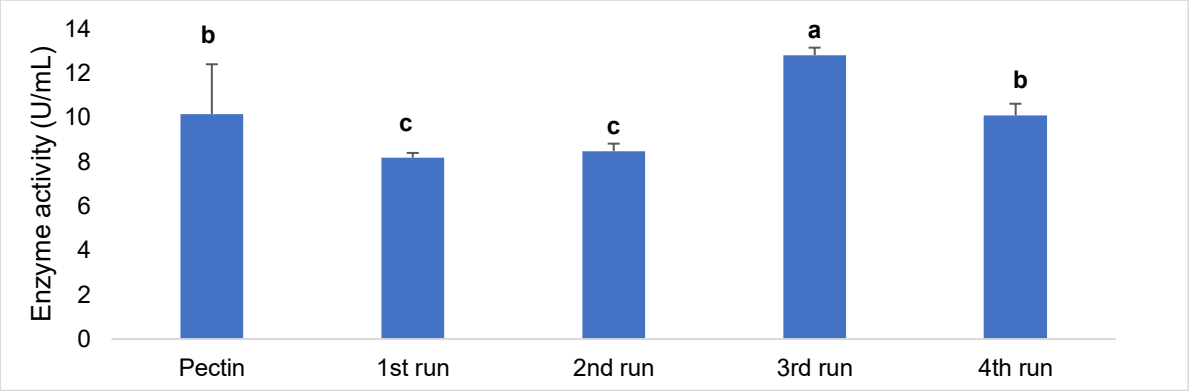


Fig. 2 Pectinase activity by free and immobilized *Streptomyces* sp. (S-5) in canola straw. The bars are represented as mean ± standard deviation, and lowercase alphabets indicate the result is statistically significant (P≤0.05).

When the different concentrations of canola straw and inoculum volume of the bacterium were used, the enzyme activity by each sample is presented in Fig. 3. Here, the maximum enzyme activity was illustrated by the sample having 1.5% w/v substrate and 1% v/v inoculum volume and lowest enzyme activity in 1% w/v substrate and 1% v/v inoculum volume in 1st run (Fig. 3).

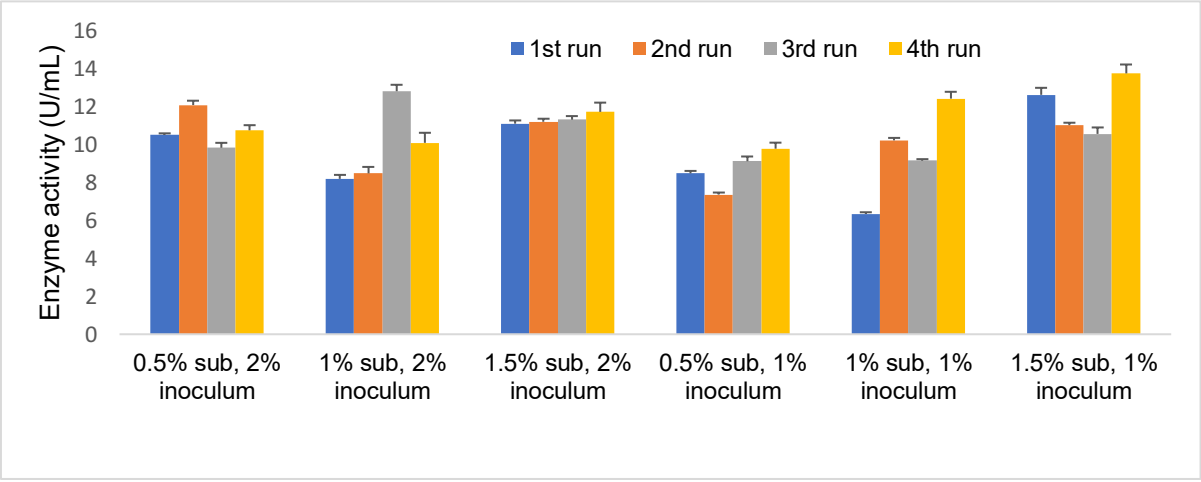


Fig. 3 Pectinase activity by immobilized *Streptomyces* sp. (S-5) in different concentration of biomass and inoculum. The bars are represented as mean ± standard deviation.

The study also demonstrated that the canola straw could be used as the carrier for whole cell immobilization for enzyme production. The substrate concentration and inoculum volume of the

bacterium play an important role during immobilization (Wang et al., 2014; Ejaz et al., 2018, 2020). Ezaj et al. (2020) demonstrated that substrate concentration affected pectinase production (Ejaz et al., 2020). Another study revealed that the different immobilization parameters, such as bath temperature, reaction time, embedding cell amount, have influenced the degradation of crude oil in the soil by the immobilized bacterium (Wang et al., 2014). Furthermore, *Geotricum candidum* yeast was immobilized in sodium alginate beads for pectinase production, and the production was higher in alginate beads than in a free cell. Moreover, when orange peel was used as the substrate instead of pectin, the immobilized cell produced higher pectinase by using orange peel comparable to pectin. Thus, immobilization and using natural biomass can increase enzyme production and reduce the production costs (Ejaz et al., 2020). In addition, immobilized cells can be used to degrade various wastewater contaminants and remove dye and color in industrial wastewater as well (Bouabidi et al., 2019).

3.3. Agro-wastes as an absorbent of crystal violet

The present study utilized different biomasses, including banana peel, orange peel, pomegranate peel, and canola straw, to evaluate the adsorption of crystal violet on those biomasses. Our study revealed the orange peel as the best adsorbent of crystal violet among the different agro-wastes studied in different concentrations (0.5, 1, and 2%). The adsorption of crystal violet was maximum for low concentrations of crystal violet and showed that the adsorption capacity (%) decreased as the concentration of the crystal violet increased (Fig. 4).

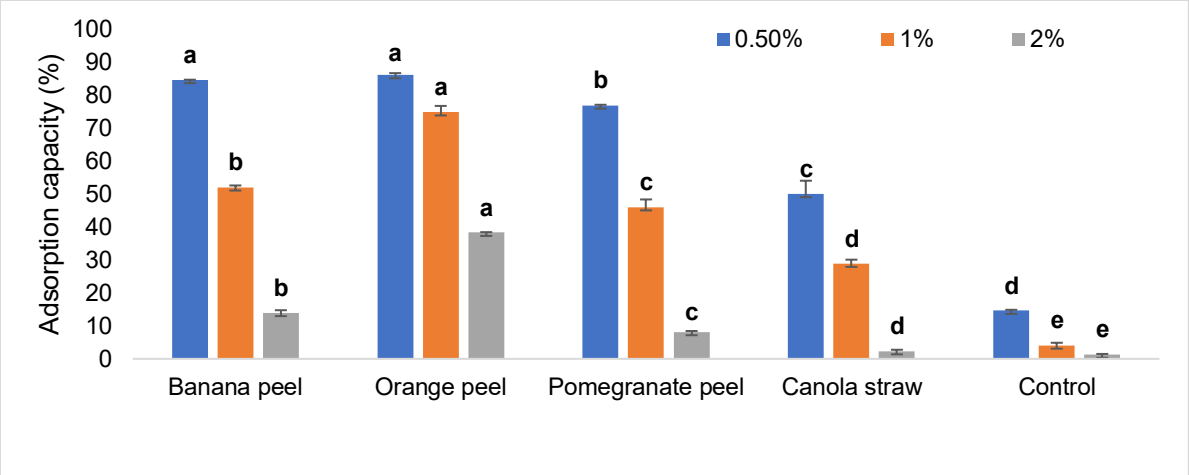


Fig. 4 Adsorption capacity (%) of different biomasses

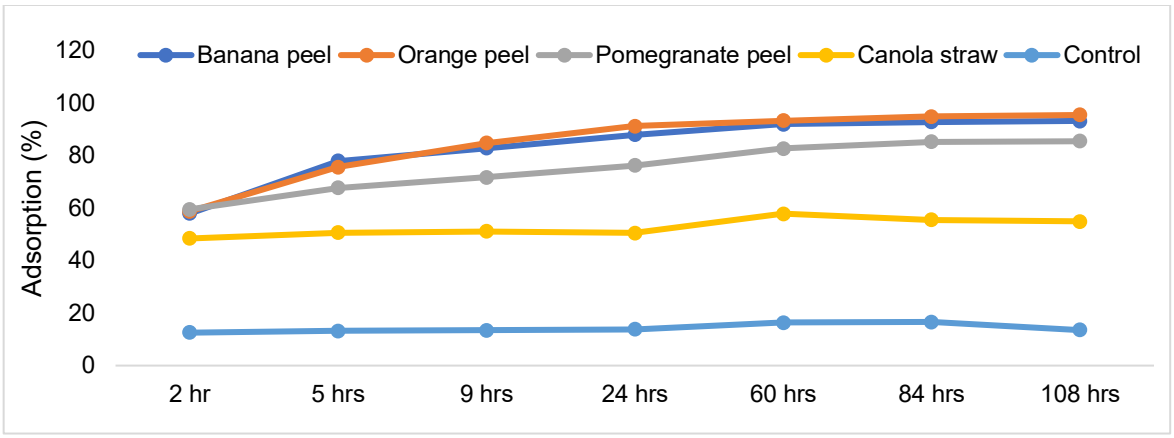
Various biomasses have been used in bioremediation process for example, 4-nitro phenol adsorption was held in groundnut shell, and biodegradation of crude oil present in soil by immobilized cell (Wang et al., 2014; Sarkar et al., 2022). Immobilization of yeast cell in corn cob for pectinase production by entrapping was explored in another study (Ahmed et al., 2020). Use of orange peel as an inducer for polygalacturonase production by *Aspergillus niger* immobilized in modified polyvinyl alcohol beads (Nighojkar et al., 2006), and production of various value-added products from different lignocellulosic biomass (Shrestha et al., 2020, 2021a). Different types of agro-wastes such as gram husk, orange peel, and tea leaves have been studied for adsorption of heavy metals including cobalt, cadmium, nickel, and zinc (Li et al., 2008). Garlic peel which is an easily available agricultural waste has been studied for methylene blue dye adsorption from an aqueous solution (Hameed and Ahmad, 2009). Different kinds of orange waste are used in the adsorption of Ni from electroplating effluent, cadmium, zinc, and lead from aqueous solution (Pérez-Marín et al., 2008), copper, lead, and cadmium adsorption onto olive pomace (Pagnanelli et al., 2005).

Adsorption of crystal violet dye is the promising conventional technique for color remediation from wastewater treatment. Therefore, the current study used different agro-wastes as absorbent

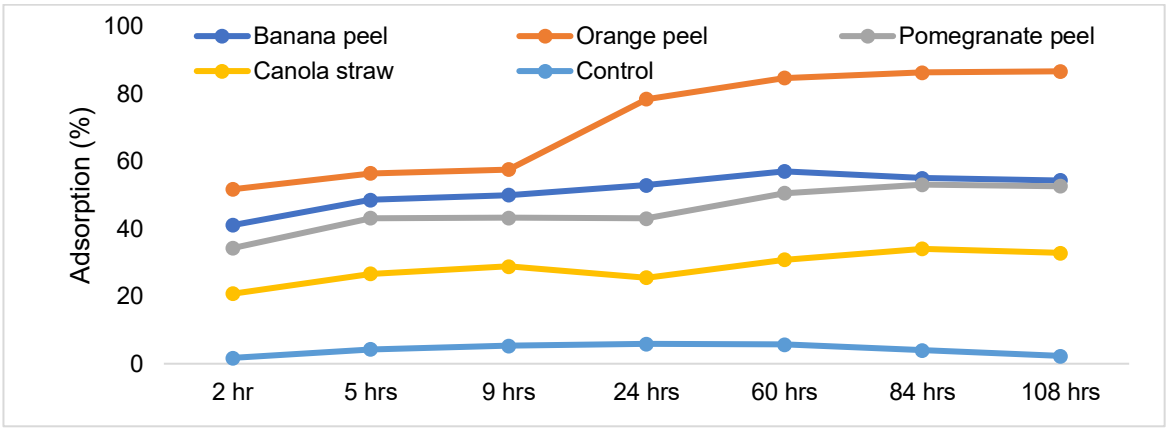
and crystal violet as adsorbate. Among them, orange peel showed as the best adsorbent of crystal violet. This may be due to the greater surface area for adsorption of crystal violet in orange peel than other agro-wastes and the exposure of many functional groups for binding of orange peel and crystal violet. The polysaccharides and proteins consisting different functional groups, such as carboxyl and amino, play a crucial role in adsorption of crystal violet in the biomass (Tahir et al., 2017).

The maximum adsorption of crystal violet in low concentration of orange peel may be due to the functional groups present in the adsorption of crystal violet and biomass was maximum/ equilibrated on low concentration (0.5%) of crystal violet. A study concluded the adsorption of cadmium onto orange waste was maximum at the equilibrium condition of functional groups participation in adsorption (Pérez-Marín et al., 2007). Our result was supported by Fernández-Pérez and Marban (Fernández-Pérez and Marbán, 2020) where they mentioned that the adsorption of H_2O_2 on the catalyst increases as the dosage of catalyst was increased but the adsorption of intermediates decreased the adsorption of H_2O_2 on catalyst.

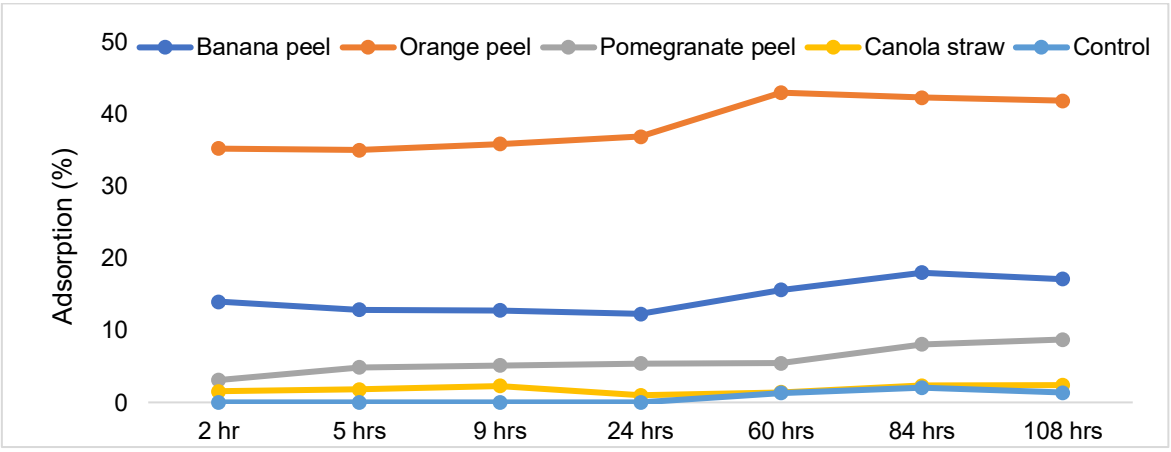
Fig. 5a depicted the adsorption of crystal violet (0.5%) by orange peel (OP) and banana peel (BP) was similar and reached almost 100% in 108 hours. The adsorption by OP and BP was continuously increasing till 24 hours but almost constant from 24 to 108 hours. The pomegranate peel showed a continuous slight increase in the adsorption of crystal violet till 84 hours. However, the adsorption by canola straw was the least among the biomass used and was not increasing (Fig. 5a).



a)



b)



c)

Fig. 5 The adsorption of crystal violet at **a**) 0.5%, **b**) 1%, and **c**) 2% (w/v) with respect to time

Fig. 5b elucidated that orange peel as the best adsorbent of crystal violet (1% w/v) among the studied agro-wastes. The orange peel showed about 50% of crystal violet adsorption in 2 hours and almost constant till 9 hours. However, the adsorption increased to 80% in 24 hours and

slightly increased till 60 hours and after almost constant (~85%) (Fig. 5b). Similarly, Fig. 5c illustrated the maximum adsorption (35%) of crystal violet (2%) was by orange peel in 2 hours which was constant till 24 hours and increased to 43% in 60 hours (Fig. 5c). Therefore, Fig. 5a, b, and c aided in concluding the adsorption of crystal violet was almost maximum within 24 hours. The lower and almost steady adsorption of crystal violet after 24 hours in different concentrations of crystal violet may be due to the less vacant sites for diffusion of dye in biomass after 24 hours. A similar supporting result was observed in the adsorption of crystal violet onto starch, chitosan aniline, chitosan pyrrole composites, polypyrrole, and peanut hull biomass (Tahir et al., 2017).

3.4. Changes in visual appearance in the composts

During the composting process, there were some changes in appearance, odor, and organism growth. The color and texture of the agro-wastes mixtures on the 1st day were clean, pale yellowish, and shiny with a slight orange odor. On the 6th day, there was prominent cottony growth observed in N3, N4, and N5 samples, and hazy growth in N2 and N6 samples with a musty odor. The cottony hazy growth was not observed later. And, on the 90th day, the agro-wastes changed into dark humus like appearance with the compost odor. All the compost samples appeared similar only with slight color change (Fig. 6). The hazy cottony growth in the initial few days may be due to the fungal growth favored by the acidic pH and lower temperature. The low pH in the beginning of the composting process is because of the pH of the waste which generally ranges from 4.5-5.1 (Sundberg and Jönsson, 2008).

Composting is aerobic, heat generating process, and active microbial system which change environmental conditions such as dryness of biomass, change in pH and temperature, and differences in microbial population (Sundberg and Jönsson, 2008; Külcü and Yaldiz, 2014). In

this study, a few milliliters of distilled water were added 3 times in the beginning of the process (within 10 days) and the agro-wastes were mixed and turned upside down every 15 days for aeration. Similarly, a study reported water was added every alternate days for first week to maintain moisture content for optimum degradation process (Hariz et al., 2013). The microorganisms use moisture and organic materials, generate heat and other products using air of pore space (Sundberg and Jönsson, 2008).

Day 1 

Days	N1	N2	N3	N4	N5	N6
6						
90						

Fig. 6 Changes in visual appearance of agro-wastes during composting

3.5. Change in temperature and pH during composting

During composting of agro-wastes mixtures using different bacterium and their consortia, there was a change in temperature. The trends of temperature change for 6 compost samples were similar with only a slight difference (Fig. 7a). On the initial day, the temperature was the temperature of the room (25°C) for all samples, on 4th day the temperature reached 35°C except

for sample N3 (32°C), and after the 4th day, the temperature decreased down to 27 to 28°C on 90 days.

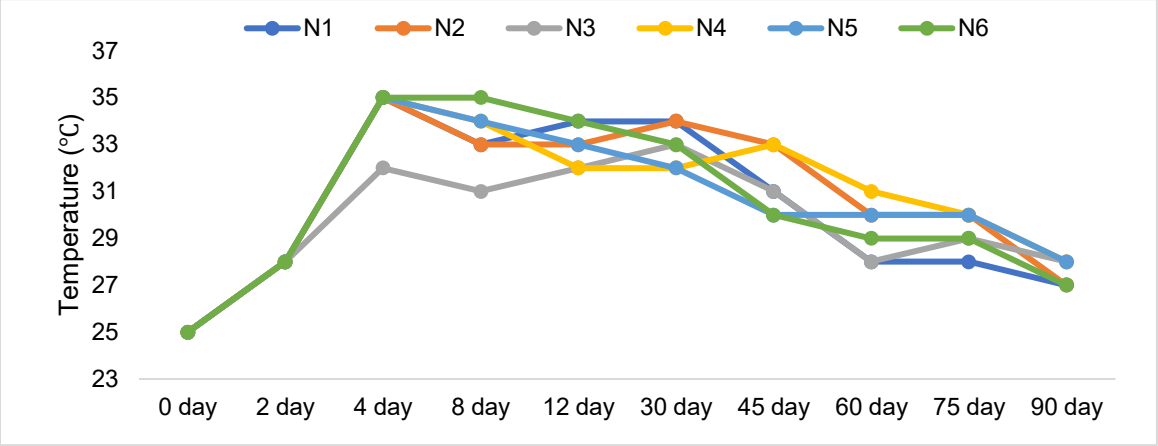


Fig. 7a Trend in temperature change during composting

The temperature of compost sample N3 was almost the same from the 4th day to 30th day which then declined until the 60th day. Similarly, the temperature from the 8th day to 30th day for N1 and N2 were almost constant but after 30th day there was a decline in temperature. While N4, the temperature was almost the same from 12th day to 45th day. Our study did not depict the thermophilic temperature (higher than 50°C) during the composting process. This may be because the temperature was not measured from 12th day to 30th day and during this period the temperature might have reached above 50°C. The temperature of compost needs to reach above 50°C for hygienisation aspect. However, higher temperature is not desirable because the higher temperature limits the microorganisms growth and activity resulting in slow decomposition process (Luskar et al., 2022).

The similar trend of temperature changes i.e increasing in initial stage and decreasing in the final stage was observed in other studies performed by Luskar et al. (2022) (Luskar et al., 2022), Zhang et al. (2018) (Zhang et al., 2018) and Hariz et al. (2013) (Hariz et al., 2013) and supported the current study. This is because the microorganisms release energy and heat by organic

materials decomposition during composting which results in rising temperature. The increase in temperature may also be due to the higher microbial activities in composting process (Zhang et al., 2018). This mechanism is useful to deactivate or reduce pathogenic microorganisms present in agro-wastes.

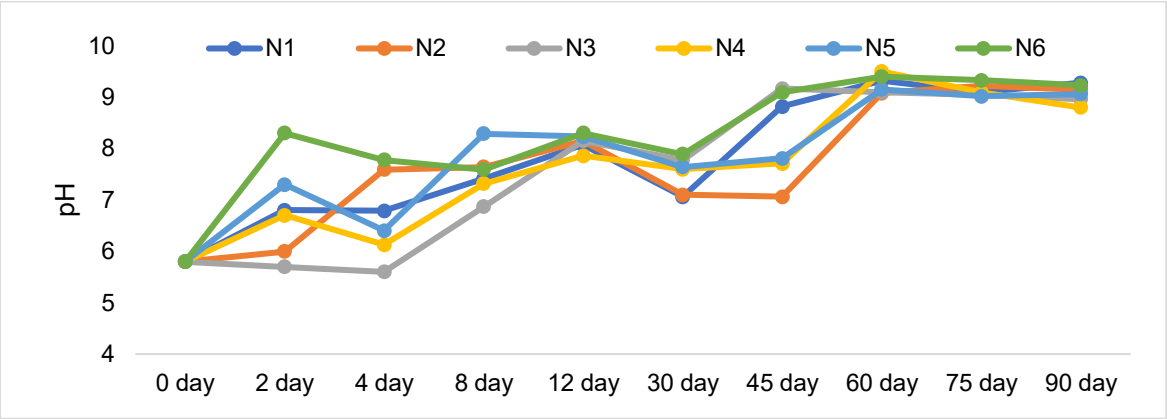


Fig. 7b Change in pH of the compost samples (N1, N2, N3, N4, N4, N6)

The low pH in the initial process is due to the formation of organic acids (commonly acetic acid and lactic acid) during composting (Sundberg and Jönsson, 2008; Luskar et al., 2022). Whereas, higher (alkaline) pH in the end indicates the high content of NH_4^+ which could be due to the decomposition of nitrogenous material and accumulation of ammonia which forms alkaline NH_4^+ when dissolves with water (Luskar et al., 2022). The temperature of the compost at the final stage is slightly higher than room temperature because of the increased aeration. The higher aeration decreases temperature which further increases pH during end of composting (Sundberg and Jönsson, 2008).

In addition, the current study illustrated almost stable temperature and pH after 60 days. Thus, this may indicate the maturity of compost after 60 days. Other study demonstrated the maturity of the compost after 38 days and the maturity depends on the wastes used as well as the environmental conditions during composting (Hariz et al., 2013).

3.6. Effects of compost on germination of seeds

The moong seeds were all germinated (100%) in all different composts (N1, N2, N3, N4, N5, and N6). However, the germinated seeds from N1 compost appeared not well grown as others. Visually, the germination of seeds appeared better in N5, N3, and N6 (Fig. 8a). This may be because the bacterial mixture added in N5 and N6, and bacterium added in N3 may have produced some essential products or they may have synergistic mechanisms that stimulate the growth of seedlings; however, no bacterium was added to the compost mixture N1.

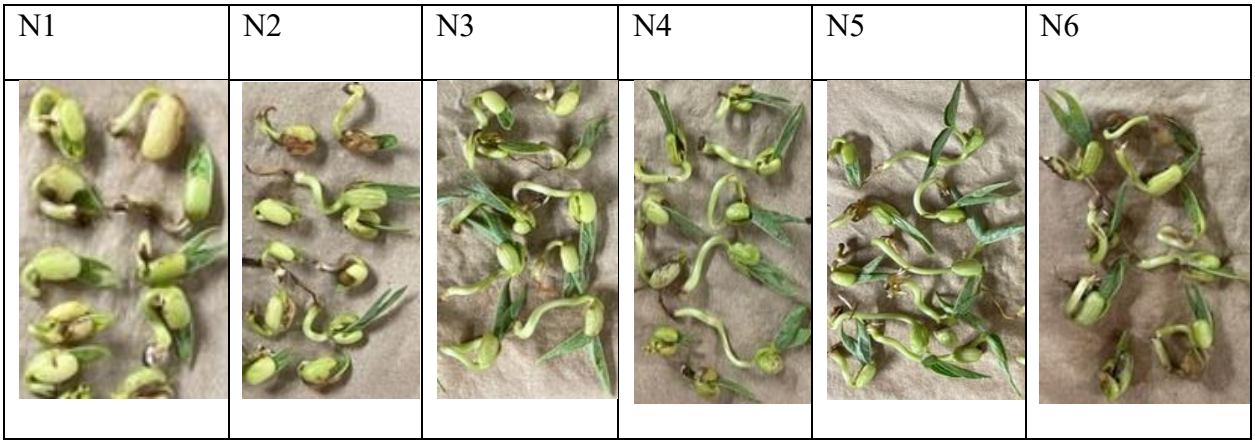


Fig. 8a Morphology of germinated seeds on the composts

The weight of those germinated seeds from N5 compost illustrated the highest weight and may indicate the best germination of seeds (Fig. 8b).

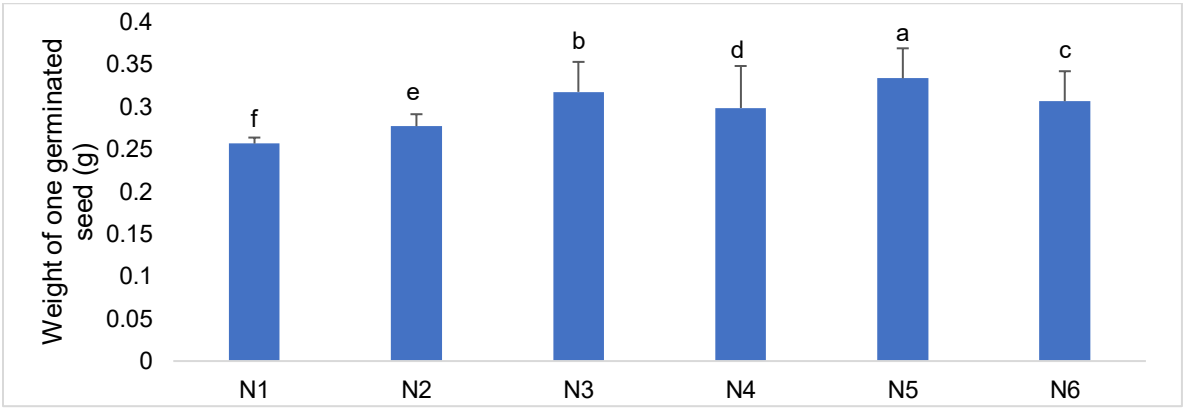


Fig. 8b Weight of the germinated seeds from the composts

3.7. Isolation of bacteria from different composts

The bacterial population of the compost formed was analyzed by counting the viable individual organisms and representing as colony forming unit per gram of compost (cfu/g) which is cultivation-based method. The highest number of bacteria (cfu/g) was illustrated from N6 compost ($6.1X10^7$ cfu/g) followed by N4 compost ($4.9X10^7$ cfu/g) and least from N3 compost ($3.3X10^7$ cfu/g), whereas other compost samples showed the bacterial population of around $4.5X10^7$ cfu/g (Table 1). A study revealed the bacterial population from the hop compost which were initially mixed with biochar, effective microorganisms and no additives ranged from 4.7×10^6 to 3.2×10^7 cfu/g (Luskar et al., 2022). The number of bacteria observed was not in accordance with the bacteria added in the initial stage of composting. This finding is supported by the conclusion made by Zhang et al. (Zhang et al., 2018) which stated that the yield of compost is not always related to the initial microbial load and diversity of materials used. The lowest number of bacteria in compost N3 may be due to presence of inhibiting indigenous bacteria, or high temperature during 12 to 30 days, or the compost not favoring the growth of bacteria. Interestingly, 7 different types of bacterial colonies were observed from compost N3. Besides, the germination of moong seed was 100% and the weight of the seedlings grown on this compost N3 were higher than the seedlings grown on compost N6, N4, N2, and N1 (Fig. 8b).

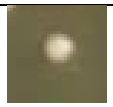
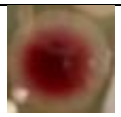
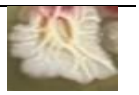
Table 1 Number of bacteria observed in each compost samples

Compost	Colony forming unit (cfu/g)	Types of colonies
N1 (control)	$4.7X10^7$	6
N2 (S-5)	$4.5X10^7$	7
N3 (S-10)	$3.3X10^7$	7
N4 (S-17)	$4.9X10^7$	6

N5 (S-5, 10, 17)	$4.5X10^7$	6
N6 (S-5, 10, 17, C-19)	$6.1X10^7$	7

The 6-7 different types of colonies were observed on the agar plates on which the different composts were spread. The colony morphologies of seven different observed bacteria are listed in Table 2.

Table 2 Different bacteria isolated from compost

S.N	**Colony morphology**	
C-1	Small, round, shiny, circular, raised, hard consistency, 1-2 mm in diameter	
C-2	Pink-center, pale periphery, circular, shiny,7-8 mm (4 mm pink circular center)	
C-3	Dark red center, pale translucent periphery, circular, shiny, 9 mm in diameter (4 mm red circular center)	
C-4	Big, whitish rough, irregular edge, wrinkled surface	
C-5	Pale, circular, translucent, flat like colony, 3-4 mm diameter	
C-6	Big, diffused colony, non-circular, rough edge, buttery in consistency	
C-7	Light pink, translucent, big, shiny, center pointed, 3-4 mm in diameter	

All these bacteria can grow in higher pH (~8.5) since the compost illustrated pH higher than 8.5. Therefore, those bacteria may have some potential to be used in industrial wastewater treatment and for other applications where pH is alkaline.

4. Conclusions

An inexpensive, easily available, natural, and sustainable canola straw has the potential to be used as the immobilizing matrix for pectinase production. The pectinase production by immobilization also depended on the concentration of substrate and inoculum volume, and number of repeated runs. The canola straw, banana peel, orange peel, and pomegranate peel can be used as the adsorbent of crystal violet. However, among four different types of agro-wastes studied in this study, orange peel was found to be the superior adsorbent and canola straw as the inferior adsorbent of crystal violet in different concentrations. The study revealed that the types of agro-waste, concentration of crystal violet and time of exposure have the effect on adsorption of crystal violet. Besides, the study also illustrated that the composts were formed within 90 days in laboratory scale. During the composting process there was a change in visual appearance of agro-wastes mixtures, temperature, and pH of composts. In the initial stage within the first week, the temperature increased and after declined. Similarly, the pH of the compost was low in the beginning, increased continuously and remained almost constant (around pH 9) at the end. The number of bacteria in all composts was 10^7 colony forming unit per gram of compost. Further, seven bacteria with different colony morphologies were observed from the compost. The germination of the moong seeds with those composts were observed 100%, however, the weights of the seedlings were observed different with the different composts. Since the pH of the composts were alkaline having around pH 9 and the germination of seeds were 100%, it can be concluded the germination of moong seeds was favored by alkaline pH and those composts can be added to the acidic soil for neutralizing soil and helping to grow the plants that require neutral pH. Thus, this study concluded the low-cost, sustainable organic agro-wastes have the potential to immobilize bacteria for enzyme production, color remediation from aqueous solution, and

enhance the soil quality by producing biofertilizer or compost. Therefore, the utilization of agro-waste can finally help in waste management and pollution reduction.

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5E Saccharification of Agro-waste by *Streptomyces* sp. and Ethanol Production from Agro-waste Mixture Hydrolysate

Abstract

The present work demonstrated the utilization of different agro-waste mixtures in producing ethanol. The forest soil bacterium (*Streptomyces* sp.) was exploited for the saccharification of the agro-waste mixture which was formulated by the extreme vertices mixture design. The best formulation contained 43.33% orange peel, 33.33 % pumpkin pulp+seeds, and 23.33% pomegranate peel which exhibited significantly high saccharification (22.36±0.54 mg/g dry weight) among all other mixtures. The hydrolysate produced from this formulated mixture was used by yeast in fermenting into ethanol. The hydrolysate when supplemented with 2% w/v fructose produced a maximum of 7.86±0.08% v/v ethanol by the yeast isolated from the brewer's spent grains. Thus, easily available waste could be a promising source for yeast isolation and agro-waste mixture as feedstock for ethanol production.

Keywords: *Streptomyces* sp., mixture formulation, agro-waste, ethanol

1. Introduction

Waste production is continuously increasing, so the different valorization processes of those wastes have drawn attention, which is crucial. In the meantime, due to the increase in population, the energy and gasoline crisis is startling and the study or search for alternate energy sources is increasing too (Jacob, 2009; Gupta and Verma, 2015; Yusuf, 2019). Ethanol production from food-based first generation lowers the production cost but at the same time, this hampers the food demands and supplies as first-generation ethanol production uses starchy and sugar crops such as corn, beets, and wheat. Thus, the second generation of ethanol production which utilizes food waste or lignocellulosic materials is more sustainable, attractive, and beneficial (Kang et al.,

2014; Zabed et al., 2016; Mensah et al., 2021). Lignocellulosic materials include non-edible food such as corncob, cornstalk, cornhusk, sugarcane bagasse, and sugarcane bark which don't have food value and do not impede the food supplies. By using those wastes for ethanol production the waste is converted into wealth on one hand and in another hand, this step helps to manage waste, decrease pollution, and decrease greenhouse gas emissions (Kurian et al., 2013; Gupta and Verma, 2015; Yusuf, 2019; Shrestha et al., 2020).

The worldwide interest in producing ethanol from lignocellulosic biomass is increasing due to the concern/focus on renewable sources and the depletion of fossil fuel reserves. Bioethanol can be used both for heat and power and recently it has been used as liquid biofuel. The bioethanol production process involves mainly pretreatment, hydrolysis, fermentation, and purification steps (Gupta and Verma, 2015; Zabed et al., 2016). All those processes are not the same for all the biomass for ethanol production because the compositions and physiochemical properties of those biomasses vary from each other. Thus, the selections of biomass, pretreatment method, microorganisms, and type of fermentation process should be taken into consideration for maximum ethanol production (Zabed et al., 2016). Different microorganisms have the optimistic potential for the process development of ethanol production. Besides, there are different strategies applied in ethanol production; separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bioprocessing (CBP) (Parawira and Tekere, 2011). Ethanol production using agro-waste following separate hydrolysis and fermentation is a conventional and easy method.

This study reports on the production of ethanol from agricultural wastes mixture following the separate hydrolysis and fermentation process. The hydrolysis of the agro-wastes mixture by *Streptomyces* sp. whereas the fermentation of the hydrolysate by using three different yeasts. The

formulation of the agro-waste was determined by the extreme vertices mixtures design (EVMD) using three agro-waste (orange peel, pomegranate peel, and pumpkin pulp+seeds) combination as this design elucidated to be the best for saccharification of biomass compared to other combinations and single agro-waste.

2. Materials and methods

2.1. Materials

Different agro-wastes which are locally available such as orange peel, pomegranate peel, pumpkin pulp+seeds, and canola straw were used. And brewer's spent grains were collected from a local brewing company (Sleeping Giant Brewing Co., Thunder Bay, Ontario, Canada). All those wastes were dried, ground with a coffee grinder, washed with warm water till the presence of impurities such as simple sugar. The presence of reducing sugar was detected by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). After agro-waste were washed, they were dried in a 50℃ oven for 48 hours till the consistent weight was noticed and kept in airtight containers for further use.

2.2. Microbial strain and inoculum preparation for saccharification

The bacterium used in the present study was *Streptomyces* sp. that was isolated from the forest soil (Thunder Bay, Ontario, Canada) and was identified based on colony morphology, biochemical characteristics, microscopic characterization, and 16S rDNA sequencing (Shrestha et al., 2021b). The bacterium was activated by sub-culturing in LB at 35°C for 24 hours and used as the seed culture for saccharification of agro-waste.

2.3. Saccharification of biomass mixture and hydrolysate preparation

Saccharification is simply the process of breaking down polysaccharides into simple sugar. Our study used bacterium for saccharification of the different agro-waste mixture formulated by the

EVMD method. Two mixture design sets were formulated; One (Set-I) used orange peel, canola straw, and brewer's spent grains and another set (Set-II) contained orange peel, pomegranate peel, and pumpkin pulp+seeds. Both sets included 40-100% orange peel and agro-waste 20-100%. The hydrolysis of the biomass mixture was undergone by using *Streptomyces* sp. at 35℃ and the cell-free supernatant was collected as hydrolysate after centrifugation. The hydrolysate collected this way was used for ethanol production.

2.4. Determination of total reducing sugar

Total reducing sugar content in the hydrolysate is vital for fermentation to produce ethanol. Thus, total reducing sugar of the hydrolysate was determined by DNS method (Miller, 1959) as glucose equivalent with some modification. In brief, 150 µL of supernatant was collected after centrifugation of the culture broth in the centrifuge tube and 450 µL of DNS solution added. The mixture solution was boiled in boiling water bath for 10 mins, cooled to room temperature, and read absorbance of the sample at 595 nm by spectrophotometer. Then the total reducing sugar of the sample was calculated in terms of glucose concentration using a standard curve prepared with different glucose concentrations and absorbance.

2.5. Yeast inoculum preparation for the fermentation process

Two grams of locally available baker's yeast, fermenting yeast, and brewer's spent grains were added into yeast peptone dextrose (YPD) broth and incubated at room temperature for 4 hours. Then, 100 µl of the suspension was spread on YPD agar plates and incubated at room temperature for 48 hours. When isolated pure colonies were observed, a speck of isolated yeast colony was taken, inoculated into YPD liquid media, and incubated for 24 hours at room temperature. From this cultured broth, 1 mL was pipetted out and inoculated into the flask containing 50 mL hydrolysate and incubated on a shaker at room temperature, 180 rpm for 5

days. Ethanol determination was performed every 24 hours. These three yeasts designated as M, Y, and BY which were isolated from fermenting yeast, brewer's spent grains, and baking yeast. The yeasts were grown in hydrolysate at room temperature without shaking for 4 days, then the ethanol concentration (%) in the hydrolysate was determined by potassium dichromate spectrophotometric method.

2.6. Ethanol determination

This study follows the spectrophotometric method for determination of ethanol concentration as described in Sriariyanun et al. 2019 (Sriariyanun et al., 2019) with slight modification. In this method, ethanol was extracted from culture broth by Tri-n-butyl phosphate (TBP) and alcoholic solvent oxidized by dichromate. In brief, one mL of TBP was mixed with 1 mL of aqueous solution/ cell free supernatant and vortex vigorously for 1 min. Then the mixture was centrifuged for 5 min at 13,300 rpm. The top clear and transparent layer of 500 µL was pipetted out and kept in another clean centrifuge tube. Then, 500 µL of dichromate reagent was added, and again mixed with vortexing. The mixture was kept still at room temperature for 10 mins and 100 µL of the oxidized solution was mixed with 100 µL of distilled water and absorbance at optical density 595 (OD_{595}) was taken. The estimation of ethanol concentration % v/v was determined by using the standard curve represented by different ethanol concentration and absorbance (OD 595).

3. Results and discussion

3.1. Isolation of yeast

Three different yeasts; from commercial fermenting yeast, baker's yeast, and barley's spent grains were isolated on YPD agar plates after subculturing many times (Fig. 1). The colonies of these three yeasts appeared to be slightly different in color and size. The colonies of M

(fermenting yeast) appeared pale and slight bigger than Y (yeast from brewer's spent grains) and BY (baker's yeast), whereas Y appeared more whitish than BY and M.

Fig. 1 Pure and isolated colonies of yeast (M: fermenting yeast, Y: yeast from brewer's spent grains, and BY: baker's yeast)

After simple staining of these yeasts with crystal violet, these three isolated yeasts illustrated different morphologies under microscope; however, the buddings of cells were observed in three yeasts. The microscopic structures revealed that these three yeasts were different.

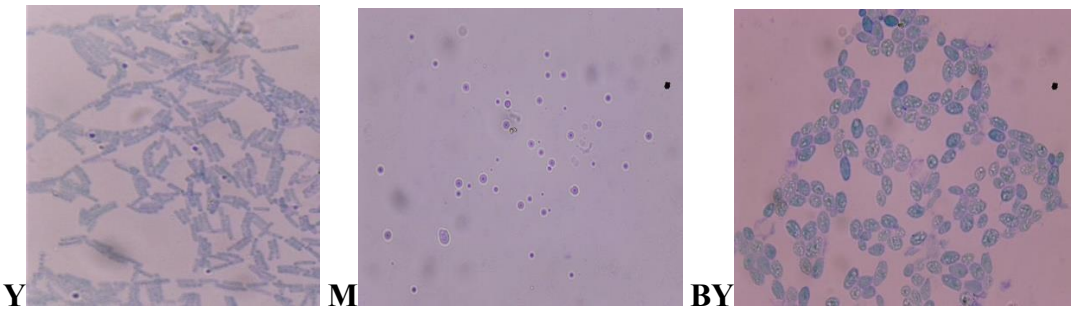

Fig. 2 Simple staining of yeasts (M: fermenting yeast, Y: yeast from brewer's spent grains, and BY: baker's yeast)

3.2. Saccharification of agro-waste

Nine different agro-wastes were used for saccharification by *Streptomyces* sp. This bacterium was selected for this study because it produces multi-enzyme, including pectinase, cellulase, xylanase, and amylase (Shrestha et al., 2021b) which are helpful to hydrolyze the biomass. The

total reducing sugar content exhibited by the bacterium using different agro-wastes is as shown in Table 1.

Table 1 Reducing sugar (mg/g dry weight) produced from different agro-wastes

Agro-waste	Reducing sugar (mg/g dry weight)
Pomegranate peel	13.87 ± 2.18^{a}
Orange peel	13.69 ± 0.55^{ab}
Barley straw	10.05 ± 0.06^{de}
Maple leaf	12.42 ± 1.24^{bc}
Banana peel	11.23 ± 0.12^{cd}
Canola straw	10.65 ± 0.17^{de}
Brewer's spent grains	12.69 ± 1.13^{ab}
Pumpkin pulp+seeds	9.68 ± 0.09^{e}
Algae	10.92 ± 0.04^{de}

Data presented as mean±standard deviation, different lowercase alphabets represent the values are statistically significant ($P\leq0.05$)

The reducing sugar from pomegranate peel was found to be maximum (13.87±2.18 mg/g dry weight) followed by orange peel (13.69±0.55 mg/g dry weight), brewer's spent grains (12.69±1.13 mg/g dry weight), maple leaf (12.42±1.24 mg/g dry weight) and other as depicted in Table 1. Furthermore, Table 1 illustrated the bacterium (*Streptomyces* sp.) was able to hydrolyze different agro-wastes and produce simple sugars. However, the ability of the bacterium to hydrolyze the agro-wastes varied with the types of agro-wastes used. This difference of reducing sugar from different agro-wastes is due to the differences in constituent compositions. In addition, the higher reducing sugar in the agro-waste hydrolysate is the result of the ability of the

bacterium to hydrolyze the agro-waste efficiently into simple sugars. Hydrolysis is possible only if the bacterium produces biomass degrading enzymes to break down the polysaccharides into simple monosaccharides. The previous study demonstrated higher pectinase, PGase, xylanase, and cellulase activities by *Streptomyces* sp. using pomegranate peel and orange peel (Shrestha et al., 2022). Thus, the different enzyme activities exhibited by *Streptomyces* sp. using pomegranate peel and orange peel might be the reason for showing higher reducing sugar from respective biomasses. Also, it may be due to the presence of higher polysaccharides on those agro-wastes. The saccharification of agro-wastes mixture (43.33% orange peel, 33.33% pumpkin pulp+seeds, and 23.33% pomegranate peel) was performed by culturing *Streptomyces* sp. at its ambient cultural conditions (35℃, 180 rpm for 50 hours).

3.3. Mixture design formulation for saccharification of agro-waste mixture

The different constitutional compositions of agro-wastes play an influencing role in enzymes production and their saccharification. The reducing sugar exhibited by each mixture formulated by EVMD is demonstrated in Table 2 and it depicts higher reducing sugar from a mixture compared to single feedstock. The highest reducing sugar observed by the single feedstock was 13.87±2.18 mg/g dry weight from pomegranate peel. However, the maximum reducing sugar obtained after saccharification from Set I (consisting of three agro-waste; orange peel, canola straw, and barley straw) was 14.59±0.15 mg/g dry weight, whereas 22.36±0.54 mg/g dry weight was observed in Set II (orange peel, pomegranate peel, and pumpkin pulp+seeds). Almost 1.5 times higher reducing sugar was observed in Set II compared to Set I, so Set II was selected in this study for saccharification of agro-wastes mixture by exploiting *Streptomyces* sp. When the data was analyzed by one way ANOVA, the data were statistically significant at 95% level of confidence. In Set II, data were observed statistically not different in Run 2, 3, 6, and 7 although

the values were slightly different, thus the mixture design formulation of Run 7 was selected for further saccharification by *Streptomyces* sp. After selection of the three agro-waste mixture (43.33% orange peel, 33.33% pumpkin pulp+seeds, and 23.33% pomegranate peel) from EVMD formulation, validation of this mixture was performed. The validation was done by determining the reducing sugar from the selected biomasses mixture and saccharified by using *Streptomyces* sp. at optimal conditions and almost similar reducing sugar (21.66±2.06 mg/g dry weight) was observed.

Table 2 Reducing sugar exhibited by the agro-waste mixture formulation (Set I: orange peel, canola straw, and brewer's spent grains and Set II: orange peel, pumpkin pulp+seeds, and pomegranate peel)

Run	Orange peel	Canola straw/ Pumpkin pulp+seeds	Brewer's spent grains/ Pomegranate peel	Reducing sugar (mg/g dry weight)	
				Set I	Set II
1	40	20	40	12.32±0.11^{d}	18.07±0.46^{b}
2	60	20	20	12.99±0.09^{c}	20.92±0.79^{a}
3	40	40	20	12.21±0.08^{d}	21.70±0.62^{a}
4	46.67	26.67	26.67	14.59±0.15^{a}	13.76±0.28^{c}
5	43.33	23.33	33.33	12.76±0.09^{c}	18.85±0.35^{b}
6	53.33	23.33	23.33	14.06±0.01^{b}	21.25±0.73^{a}
7	43.33	33.33	23.33	12.79±0.04^{c}	22.36±0.54^{a}

Data presented as mean±standard deviation, different lowercase alphabets represent the values statistically significant ($P \leq 0.05$)

Besides, different compositions of agro-wastes were studied (supplementary Table 1, 2) such as 4 agro-wastes (Brewer's spent grains, orange peel, canola straw, barley straw) mixture and nine different agro-wastes (orange peel, pomegranate peel, barley straw, banana peel, canola straw, brewer's spent grains, pumpkin pulp+seeds, maple leaf, and algae) mixture compositions. However, the reducing sugars from those mixture compositions were lower than the three agro-waste mixture design. The maximum reducing sugar observed from four and nine agro-wastes mixture were 12.26 and 15.79 mg/g dry weight, respectively (Supplementary Table 1, 2). Therefore, among different mixture compositions, 3 agro-wastes mixture composition of 43.33% orange peel, 33.33% pumpkin pulp+seeds, and 23.33% pomegranate peel were found to be the best and used for saccharification by *Streptomyces* sp. and the hydrolysate produced was further used in ethanol production.

As per our knowledge, there are many studies regarding ethanol production by using single biomass whereas there is no such study regarding the formulation of agro-wastes for saccharification by *Streptomyces* sp. and ethanol production. For instance; a study included duckweed (*Landoltia punctata*), and hemicellulose waste such as pomegranate peel, paddy straw, sugarcane bagasse, corn cob, rice flour, wheat flour, finger millet flour, sweet lime peel, banana peel, groundnut oil cake, sesame oil cake, and coconut oil cake as a feedstock for ethanol production (Chen et al. 2012; Barathikannan et al. 2016). In addition, pectin rich biomass like sugar beet pulp, citrus waste, and apple pomace, and corn stalk juice have been studied for bioethanol production (Edwards and Doran-Peterson, 2012; Bautista et al., 2022).

3.4. Spectrophotometric determination of ethanol concentration

The cell free hydrolysate obtained after centrifugation at 13,300 rpm for 10 mins was used for ethanol production by using three different yeasts; Y: yeast isolated from brewer's spent grains,

M: commercially available fermentation yeast, and BY: baking yeast. The study revealed the highest of 5.59±0.15% ethanol from M followed by BY (4.63±0.12%) and Y (3.47±0.19%) respectively (Fig. 3).

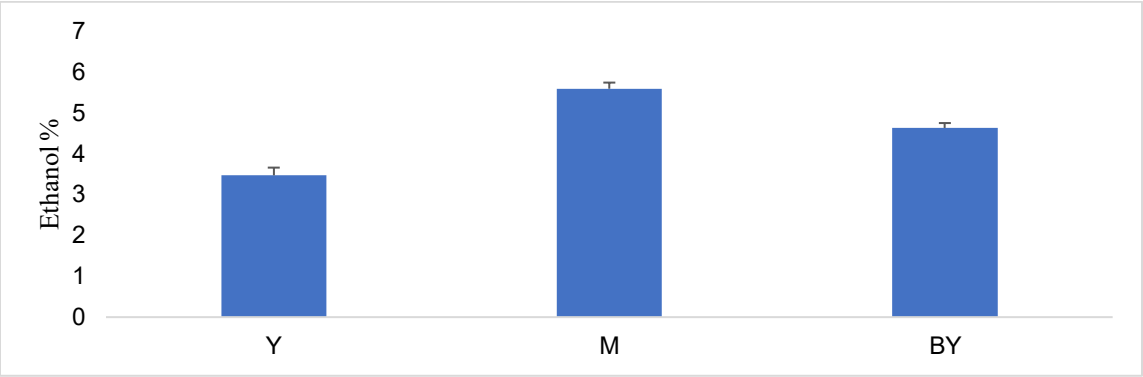

Fig. 3 Ethanol % v/v at 30℃ without shake from three yeasts: Y, M, and BY

A higher ethanol concentration than our study was observed in other studies. A study illustrated the maximum of ethanol 10.26±0.24% from medicinal plant (Draksharistha) following the potassium dichromate oxidation method (Sayyad et al., 2015). Another study illustrated the maximum of 7.87% v/v ethanol concentration produced from corn stalk juice by immobilized *Saccharomyces cerevisiae* in 1st batch of fermentation (Bautista et al., 2022). The variation in the concentration of ethanol observed from those different feedstocks may be due to the difference in the constitutional compositions in feedstock, microorganisms used, methods and types of fermentations applied. Zabed et al. (2016) (Zabed et al., 2016) had explained about some factors that affect ethanol production from lignocellulosic biomass. They have mentioned that the sources, nature, age, stages and chemical compositions of biomasses, different biotechnological approaches, and microbial impacts play very crucial role in bioethanol production (Zabed et al., 2016).

The spectrophotometric method is easy, rapid and does not require expensive instruments. In addition, this method is based on the oxidation of ethanol by acidic potassium dichromate which

results in color change and is measurable in spectrophotometer (Sayyad et al., 2015). Besides, dichromate oxidation method is reliable and a consistent method for quantitative determination of ethanol (Sayyad et al., 2015) and Seo et al. (2009) (Seo et al., 2009) stated that potassium dichromate oxidation method is similar to gas chromatography. The non-alcoholic solvent (tri-n-butyl phosphate (TBP)) selectively extracts ethanol from culture media and forms the distinct interface between water and TBP layer. Ethanol present in the aqueous TBP solution is oxidized by chromium changing the color from orange to green (Seo et al., 2009; Sayyad et al., 2015). Therefore, this study we employed the simple, rapid, cheap and reliable potassium dichromate oxidation method for ethanol concentration determination (Seo et al., 2009; Sriariyanun et al., 2019).

3.5. Effect of temperatures and shaking in bioethanol production

When the cell free hydrolysate was inoculated with three different yeasts and incubated at 30 and 35℃ with shaking (150 rpm) and without shaking, the higher ethanol concentration was observed at 35℃ without shake by all yeasts and low at 30℃ with shake (Fig. 4). Hence, it was obvious that 35℃ without shaking was the favorable condition for maximum ethanol production.

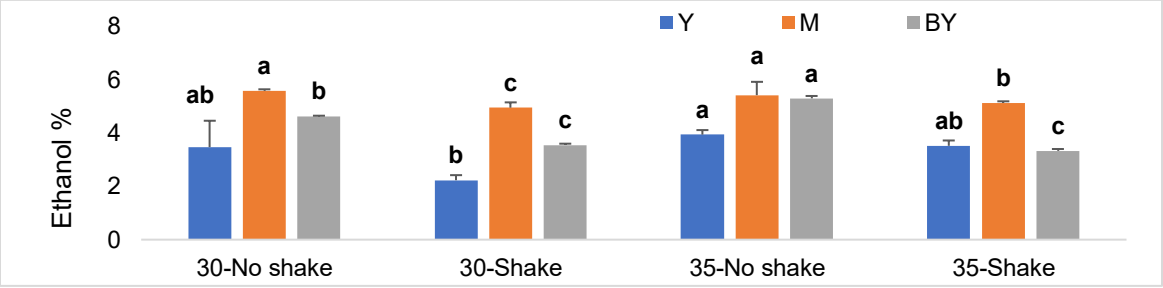

Fig. 4 Ethanol concentration (%) at 30 and 35℃ and with shaking and without shaking

When ethanol concentration at different hours were studied (Fig. 5 a, b, and c), it was revealed that three yeasts exhibited maximum ethanol concentration at different incubation hours and temperatures. The yeast Y exhibited the highest ethanol concentration (6.59±0.20% v/v) in 48

hours at 35℃ without shaking and after 48 hours the concentration continuously decreased (Fig. 5a). Similarly, the maximum ethanol concentration (7.28±0.58% v/v) was observed for yeast BY in 72 hours at 35℃ without shaking (Fig. 5b). Furthermore, yeast M showed the maximum ethanol concentration (8.21±0.16% v/v) in 24 hours at 35℃ without shaking which continuously decreased after 24 hours (Fig. 5c).

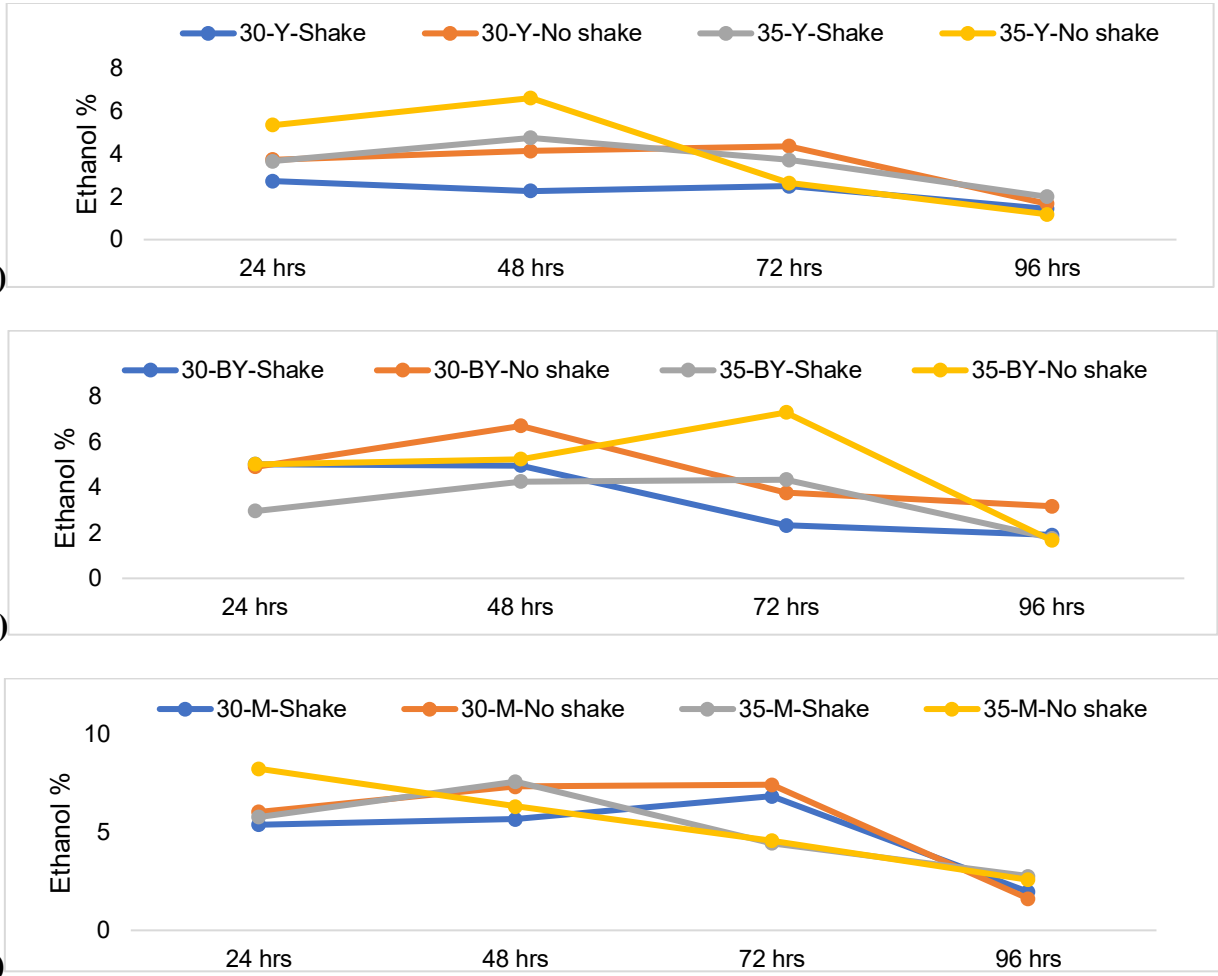

Fig. 5 Ethanol concentration at different hours by different yeasts a) Y (yeast isolated from brewer's spent grains) b) BY (baker's yeast) and c) M (yeast isolated from commercial fermenting yeast powder

At 35℃ without shaking, Y, BY, and M yeast gave the highest ethanol production at 48, 72, and 24 hours, respectively. The reason for the hours difference in maximum ethanol production may

be due to the difference in the yeast used and their fermenting abilities. The yeasts (Y, BY, and M) may have an exponential growth phase during 48, 72, and 24 hours respectively and later they may go to stationary phase. The decrease in ethanol production after a certain time may be due to the depletion of nutrient and/or change in environmental conditions in the media. Fig. 5 also indicates that the shaking or high aeration was not suitable for ethanol production by these three yeasts.

3.6. Effects of glucose and fructose in ethanol concentration

The addition of simple sugars in the hydrolysate and the types of sugar may affect the ethanol production. Thus, 2% of glucose and fructose were added in the hydrolysate and incubated at 35°C without shaking and their effect on ethanol concentration were studied (Fig. 6). Fig. 6 illustrated fructose significantly enhanced ethanol production by Y and M yeasts. Similarly, addition of glucose increased the ethanol production by Y, however, BY and M decreased the production.

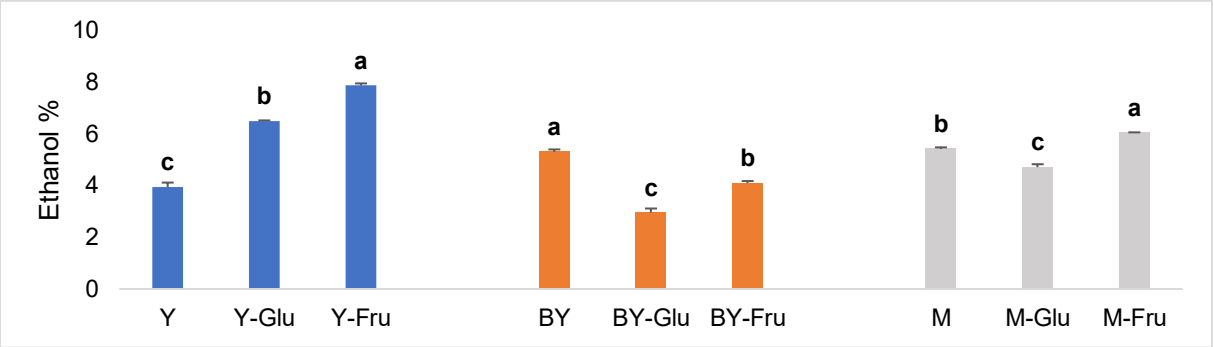

Fig. 6 Effects of fructose and glucose in ethanol production by three yeasts (Y, BY, and M)

Fig. 6 depicts the highest ethanol (7.86±0.08% v/v) was produced by Y when 2% w/v fructose was supplemented in the hydrolysate and lowest (2.95±0.16% v/v) was produced by BY when glucose was added. This finding also showed that the addition of simple sugar has an effect on ethanol production and the effect depends on the yeast used.

Ethanol production via fermentation follows hexose monophosphate pathway which is an important process for yeast to utilize sugar and produce ethanol with carbon dioxide. Although ethanol fermentation is known to be an anaerobic process, some yeast can produce ethanol aerobically from high sugar concentration. This process is known as the Crabtree effect. However, the range of sugar has different effect on growth of yeast and metabolisms (Pronk et al., 1996; Mori et al., 2019). In this aspect, yeast Y showed the Crabtree effect producing significantly higher ethanol by using additional glucose and fructose. In contrast, BY did not show such effect. However, M illustrated such an effect only when fructose was added in the hydrolysate. This finding also cleared that all these three yeasts prefer ketonic hexose sugar (fructose) to glucose (Fig. 6).

4. Conclusions

The agro-wastes like pomegranate peel, orange peel, pumpkin pulp+seeds, barley straw, and brewer's spent grains are the potential feedstocks for ethanol production. The formulation of agro-wastes mixture by EVMD of orange peel (43.33%), pomegranate peel (23.33%) and pumpkin pulp+seeds (33.33%) exhibited the best formulation for saccharification of agro-wastes mixture by *Streptomyces* sp. compared to other mixture and single feedstock. The hydrolysate produced by this formulation of agro-wastes was used for fermentation by three yeasts (yeast isolated from brewer's spent grains, baker's yeast, and commercial fermenting yeast) to produce ethanol. This study revealed that the yeast can be isolated from brewer's spent grains and be used in fermentation for ethanol production. In addition, the study illustrated glucose and fructose play influencing effect in ethanol production. Further research to improve and increase the ethanol production and yield is recommended by using different agro-wastes and fermentation conditions. Also, other chemicals produced during fermentation are needed to be detected.

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Chapter 6

Cloning and Expression of Pectinase Gene from Two Forest Soil Bacteria Identified as *Streptomyces* sp.

Abstract

The gene encoding a thermostable pectinase from two forest soil bacteria (S-5 and S-14) identified as *Streptomyces* sp. was cloned and expressed in *Escherichia coli* BL21(DE3). The pectinase gene illustrated about 750 bp on the gel after electrophoresis and encoded a product of approximately 25 kDa molecular weight. Both expressed pectinases showed optimal activity at 2 different pHs (pH 5 and 9) and at 50°C. When both recombinant pectinases were exposed to different temperatures, they were able to retain their activity for 120 mins indicating both have important thermostable properties. These enzymes could be of great potential in industrial biotechnological processes due to their stability over broad pH and high temperatures.

Keywords: *Streptomyces* sp., Cloning and expression, pectinase

1. Introduction

Pectin is the complex heteropolysaccharide found mostly in the middle lamella and cell wall of vegetables, cereals, and fruits. This complex carbohydrate is acidic in nature, have high molecular weight, and is broken down by pectin degrading enzymes named pectinases (Guan et al., 2020; Shrestha et al., 2021c). Due to the complex structure of pectin, various forms of pectinases appear which are classified as protopectinases, pectinesterases, and depolymerases. Protopectinases solubilize the insoluble protopectin into highly soluble pectin, pectinesterases deesterify the methoxyl group of pectin producing pectic acid, and depolymerases degrade pectin by cleaving or hydrolyzing glycosidic linkage. In the nutshell, pectinases belong to the

polysacharidases family and degrade pectin through de-esterification and depolymerization reactions (Garg et al., 2016; Nighojkar et al., 2019; Shrestha et al., 2021c).

Pectinases have great commercial importance because these enzymes have been used in various industries from juice industries, animal feed industries, and pharmaceutical industries to wastewater treatment plants, etc. In addition, the applications and demand for pectinases are continuously growing up (Amin et al., 2019). Therefore, there is necessary to produce pectinases having some characteristics including stability at different temperatures and pHs for various industrial applications. There have been cloning works to mitigate the enzyme demand after the successful expression of a specific pectinase gene. Li et al., 2014 (Li et al., 2014) cloned a pectate lyase from *Paenibacillus* sp. and expressed it in *E. coli*. Mei et al., 2013 (Mei et al., 2013) cloned a pectinase from *Bacillus halodurans* and expressed it in *E. coli* JM109. The expressed pectinase from *B. halodurans* illustrated high thermostability. Similarly, Zhou et al (Zhou et al., 2017a) and Guan et al (Guan et al., 2020) expressed pectate lyase from *Bacillus* sp. in *E. coli* which showed maximum activity at pH 10.5 and 70℃, and 8 and 50℃ respectively. And Yuan et al (Yuan et al., 2019) illustrated the maximum activity of recombinant pectate lyase from *Paenibacillus polymyxa* in *E. coli* at pH 10 and 40℃. However, there are very few articles for cloning and expression of pectinase gene from *Streptomyces* sp. Thus, this study is focused on cloning of the pectinase gene from *Streptomyces* sp. and expression in *E. coli* BL21 (DE3) and further characterizing the expressed pectinase. Furthermore, 3D computational structures of expressed pectinases and docking analysis were performed and some properties of these recombinant pectinases were compared.

2. Materials and methods

2.1. Bacterial isolates

Two forest soil bacteria isolated and identified earlier as *Streptomyces* sp. (Shrestha et al., 2021b) were stored at -80℃. They were revived by culturing in Luria-Bertani (LB) broth at 35℃ for 18-20 hours. Genomic DNAs of two *Streptomyces* sp. (S-5 and S-14) were extracted by using the alkaline SDS method.

2.2. PelA (pectinase A) gene cloning

The specific primers for amplifying the open reading frame (ORF) of PelA used in this study was without signal peptide which was designed in other study (Tonouchi et al., 2010). The forward primer was PelA-F 5'-CCCAAACATATGACATCCGCGACACGACC-3' (Ndel digestion site) and the reverse primer was PelA-R 5'-CCCCTCGAGCCGGTAGGTGATGTCGGACG-3' (Xhol digestion site). Using the genomic DNA of *Streptomyces* sp. as the template, PelA was amplified with PelA-F and PelA-R. The polymerase chain reaction system composed of genomic DNA 2 μL (500 ng), 2X Taq mix 12.5 μL, 10 μM forward and reverse primer 1 μL each, and distilled water 8.5 μL making the final volume of 25 μL. The PCR cycles were programmed as pre-denaturation for 3 mins at 94℃, followed by denaturation at 94℃ for 30 sec, annealing at 59℃ for 30 sec, extension at 72℃ for 60 sec for 33 cycles, final extension at 72℃ for 10 mins, and finally preserving at 12℃. Then the PCR products were tested by 1% (w/v) agarose gel electrophoresis.

2.3. Plasmid construction and prokaryotic expression

The PCR products from the gel were purified using a gel extraction kit and they were double digested. The digestion system was composed of digestion enzymes 1 μL each, 10X Tango buffer 4 μL, and purified PCR products 14 μL making a final volume of 20 μL mixture. The

system was incubated at 37℃ for an hour, then St-PelA digested products were purified. The digested products were ligated into pET21a which is double digested with the same enzymes to obtain recombinant vector pET21a-PelA. After transformed into *E. coli* JM109, the mixture was plated on LB agar plate containing 100μg/mL of ampicillin to screen the positive clones.

PCR was performed to confirm the positive clones. The PCR reaction system consisted of 10 μL of 2x Taq PCR Master Mix, 0.5 μL of 10μM forward and reverse primer, 1 μL of liquid bacteria (liquid culture of cloned isolate), and 8 μL of distilled water making the final volume of 20 μL. The PCR was programmed as 3 mins pre-denaturation at 94℃, followed by 30 sec denaturation at 94℃, 30 sec of annealing at 59℃, 1 min extension at 72℃ for 35 cycles, and final extension at 72℃ for 10 mins. When the PCR was completed, the products were tested by 1% (w/v) agarose gel electrophoresis.

After verification, the recombinant plasmids (PET 21a-PelA-S5/S14) were extracted using a Plasmid DNA Miniprep Kit (Biobasic, Canada). Then, the plasmids were transformed into the expression host *E. coli* BL21 (DE3). Both of the recombinant enzymes were expressed with a C-terminal His_6-Tag.

For enzyme, *E. coli* BL21 (DE3) cells harboring the recombinant plasmids were grown in 10 mL of LB broth containing 0.01 g/L ampicillin and incubated at 37°C, 200 rpm overnight. Then 1 mL of overnight grown culture was inoculated into 100 mL LB medium without ampicillin. Cultivation was carried out in 250 mL flasks at 37°C and 200 rpm in a shaker incubator for 2-3 hours until the OD_{600} reached 0.3 to 0.5. Next, the cells were induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Then those flasks were incubated at 25°C and 160 rpm for up to 24 hours, and the cells were harvested by centrifugation (8,500g at 4 °C for 10 min). The cell pellet obtained was re-suspended in

the phosphate buffer (pH 7.4) and disrupted by an ultrasonic sonicator with alternate 10 sec ON and 15 sec OFF for 15 min. Then the cell suspension was centrifuged (12,000g at 4 °C for 15 min) and the clarified cell lysate obtained was kept at 4 °C for further study including protein quantification, SDS-PAGE, enzymatic activity assay, and purification.

2.4. Pectinase activity

The pectinase activity was measured as described by Shrestha et al. (Shrestha et al., 2021b) following 3,5-Dinitrosalicylic acid (DNS) reagent using Miller method (Miller, 1959) that quantifies reducing sugar expressed as GalA units. In brief, a volume of 10µL of the enzyme was added to 30µL of 1% (w/v) citrus pectin in phosphate buffer at pH 7.4. After 10 min of incubation at a 50 °C water bath, 60µL DNS of was added. Then the mixture was heated for 10 mins in boiling water bath, brought to room temperature, and 200µL of distilled water was added after the reaction mixture cool down. The reducing sugars were quantified by reading the absorbance of the reaction solution at 540 nm. Similarly, the blank reaction was carried out using only a buffer with no enzyme solution. One unit (U) of pectinase activity was defined as the amount of enzyme that releases 1 µmol of GalA equivalent per min at given conditions.

2.5. Purification of protein by Ni-NTA chromatography and SDS-PAGE

Both the enzymes were purified by affinity chromatography using a His-Tag Ni-affinity resin (Ni-NTA Agarose, Thermo Fisher Scientific Inc, UK). Ten mM phosphate buffers containing 10, 20, and 300 mM imidazole and 60 mM NaCl were used as binding, washing and elution buffer, respectively. The purified enzymes were stored at 4 °C to be used for characterization assays. The purity of the protein samples was also analyzed by SDS-PAGE gel electrophoresis with 5% (w/v) stacking gel and 12% (w/v) separating gel. Electrophoresis was carried out at 80 volts for 20 mins and 100 V constant voltage for 120 min on a vertical slab. The marker protein with

molecular weight ranging from 10 to 250 kDa was used as the reference to estimate the molecular weight of purified recombinant pectinase. The gel was stained with Coomassie Brilliant Blue R-250 and destained with methanol-acetate-water (1:1:12).

2.6. Enzyme characterization

2.6.1. Optimal pH and temperature

The optimum pH of the pectinases was determined at 50 °C using 1% (w/v) citrus pectin in the buffers having different pHs; sodium acetate (pH 4 and 5), sodium phosphate (pH 6 and 7), TRIS–HCl (pH 8 and 9) and sodium bicarbonate (pH 10). Similarly, the optimum temperature for pectinase was determined at different temperatures from 40 °C to 90 °C in 10 °C increments. The pectinase activities were determined following the standard assay procedures as described earlier and the relative activity was expressed as a percentage of the maximum activity.

The thermal stability was determined by incubating the enzymes in phosphate buffer of pH 7 in a water bath arranged temperature from 40 to 90 °C in 10 °C increments for up to 2 hours. Aliquots of the enzyme solutions were taken periodically, and enzyme activity was measured under the standard assay conditions at 50 °C, as detailed and expressed as relative activity.

2.6.2. Kinetic parameters

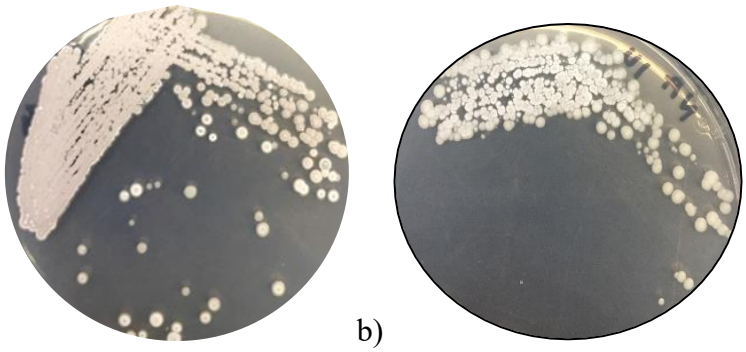
Kinetic parameters were determined by measuring the initial velocity of reaction at different concentrations of substrate (citrus pectin) from 0.25 mg/mL to 6 mg/mL. The reactions were performed at 50 ℃ for 10 mins for each enzyme. The kinetic data the Michaelis-Menten constant (K_m) and maximum velocity for the reaction (V_{max}) with citrus pectin (1% w/v) were obtained by non-linear regression using GraphPad Prism 5 software.

2.7. Computational modeling of the expressed PelA and its docking analysis

DeepFold from Zhang lab (https://zhanggroup.org/DeepFold/) was used for constructing the 3D model of the expressed PelA. With the computational 3D structure of the PelA, AutoDock4 (Morris et al., 1998) was used for docking analysis and explaining the catalytic domain. The 3D structure of the polygalacturonic acid (PubChem CID: 92023580) was obtained from PubChem. The original structure was modified (removing sodium ions) and its energy was minimized by Chem3D (PerkinElmer Informatics, United States) with a minimum RMS gradient of 0.01. The PelA enzyme was selected as the rigid macromolecule and the polygalacturonic acid was selected as the ligand. The maximum number of evaluations in the Genetic Algorithm was set as "Long" with 2500000 steps and the Lamarckian genetic algorithm was used for the structure docking. The optimal docking structure was determined based on the lowest binding energy and the visualization was performed using the open-source PyMOL (Schrodinger, LLC. 2010. The PyMOL Molecular Graphics System, Version 2.6 0a0).

3. Results and discussion

Two bacteria (S-5 and S-14) used in this study were isolated from forest soil in Thunder Bay, Ontario. They were both identified as *Streptomyces* sp. after their morphological, biochemical characterization, and 16S rDNA sequences analysis. S-5 had gray color morphology and a fast grower, but S-14 was whitish in color and a slow grower compared to S-5. Since both bacteria were identified as *Streptomyces* sp. (Shrestha et al., 2021b) and were positive for pectinase screening tests, they were used for pectinase cloning.

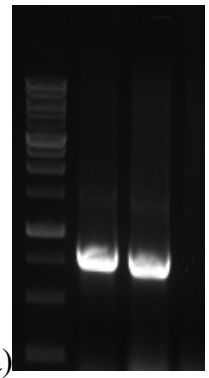


a) b)

Fig. 1 Colony morphology of soil bacterial isolates a) S-5 and b) S-14

3.1. Pectinase gene cloning, vector construction, and prokaryotic expression

The primers used in this study for pectinase gene cloning were designed by Tonouchi et al. (Tonouchi et al., 2010) from *Streptomyces* sp. genome. The extraction of genomic DNA of *Streptomyces* sp. was the very first step for cloning and DNA was extracted following the alkaline SDS method. The amplification, the PCR product illustrated the band approximately 750 bp which confirmed that the desired pectinase has been amplified. Then the PCR products and PET 21a both were digested with Nde I and Xho I. The double digested products were ligated and transformed into the competent cell *E. coli* JM109. Then a colony PCR followed by gel electrophoresis verified the gene of interest was inserted. A single band observed at around 750 bp made sure that the cloned pectinase gene had been inserted (Fig. 2a). Further the inserted sequences of the plasmid pET21a-PelA-S5/S14 were sequenced and aligned with the available sequences of pectinase in the National Center for Biotechnology Information (NCBI) database by using the basic local alignment search tool. We observed the nucleotides sequence of the cloned plasmid was 99% identical to the pelA gene of *Streptomyces thermocarboxydus* (AB513441) (Fig. 2b). Thus, it was confirmed that the cloned plasmid has the pectinase gene.

a) 

b)

Score	Expect	Identities	Gaps	Strand
1094 bits(592)	0.0	607/614(99%)	2/614(0%)	Plus/Plus

```
Query  1    GGCAGTCAGGGCGTCTCCTCCACCATCTCCGTGTCCGGCACCAAGGACTACGGCATGAAG  60
Sbjct  333  GGCAGTCAGGGCGTCTCCTCCACCATCTCCGTGTCCGGCACCAAGGACTACGGCATGAAG  392

Query  61   CGCCTCTACGGCACCGGCGACCTGGGCTCCGGCAGCCAGGACGAGGACCAGGGCCCGATC  120
Sbjct  393  CGCCTCTACGGCACCGGCGACCTGGGCTCCGGCAGCCAGGACGAGGACCAGGGCCCGATC  452

Query  121  CTGAAGCTGTCCGACGGCGCGGTCCTGAAGAACGTGATCATCGGCGCCCCGGCCGCCGAC  180
Sbjct  453  CTGAAGCTGTCCGACGGCGCGGTCCTGAAGAACGTGATCATCGGCGCCCCGGCCGCCGAC  512

Query  181  GGCATCCACTGCGAGGGGCTCCTGCACCCTGCAGAACGTCTGGTGGGAGGACGTCGGTGAG  240
Sbjct  513  GGCATCCACTGCGAGGGCTCCTGCACCCTGCAGAACGTCTGGTGGGAGGACGTCGGCGAG  572

Query  241  GACGCGGCCACCTTCAAGGGCAAGTCGTCCGGGGCGGTGTACACCGTCAGCGGCGGCGGC  300
Sbjct  573  GACGCGGCCACCTTCAAGGGCAAGTCGTCCGGGGCGGTGTACACCGTCAGCGGCGGCGGC  632

Query  301  GCGAAGGACGCCAGTGACAAGGTCTTCCAGTTCAACGGCGCGGGCACGCTGAACGTGTCC  360
Sbjct  633  GCGAAGGAGGCCAGTGACAAGGTCTTCCAGTTCAACGGCGCGGGCACGCTGAACGTGTCC  692

Query  361  AACTTCGCCGTCCAGAACTTCGGCACCTTCGTCCGCTCCTGCGGCAACTGCTCCTCGCAG  420
Sbjct  693  AACTTCGCCGTCCAGAACTTCGGCACCTTCGTCCGCTCCTGCGGCAACTGCTCCTCGCAG  752

Query  421  TACAAGCGGACGATCAACCTCAACTCCATCGAGGTGACCTGGAAGGGCGGCCGTATCGCC  480
Sbjct  753  TACAAGCGGACGATCAACCTCAACTCCATCGAGGTGACCTGGAAGGGCGGCCGTATCGCC  812

Query  481  GGCATCAACACCAACTACGGCGAC-AGCGCGACCCTGCGCAACATCACGATCGTCGGCGA  539
Sbjct  813  GGCATCAACACCAACTACGGCGACCAG-GCGACCCTGCGCAACATCACGATCGTCGGCGA  871

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Sbjct  872  CACGAGCAAGAAGATCGTGCCGTGCCAGAAGTACATCGGCAACAACACCGGCAAGGAGCC  931

Query  600  CAGCAGCAACGGCT  613
Sbjct  932  CGGCAGCAACGGCT  945
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Fig. 2 Gel electrophoresis of a) colony PCR after transforming into the competent *E. coli* JM109 and b) aligned cloned PelA-S5 and PelA gene of *Streptomyces thermocarboxydus* (AB513441)

Further, a single white colony grown on the LB agar plate containing ampicillin was picked and cultured in 5 mL LB broth with 100 µg/mL ampicillin for 14 to16 hours and its plasmid was extracted. The extracted plasmid was then transformed into one of the well-known expression vectors, *E. coli* BL21 (DE3). In addition, IPTG was added into the medium to induce the pectinase production. The C-terminal of expressed pectinase contained His-tag to make the purification process easy. The expression of enzyme in the medium was checked but no activity was observed so it was supposed the enzyme was expressed intracellularly but not extracellularly. Thus, the cells were ultra-sonicated to release the expressed pectinase from the cells after centrifugation. This study used one-step affinity chromatography to purify the recombinant PelA using a His-Tag Ni-affinity resin which eluted the expressed pectinase in a pure form. The cell-free purified pectinase was then used for further studies including SDS-PAGE, enzyme assays and characterization.

The unpurified enzyme depicted a few bands on SDS-PAGE indicating there were other impurities, whereas the eluted purified protein fraction showed a single electrophoretic band on 12% SDS-PAGE gel as illustrated in Fig. 3a. The single clear band helped to estimate the

molecular weight of both expressed PelA (PelA-S5 and PelA-S14) to be around 25 KDa (Fig. 3b).

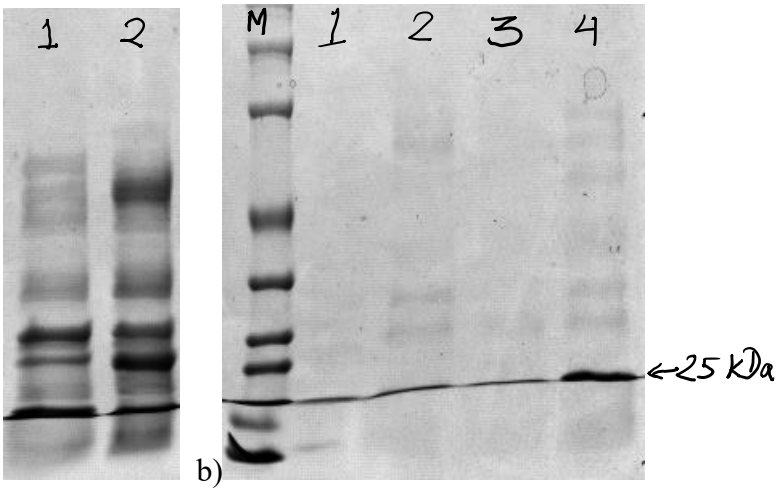

Fig. 3 SDS-PAGE analysis with 12% denaturing acrylamide separating gel. a) extracted enzymatic solution of induced BL21 harbouring pET 21a-PelA cell lysate (IPTG induced protein) before purification. b) Lanes: M (the protein marker), 1& 2 (the cell lysate of an induced *E. coli* BL21 after Ni-NTA purification of PelA-S5), and 3 & 4 (the cell lysate of an induced *E. coli* BL21 after Ni-NTA purification of PelA-S14)

The molecular weight of these expressed pectinase was slightly lower than the molecular weight of pectate lyase (28 KDa) which was cloned by using same primers (Tonouchi et al., 2010) as in the present study.

3.2. Effects of temperature and pH on the enzyme activity and thermostability

When the effects of different temperatures and pHs on the enzyme activity of the purified pectinase were studied, the maximum enzyme activities were observed at 50°C and two pH (pH 5 and 9). Pectinases (PelA-S5 and PelA-S14) obtained from both bacteria illustrated the similar trends of activities as shown in Fig. 4. The Fig. 4a depicted the activity of recombinant PelA increased from room temperature to 50°C but after 50°C the activity declined. Both expressed

pectinases illustrated the optimal activity at 50°C. PelA-S5 was able to retain about 20% of the activity at 80°C and completely lost at 90°C. However, PelA-S14 retained 20% of the activity at 70°C and totally lost at 80°C (Fig. 4a).

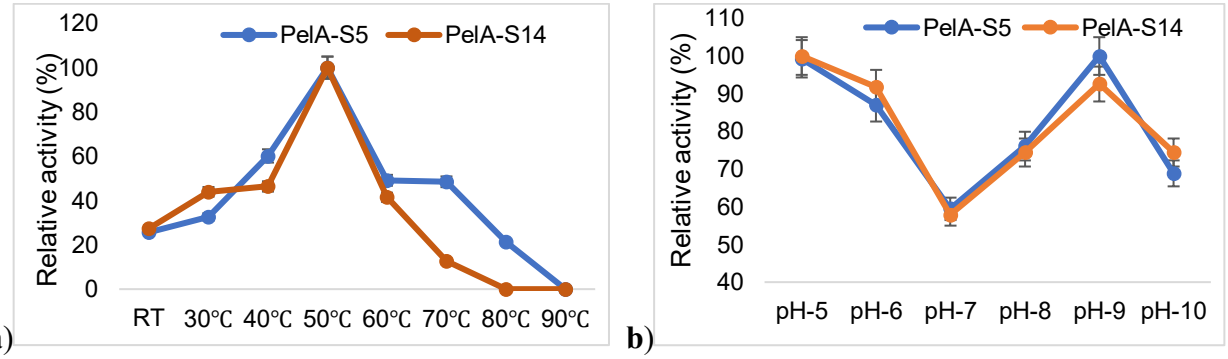

Fig. 4 Effect of **a**) different temperature **b**) different pH on the enzyme activity of expressed pectinase (Pel-S5 and PelA-S14)

Similarly, Fig. 4b illustrated that PelA-S5 and PelA-S14 retained their activity about 60% at pH 7. The maximum activity was observed at pH 5 and 9 showing they can retain their activities (about 100%) at both acidic and alkaline pH. This unique property of pectinase can be very important for industrial applications.

pH is a very essential parameter that affects the properties of enzymes and proteins; thus, pH is not only crucial for industrial applications but also in all biological and chemical processes or mechanisms. Generally, the enzymes show good activities at neutral pH (pH 7). However, some amino acids present in protein or enzymes interfere with the activity and change the optimal values. The positive and negative charges of amino acids present on the surface of enzyme play crucial role in their activity both in acidic and alkaline pH. The different pH can also affect the confirmation of the substrate affecting the ionization state of acidic or basic amino acids and finally the enzyme substrate reactions. The active site of the enzyme, charge and shape of substrate are also affected by the pH (Okamoto et al., 2017). If the enzyme shows the acidic

property, then the protein has an excess of acidic amino acids on its surface. In this case the negative charges are neutralized by tightly bound water dipole and make enzyme soluble. Thus, the amino acid residues, the side chain packaging, different bonding and translational modifications and many other factors play indirectly or directly in the stability of enzyme (Alponti et al., 2016). A study by Zhou et al. 2017 (Zhou et al., 2017) demonstrated the pectate lyase activity from *Bacillus subtilis* in a wide range of pH (pH 5 to pH 11) but the optimal pH was 9.5. The basic residues such as Lys, Arg, or His act as a Bronstead base and accept proton from galacturonate residue. Also, a divalent calcium cation might bind to the carboxylate of substrate and acidifies at the C-5 position. These mechanisms might be the reason for different characteristics of enzymes (Zhou et al., 2017).

The thermostability was evaluated by determining the residual activity after incubating the enzyme in buffer for various duration at different temperatures of 40 to 80℃. The relative activity was calculated with reference to enzyme activity at 50℃ for 10 mins in reaction conditions. Fig. 5 illustrates the enzyme activity was enhanced with increase in incubation duration at different temperatures. The activity of PelA-S5 was observed maximum when incubated for 60 mins at 70℃, and 90 mins at 40℃. It also demonstrated the PelA-S5 could retain its total stability when incubated at 80℃ for 2 hours (Fig. 5a).

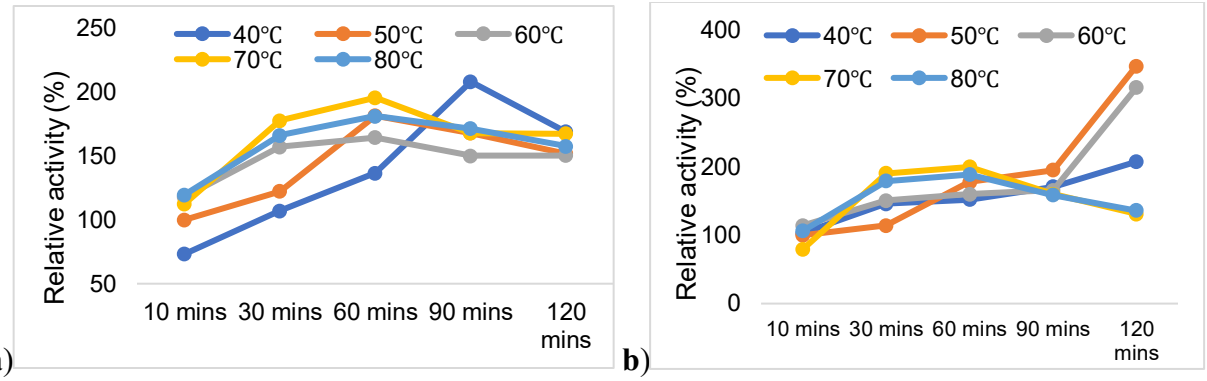

Fig. 5 Thermostability of expressed pectinase **a**) Pel-S5 and **b**) PelA-S14

Similarly, PelA-S14 elicited its thermostability property as in Fig. 5b. Fig. 5b illustrated the activity was completely retained even when it was incubated at different temperatures for 2 hours. At 70 and 80°C, the activity was maximum for 60 mins and started to decline when incubated for longer duration, but the activity was still increasing when incubated at 40, 50, and 60°C. These results showed that the stability was retained at low temperatures for longer time and in case PelA-S14, the activity was still increasing (Fig. 5b). Like our study, another study has shown that the pectate lyase cloned from *Bacillus* sp. and expressed into heterogenous *E. coli* had optimal temperature of 50°C and pH 8. The expressed pectate lyase was highly thermostable and retained about 51% of activity when incubated at 40, 50, and 60°C for 1 hour (Guan et al., 2020). Pectate lyase cloned from *B. subtilis* (BsPel-PB1) showed the optimal temperature of 50°C and pH 9.5 (Zhou et al., 2017). Pectinase from *B. halodurans* illustrated the optimal temperature of 80°C and pH 10, and the enzyme was stable at pH 9.5 to 10.5 (Mei et al., 2013). The thermostability of proteins mainly depends on the various structural features including the bonding formed during intra and intermolecular interactions, post-translational modifications, packing of side chain in hydrophobic core of protein, and amino acid sequences (Alponti et al., 2016). In the present study, both expressed PelA-S5 and PelA-S14 illustrated the good stability at higher temperature and extreme pH making these pectinases of great industrial importance.

The enzymes having acidic or alkaline optimal pH and thermostable properties pose numerous opportunities for biological economic development.

3.3. Michaelis–Menten kinetics parameters

K_m and V_{max} were determined using different substrate concentrations of 0.1, 0.25, 0.50, 1.00 and 1.50 mg/mL (w/v) in phosphate buffer of pH 7.4. The K_m values of expressed pectinases were: 2.112 and 13.816 mg/mL, and V_{max} 9.843 and 23.529 U/mg, respectively (Table 1). The K_m value is a measure of affinity or specificity of an enzyme to the substrate and the high V_{max} is reflective of the limiting rate of the enzymatic reaction.

Table 1 The kinetic parameters (K_m and V_{max}) of two expressed pectinase

Expressed pectinase	K_m (mg/mL)	V_{max} (U/mL)	R^2
PelA-S5	2.112	9.843	0.87
PelA-S14	13.816	23.529	0.93

Another study illustrated the thermostable pectinase when expressed revealed the kinetic parameter; K_m and V_{max}, values of 4.1 g/L and 352 U/mg when polygalacturonic acid was used as substrate and reaction was undertaken at 80°C (Mei et al., 2013). Pectate lyase PelB-B2 expressed in *E.coli* showed K_m of 1.64 g/L and V_{max} 232.56 mol/(L min) illustrating the higher affinity to citrus pectin (Guan et al., 2020) which is similar to our study.

3.4. Computational modeling of the expressed PelA and its docking analysis

DeepFold, a deep-learning-based method for predicting protein structure, was used for constructing the computational 3D model. The docking result showed that PelA-S5 has lower binding energy with the ligand polygalacturonic acid (-9.76 kcal/mol) compared to PelA-S14 (-7.09 kcal/mol). Furthermore, the docking analysis indicated that Ser124, Asn145, Gly147, Thr148, Lys174, Gly175, Arg177, and Lys203 are involved in the substrate binding in PelA-S5

and among them, Lys174, Arg177, and Lys203 would be responsible for the major catalysis. Similarly, Ser150, Lys200, Arg203, and Lys228 would be involved in the substrate binding in PelA-S14, and Lys200, Arg203, and Lys228 would be involved in major catalysis.

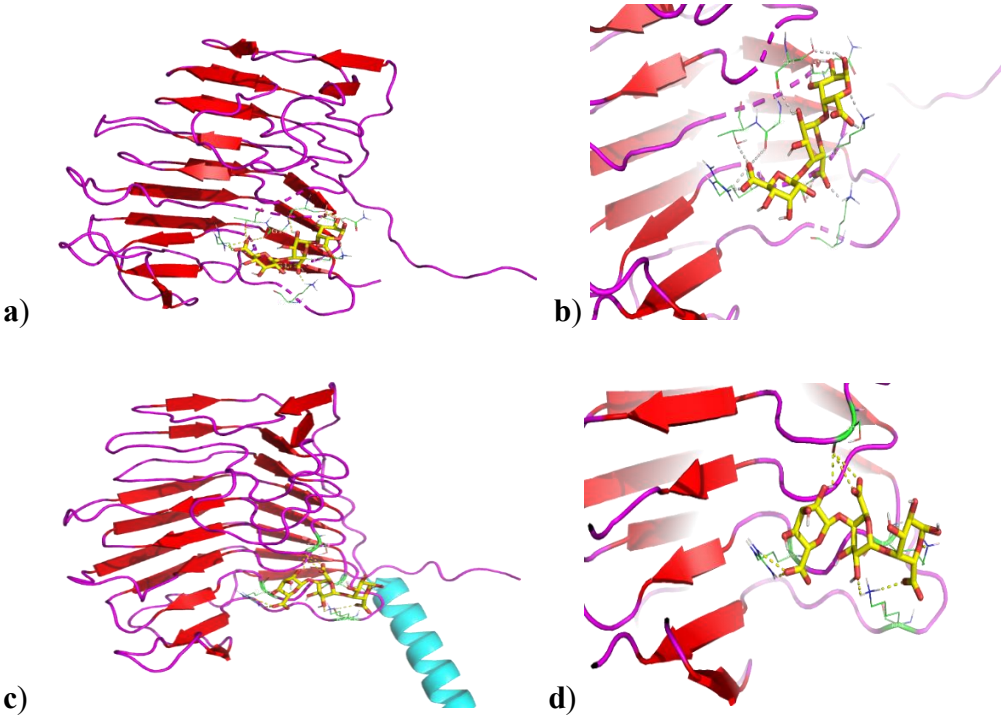

Fig. 6 Structure overview **a**) and **c**) and binding pockets **b**) and **c**) of expressed PelA-S5 and Pel A-S14, respectively

The crystal 3D structure of the pectate lyase family 3 is rarely reported. Therefore, when constructing the computational homologous 3D model via SWISS-model, there was only 33.94% sequence identity with the template 4u4b. 1. A from *Pectobacterium carotovorum*. Therefore, in terms of avoiding the potential error due to the low sequence identity, an *ab initio* protein prediction DeepFold was used for predicting the protein structure. Based on the docking analysis and binding energy, PelA-S5 would have a better substrate affinity compared to the PelA-S14. Also, lower Km value of PelA-S5 indicated better substrate affinity than Pel-A-S14 in the experiment. Furthermore, Lys and Arg are commonly considered the basic amino acid in the

active site of the pectate lyase family 3 (Creze et al., 2008; Pineau et al., 2021) and with the operation with a water molecule, the polysaccharide would be hydrolyzed.

3.5. Sequence alignment of expressed pectinase proteins

The expressed pectinase protein (PelA-S5 and PelA-S14) from both isolates when aligned with the pectate lyase sequences found in NCBI database, their alignments were observed as Fig. 4. Fig. 4 was generated by using the free website (Robert and Gouet, 2014). The letters in BLACK mean that the similarity is lower than 70% (not even a similar amino acid). The letters in the BLUE frame are having similarity higher than 70% and within it, the letters in RED mean they are identical or similar (acidic/phenolic amino acid). However, the letters in the WHITE word and RED frame are identical amino acids.

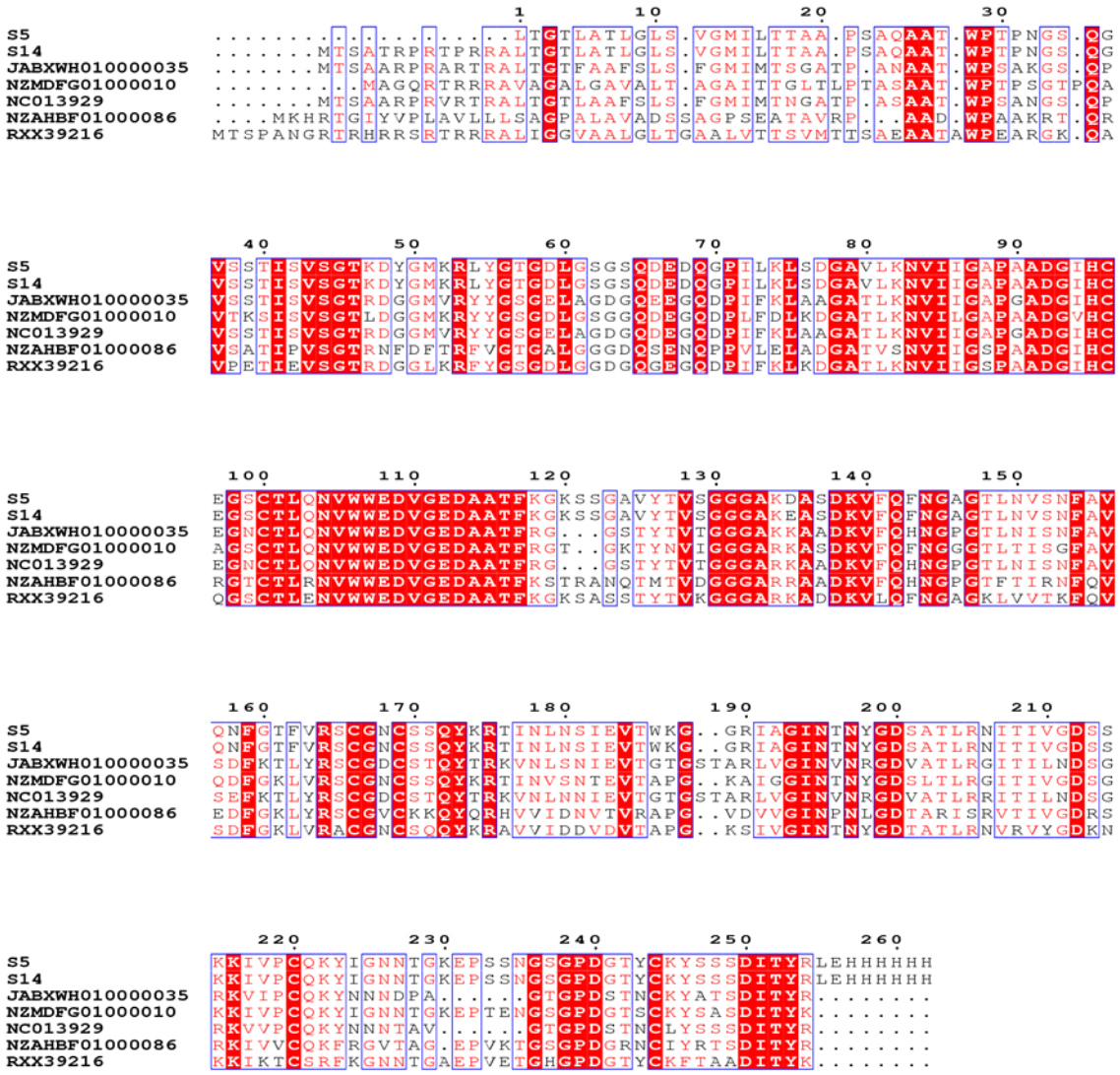

Fig. 7 Sequence alignment of expressed PelA-S5 & PelA-S14 with other pectin lyase from the NCBI database

4. Conclusion

In summary, pectinase gene from two soil isolates identified as *Streptomyces* sp. (S-5 and S-14) was cloned and expressed in a very common expression vector *E. coli* BL21 (DE3). Both expressed pectinases, PelA-S5 and PelA-S14, showed maximum activity at 50°C and in two pH (pH 5 and 9). Furthermore, both expressed pectinases illustrated the thermal stability which is a

very important property for the various industrial applications. This study indicates the expressed pectinases are more applicable in harsh environmental conditions and in different biotechnological processes. In addition, this study illustrated a very slight differences in some molecular properties of these two expressed pectinases (PelA-S5 and PelA-S14) such as their activity at different pH, temperature, thermostability, and their structures and binding pockets.

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Chapter 7

Conclusions and Future Recommendations

Conclusions

The importance and implications of pectinases are rising in diverse areas, including bioethanol production, extraction of DNA, and protoplast isolation from a plant, not limited to juice and wine industries, pulp and paper industries, tea and coffee fermentation, and animal feed industries. To mitigate the increasing demand for pectinase in various avenues and industries, it is essential to isolate and identify the organisms that can produce pectinase with a wide range of stability factors like temperature and pH. At the same time, due to the high cost of the substrate used for the microorganism's growth, pectinase production using microorganisms is limited. The low-cost or no-cost substrates, such as various agricultural biomasses, can be used as feedstock for microorganisms' growth and produce enzymes. Utilizing those agricultural residues can reduce the production cost on the one hand and, on the other hand, can reduce pollution and greenhouse gas emission and help in waste management.

Therefore, this study focuses on isolating pectinase-producing bacteria, optimizing the cultural condition for maximum pectinase production, and exploring the possible ways to valorize agricultural residues. In addition, the study utilized the various locally available agricultural waste for pectinase production by the local forest soil bacteria. Furthermore, the pectinase gene from *Streptomyces* sp. was expressed to *E. coli* and characterized the pectinase enzyme.

At the end of experiments directed towards specific objective 1, the pectinase-producing bacteria from forest soil was screened, isolated, and identified. This study illustrated that 17 of 29 bacteria (58.62%) from forest soil samples were pectinolytic. Four bacteria (S-5, S-10, S-14, and S-17) showing high pectin hydrolysis zones were processed for identification and designated as

Streptomyces sp. (S-5, S-14), *Cellulomonas* sp. (S-10), and *Bacillus* sp. (S-17). They were Gram-positive, non-hemolytic, not proteolytic (based on gelatin hydrolysis), non-capsulated, and lipase producers. Interestingly, bacteria showed multi-enzyme (pectinase, cellulase and xylanase) activities. The isolates exhibited auto-aggregation capacity in an order S-5 (66.67%) > S-14 (56.86%) > S-17 (49.77%) > S-10 (27.06%). Further, the bacteria were found to be weak biofilm producers and non-hydrophobic. S-5 illustrated the highest macerating capacity for both potato and cabbage (18.49 and 42.59%, respectively) among the isolates, and UV radiation increased pectinase activity. The soil bacteria isolated are not pathogenic and have the potential to be used as probiotic. The crude enzyme extracts of those bacteria were applicable in oil and juice extraction from sesame seeds and apples, respectively.

To address specific objective 2 of this thesis, the cultural parameters of *Streptomyces* sp. for pectinases production were optimized using the Box-Behnken design. The bacterium produced maximum pectinases at 35℃, pH 7, and 58 hours upon submerged fermentation in yeast extract-containing media. On SDS-PAGE and zymogram, the protein illustrated the molecular mass of 25 and 75 kDa. The pectinase activity was detected in a wide range of temperatures (30℃ to 80℃) and pH (7 to 9), and the maximum pectinase activity was observed at 70℃ and two different pHs (5 and 9). The residual activity of the enzyme was about 30 to 40% at different temperatures, even after 120 mins. Also, the pectinase activity of *Streptomyces* sp. was enhanced in the media containing 1.5% pectin, 1% casein as a nitrogen source, 0.5 mM $MgSO_4$, and 5 mM NaCl. Further, the bacterium illustrated the ability to decolorize crystal violet dye efficiently, showing its potential in treating wastewater produced by industries like textile industries. While optimizing cultural conditions for *Streptomyces* sp. (S-5) to maximize pectinase production, we found significantly high pectinase activity from the contaminated broth, so we isolated and

identified the contaminated bacterium as *Bacillus* sp. The bacterium produced different polysaccharides degrading enzymes, such as pectinase, polygalacturonase, xylanase, amylase and cellulase. The response surface methodology was applied to know the optimal conditions for maximum enzymes production. Furthermore, the unique colony character (clear water-like droplets in the colony) encouraged us to explore its lipid producing ability. And the bacterium depicted more than 20% lipid content indicating the bacterium potential to produce bacterial lipids and it can be the potential feedstock in producing renewable biofuels and environmental resilience. Also, the bacterium is a good candidate for producing biotechnologically important multi-enzymes and agro-waste degradation. The study helped us to conclude that the contaminant or unexpected results should not be ignored.

We subsequently studied the use of that agro-waste in producing enzymes, pectin, polyphenols, and compost (objective 3). In addition, agro-wastes were explored for immobilization of whole cells, adsorption of dye, saccharification and ethanol production. The novel bacterium (*Streptomyces thermocarboxydus)* was exploited to use eight agro-wastes (banana peel, barley straw, canola straw, pomegranate peel, orange peel, pumpkin pulp+seeds, maple leaf, and brewer's spent grains) for enzymes production. Scanning electron microscopy images of eight agro-wastes before and after bacterial treatment and weight loss of agro-wastes revealed the bacterium degraded the biomasses. Extreme vertices mixture design was used in formulating the agro-waste mixture for multi-enzyme production. The study illustrated the bacterium produced the highest pectinase, polygalacturonase (PGase), xylanase, and cellulase by formulating the agro-waste mixture of orange peel, pomegranate peel, and pumpkin pulp+seeds. Also, the mixture of agro-waste enhanced enzyme production rather than the single feedstock, and the enzyme production depends on the agro-waste mixture.

Of the various agro-wastes studied, pomegranate peel and maple leaf illustrated higher flavonoid, total phenolic content, and antioxidant capacity. However, different solvents used in extraction showed different potentials for evaluating total phenolic content, total flavonoid, and antioxidant capacity. The inhibition zone varied on the agro-wastes extract and the bacterial strains. The aqueous extract of pomegranate peel exhibited the highest inhibition zone against *Cellulomonas* sp. (S-10) and *Bacillus* sp. (S-17), while pumpkin pulp+seeds extract did not show any inhibition. Besides, the study revealed higher pectin yield from pumpkin pulp+seeds followed by orange peel, banana peel, pomegranate peel, and others. The traditional and microwave-assisted methods showed almost similar pectin yield from most of the agro-wastes used. This study supports different agro-wastes as potential low-cost resources for innovative and competitive production of phytochemicals, including total flavonoid, phenolic content, and pectin. In addition, those agro-wastes exhibited antibacterial potency and have the potential to be used in the pharmaceutical industries.

The study revealed orange peel as the best adsorbent and canola straw as the inferior adsorbent of crystal violet. The adsorption of crystal violet may be influenced by the agro-wastes, the concentration of crystal violet, and the time of exposure. Further, the study illustrated that laboratory-scale composts were formed within 90 days. During the composting process, there were changes in the visual appearances of agro-wastes mixtures, temperature, and pH of composts. The temperature increased in the initial first week of composting and later declined. Similarly, the pH of the compost was acidic in the beginning and alkaline (around pH 9) at the end. The number of bacteria in all composts was 10^7 colony-forming units per gram of compost. Further, seven bacteria with different colony morphologies were isolated from composts. 100% moong seeds germinated with those composts; however, the weights of the seedlings were

different with the different composts. Thus, valorizing agro-wastes can finally help in waste management and pollution reduction.

The best formulation contained 43.33% orange peel, 33.33 % pumpkin pulp+seeds, and 23.33% pomegranate peel which exhibited significantly high saccharification (22.36±0.54 mg/g dry weight) among all other mixtures. The hydrolysate of this mixture supplemented with 2% w/v fructose produced a maximum of 7.86±0.08% v/v ethanol by the yeast isolated from the brewer's spent grains. Thus, easily available waste could be a promising source for yeast isolation and feedstock for ethanol production.

While addressing the 4th objective of this research, the pectinase gene illustrated about 750 bp on the gel after electrophoresis and encoded a product of approximately 25 kDa molecular weight. Both expressed pectinases showed optimal activity at 2 different pHs (5 and 9) and at 50℃ and retained their activity for 120 mins indicating both have important thermostable properties. These enzymes could be of great potential in industrial biotechnological processes due to their stability over broad pH and high temperatures. This study revealed very slight differences in these two expressed pectinases (PelA-S5 and PelA-S14) like their activity at different pH, temperature, thermostability, and their structures, binding energy and binding pockets.

Future recommendations

The lignocellulolytic enzymes have humongous applications in different avenues; thus, there is a need for a green and economical process for maximum enzyme production. However, the primary consideration is the stability of the enzyme in a wide range of industrial environmental conditions and making the process cost-effective. The stability of enzymes over a wide range of temperatures and pH is the most crucial factor that gives an additional advantage to a microbial strain. So, new microorganisms with high enzyme activity, stable over a wide range of

temperatures and pH for a more extended period, and cost-effective production, need to be emphasized. In addition, genetically modified or engineered microorganisms are encouraged to be developed to resist a wide range of temperatures and pH and are applicable in huge industries and have important implications.

The maximum utilization of organic wastes as raw materials in producing industrially relevant compounds like enzymes via fermentation, single-cell proteins, and many other products with appropriate technologies should be emphasized.

More studies in the valorization of agricultural residues are recommended, not limiting only pectin and phytochemical extraction, and using them as a substrate for enzyme production. Immobilization and re-immobilization of whole cells or enzymes onto low-cost material can have great potential for making the process more cost-effective; hence further research should also be focused on this area. Moreover, the sustainable, reliable and efficient procedure to reduce or decompose wastes producing various value-added products should be explored.

The powerful and versatile enzymes need to be developed through protein engineering and recombinant DNA technologies and discover the combined effects of different enzymes.

New and exciting enzymes which drastically decrease the production cost for specific applications are beneficial in many areas, lower energy consumption, and enhance the quality of products. Besides, the technologies applied need to be environment-friendly for waste management. More extensive research is required to commercially produce various valuable products through a resourceful, assimilated, friendly, and low-cost biotransformation process.

The extraction of products depends on the types of agro-wastes, methods and conditions of extraction, and solvents used. In addition, extracting value-added products such as polyphenols and pectin from different agro-wastes is a remarkable way to help to manage waste, decrease

greenhouse gas emissions, and take economic advantages. Furthermore, the biopolymer from agro-wastes having antibacterial properties can be developed and applied in bioplastic and food packaging material production. However, a detailed study of the extraction process for optimal output of different health-beneficial and economically important compounds from agro-wastes is recommended, and the process should be environmentally friendly, efficient, and cost-effective. Further research to improve and increase ethanol production and yield is recommended by using different microorganisms, consortia of microorganisms, agro-wastes and fermentation conditions. Also, other chemicals produced during fermentation need to be analyzed.

Appendix

Appendix 1: Supplementary Materials for Chapter 3A

A. Different tests for identification of bacteria

Gram's staining: This test distinguishes organisms into two large groups; gram-positive and gram-negative. In this test, there are basically four steps; primary staining with crystal violet to a heat-fixed smear, followed by the addition of mordant (gram's iodine), rapid decolorization with alcohol or mixture of alcohol and acetone, and finally counterstaining with safranin. Gram-positive bacteria appear as purple, and gram-negative as pink or red.

Spore staining: This staining helps to know if the bacterium is a spore producer or not. The staining method uses malachite green as the primary stain and safranin as counterstain. If the spore is present, it appears as bright green and vegetative cells as brownish red to pink.

Catalase test: It determines whether the bacterium contains a catalase enzyme or not.

For this, the bacterium inoculum is taken with the non-iron loop and smeared on the glass slide's surface. Then 2-4 drops of hydrogen peroxide are added to the smear. If the bubbles are observed, then the bacterium is catalase-positive, otherwise regarded as catalase-negative.

Oxidase test: This test determines the presence of cytochrome oxidase enzyme.

For this, an inoculum of pure bacterial culture is added to the test strip's surface impregnated with the reagent. The purple color observed within 5-10 seconds indicates the bacterium has the oxidase enzyme.

Methyl red- Voges Proskauer (MR-VP) test: It consists two tests.

1. MR test is useful in determining the fermentation pathway for glucose utilization.
2. If bacteria use a mixed acid fermentation pathway, glucose is fermented and produce different organic acids. This results in converting the color of the media yellow to red.

3. If bacteria use 2,3 butanediol fermentation pathways, glucose is fermented to produce 2,3 butanediol and increase the pH making alkaline, and color is not changed.
4. VP test determines the presence of acetoin or precursor of 2,3 butanediol. When α-naphthol and potassium hydroxide are added to the medium, shaken vigorously, and set aside for few minutes, brownish-red to pink color, if observed, denotes that acetoin is present in the culture and brownish-green to yellow color denotes an absence of acetoin.

Indole test: This test helps to determine if a bacterium can break tryptophan into indole or not. For this, the bacterium is inoculated into tryptone broth and incubate for 24 hours. Then, five drops of Kovac's reagent is added to the medium. A cherry red ring at the surface of the medium denotes the positive test. Sometimes orange color, which is the precursor of indole is observed.

Citrate test: This test is used to identify if the organism has the ability to use citrate as a source of carbon. When the bacteria are grown on the citrate agar, bacteria having citrate permease enzyme can use the citrate and break down the ammonium salts into ammonia. The ammonia results in increasing the pH of the media and changes the color of the media from green to blue. The color change is due to the bromothymol blue indicator, which changes color to blue at alkaline pH.

Sulfur, indole motility (SIM) test: Some bacteria can utilize tryptophan and produce indole, ammonia, and pyruvic acid. And, some bacteria can hydrolyze sulfur-containing amino acids or compounds to produce hydrogen sulfide.

In this test, bacteria are stabbed onto the medium with sulfur-containing compound and iron salts and incubated at 37℃ overnight.

1. If any black precipitate or color is observed in media, it refers the bacterium hydrolyzed sulfur-containing compound into visible ferric sulfide.

2. If there is hazy growth observed around the stabbed area or all over the media, it shows that the bacterium is motile.
3. When Kovac's reagent is added to the medium and the color changes to red, it denotes that amino acid-like tryptophan is converted into indole and turned the color into red.

Here, three properties of bacteria (sulfur production, motility, and indole production) are checked.

Triple sugar iron (TSI) test: It determines if the bacterium can utilize one or all saccharides (glucose, lactose, and sucrose). Glucose is a monosaccharide, and lactose and sucrose are disaccharides.

For this, slants with the pH indicator phenol red are used. The bacteria are stabbed and streaked on the media and incubated.

1. If the color of slant/butt is red/ orange, it refers that the bacterium only utilized peptone.
2. If the slant/butt color is red/ yellow, it indicates the bacterium used only glucose.
3. If the color of slant/ butt is yellow/ yellow, it denotes that the bacterium utilized glucose plus lactose and/or sucrose present in the media.

If any bubbles or black precipitate or color are observed, it shows that the bacterium is a gas and hydrogen sulfide producer.

DNase test: The test determines the microorganism if it can hydrolyze DNA or not. For this, the bacterium is grown in DNase agar plate, which is pale green. If the colorless zone is observed around the colony, it denotes the bacterium produces DNase.

Urease test: This test determines an organism's ability to hydrolyze urea and produces ammonia and carbon dioxide. Due to ammonia production, the pH of the media shifts to alkaline and changes the color of the media yellow to pink.

Starch hydrolysis test: This test helps identify if the bacteria can hydrolyze starch as a carbohydrate source. The bacteria secrete an exoenzyme that hydrolyzes the starch by breaking down the bonds between glucose molecules of starch.

For this, the bacterium is streaked on a starch agar plate and incubated at 37℃ overnight. Then iodine is added over the growth. If bacteria hydrolyze the starch, there will be a clear zone around the bacterial growth otherwise, the agar will remain a dark brown or blue/black color.

Gelatin hydrolysis: It helps to recognize if the bacterium can liquefy gelatin or not.

For this, the bacterium is stabbed into the nutrient gelatin media and incubated for 24 hours at 37℃. If the media is liquefied and any drops are seen, the test is considered positive.

Lactose fermentation test: This test determines if the bacteria can utilize lactose as a carbon source.

For this, the bacterium is inoculated into the media containing lactose and incubated at 37℃ for 24 hours. A color change in the media denotes a positive result.

Violet red bile test: This test is used to identify if the bacterium is of the Enterobacteriaceae family. Based on lactose fermentation, they are classified into lactose fermenter and non-lactose fermenter. Lactose fermenter gives pink to red color, and non-lactose shows colorless or transparent colonies.

Amylase test: The test is used to identify bacteria that can hydrolyze starch using amylase enzyme. The iodine is flooded over the colonies in agar plate containing starch. A clear zone around the colony denotes the bacterium has the amylase enzyme.

Capsule staining: This staining is performed to identify if the bacteria is capsule producer or not. For this staining, a smear is made in a slide with a drop of crystal violet. After drying of

smear, it is rinsed with 20% $CuSo_4$, air dry and observe under microscope. The clear zone around microbes and violet background represents presence of capsule.

Biofilm test: Bacteria are grown overnight in broth media at 30℃ and diluted to 1:100 w/v in media with 2% glucose. Then the bacterial suspension of about 200µL were transferred into 96-well microtiter plates and incubated at 30℃ for 24 hours. The plates with bacteria was washed twice with phosphate buffer saline and dried in inverted position. Then after, the adherent bacteria were fixed with 95% ethanol and stained with 1% w/v crystal violet for 5 mins. Again, the plate was washed with distilled water and air dried, and optical density of each well was measured at 570 nm.

The result interpretation; OD570≥ 1: highly positive, 0.1≤OD570<1: moderately to weakly positive, and OD570≤0.1: negative.

Antibiotic susceptibility test: The bacteria were cultured at 35℃ for 16 hours, and 200 µL of bacterial suspensions were evenly spread on the Muller-Hinton agar plat (MHA) with the sterile disposable spreader and left to dry for 20 minutes. Seven different antibiotic discs used in this experiment are ampicillin (10 µg), bacitracin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), tetracycline (30 µg), penicillin (10 µg), and novobiocin (10 µg). The antibiotic discs were gently pushed on the agar plate with sterile forceps to ensure the proper contact before incubation. Then the plates were incubated at 35℃ for 24-48 hours. The diameters of the inhibited zone were measured and interpreted according to the measurements provided by the Clinical and Laboratory Standard Institute (CLSI) guidelines.

Supplementary Table 1: Antibiotic susceptibility ranges for different antibiotics as CLSI

Antibiotics	**Diameter of inhibition range in mm**		
	Resistant	Intermediate	Susceptible
Ampicillin (10µg)	≤11	12≤DI<13	≥14
Bacitracin(10µg)	≤6	7≤DI<10	≥11
Erythromycin(15µg)	≤13	14≤DI<22	≥23

Tetracycline(30µg)	≤14	15≤DI<18	≥19
Chloramphenicol(30µg)	≤12	13≤DI<17	≥18
Penicillin(10IU)	≤28	-	≥29
Novobiocin(30µg)	≤12	13≤DI<15	≥16

[DI: Diameter of inhibition]

B. Composition of media

Pectinase screening media

Ammonium sulphate	2 g/L
Yeast extract	1 g/L
Na_2HPO_4	6 g/L
KH_2PO_4	3 g/L
Citrus pectin	5 g/L
Agar	20 g/L

Nutrient agar

Peptone	0.5% (w/v)
Beef extract/ yeast extract	0.3% (w/v)
NaCl	0.5% (w/v)
Agar	1.5% (w/v)
pH	6.8

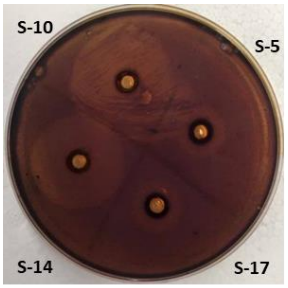
Pectinase production media

Yeast extract	0.3% (w/v)
K_2HPO_4	0.2% (w/v)
KH_2PO_4	0.2% (w/v)
K_2NO_3	0.2% (w/v)
Citrus Pectin	1% (w/v)

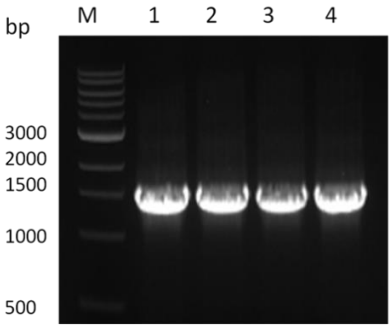
Luria-Bertani (LB) broth

Yeast extract	1% (w/v)
Peptone	1% (w/v)
NaCl	0.5%(w/v)

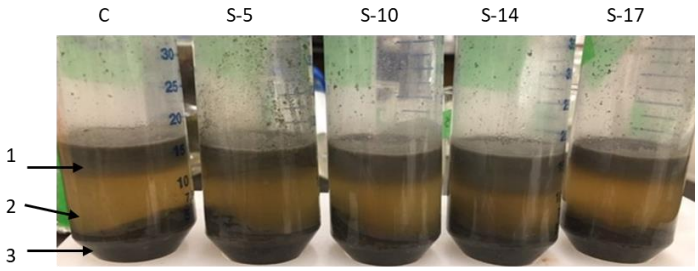
C. Supplementary Figures



Supplementary Fig. 1: Screening of pectin hydrolysis of isolates

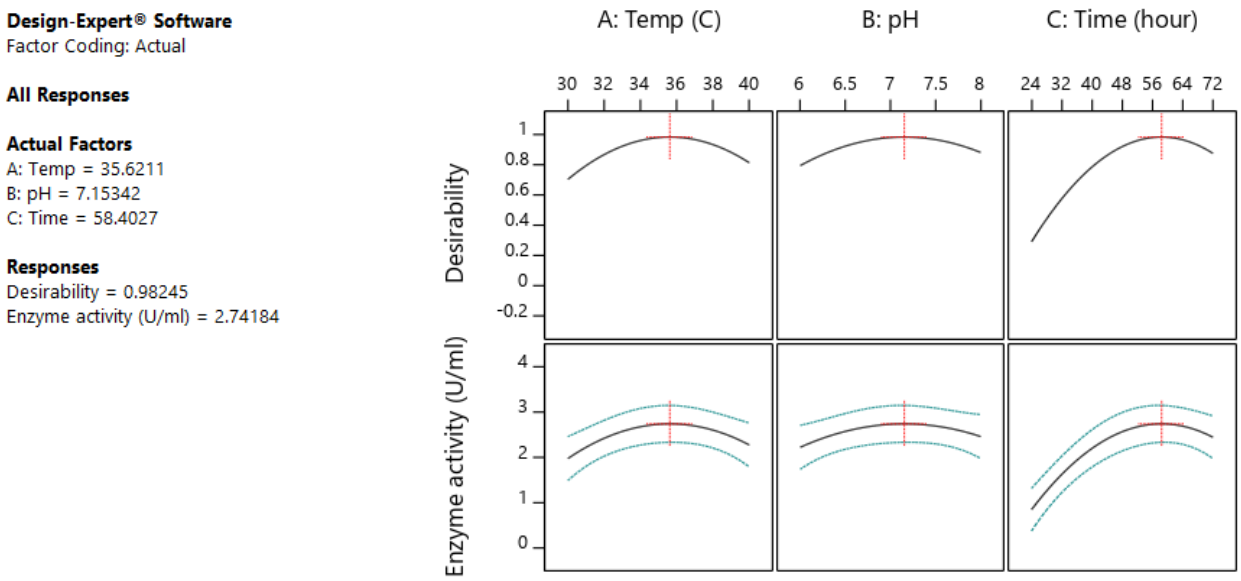


Supplementary Fig. 2: PCR results of 16s rDNA for the isolates after PCR in gel [M: DNA marker, 1,2,3,4: 16s rDNA of isolates]

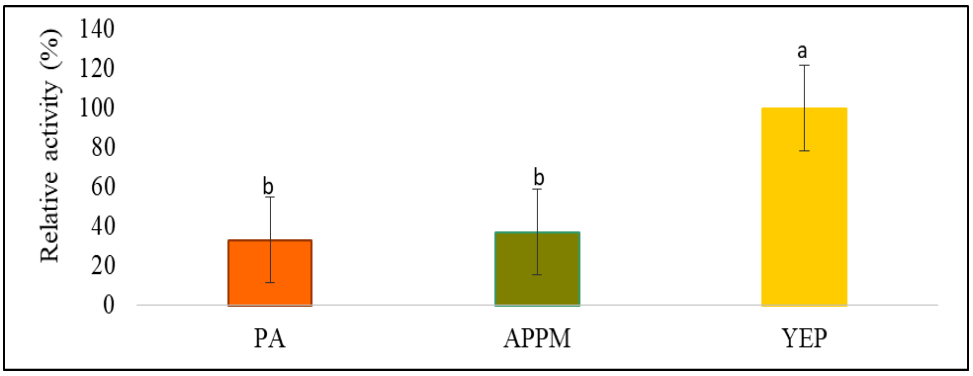


Supplementary Fig. 3: Oil extraction from sesame seeds with enzyme extracts of isolates [C: control, S-5, S-14, S-10, S-17: isolates, 1: emulsified oil, 2: skim, 3: residue

Appendix 2: Supplementary Materials for Chapter 4A



Supplementary Fig. 1 Model Projection of optimal condition

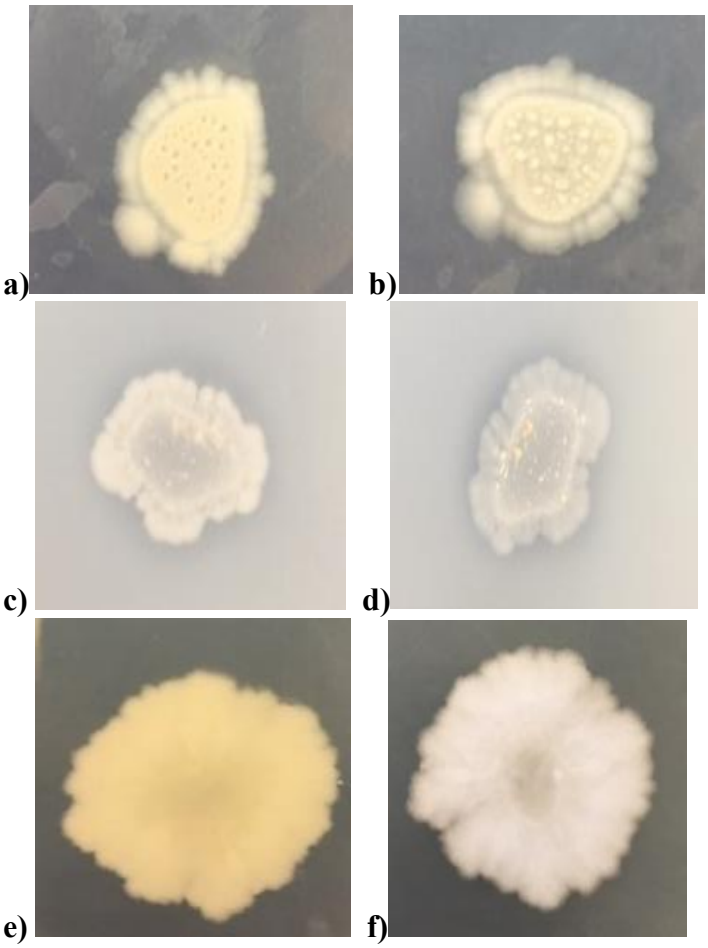


Supplementary Fig. 2 Effects of different fermentation media for pectinase activity (different lowercase letters above the bar indicate the significant difference between media used)

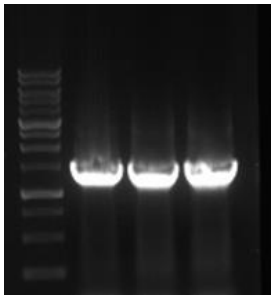
Appendix 3: Supplementary materials for Chapter 4B

Supplementary Figures

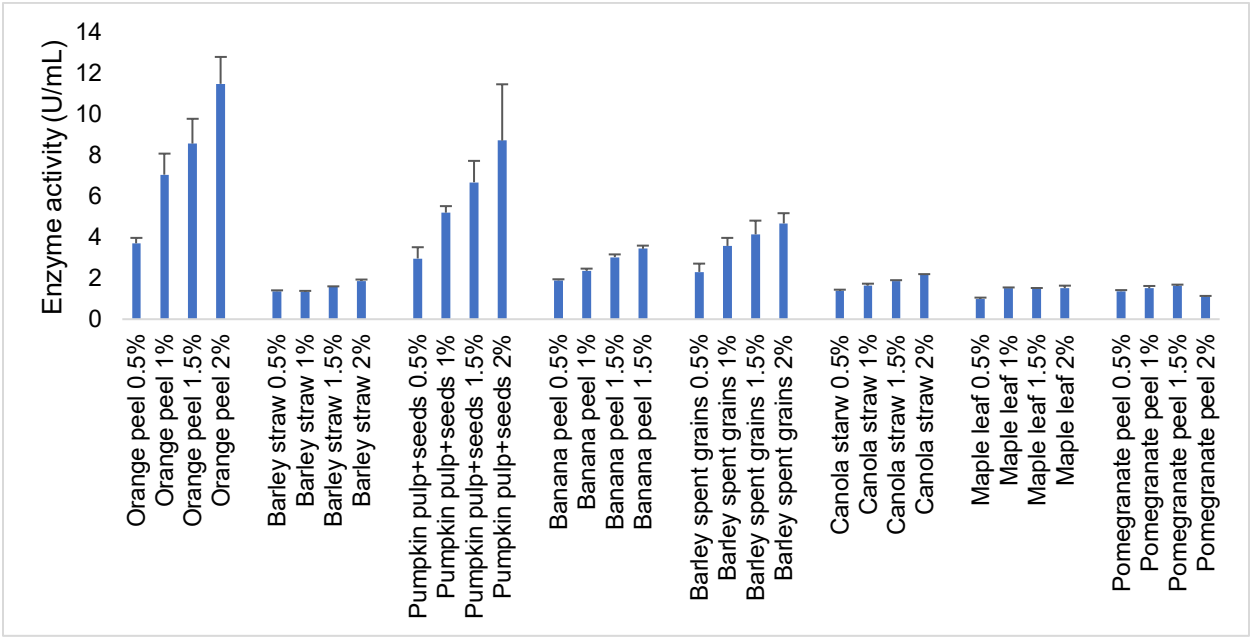
Supplementary Fig. 1 Colony morphology from the back (**a**, **c**, and **e**) and top (**b**, **d**, and **f**) of the plate on Sabouraud Dextrose Agar (SDA), Casein starch agar (CSA), and Nutrient agar (NA), respectively



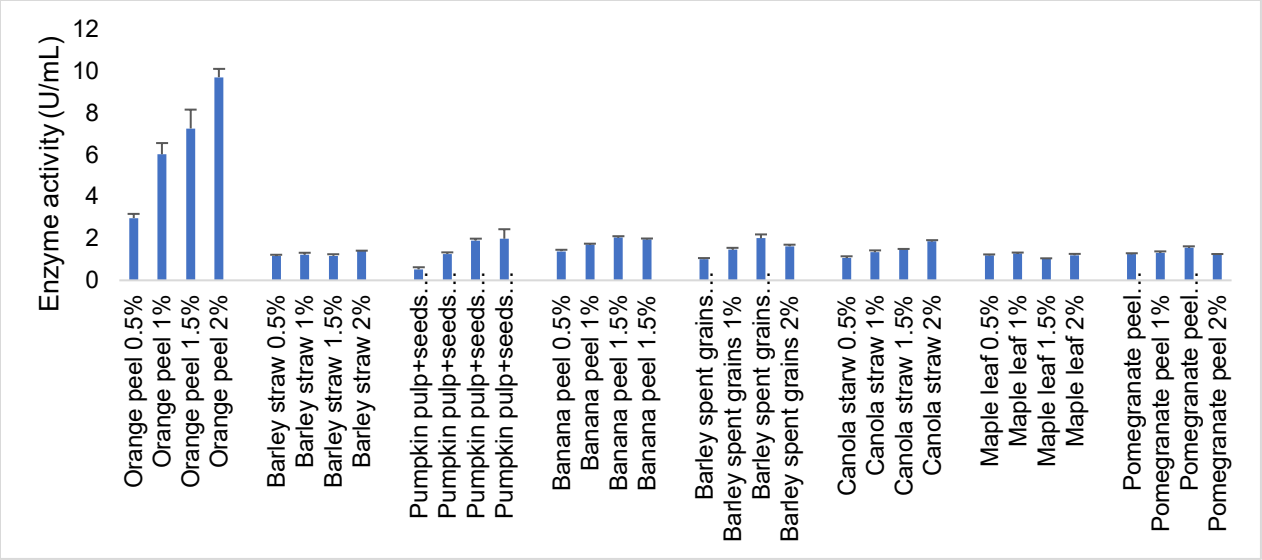
Supplementary Fig. 2 16S rDNA on Gel electrophoresis



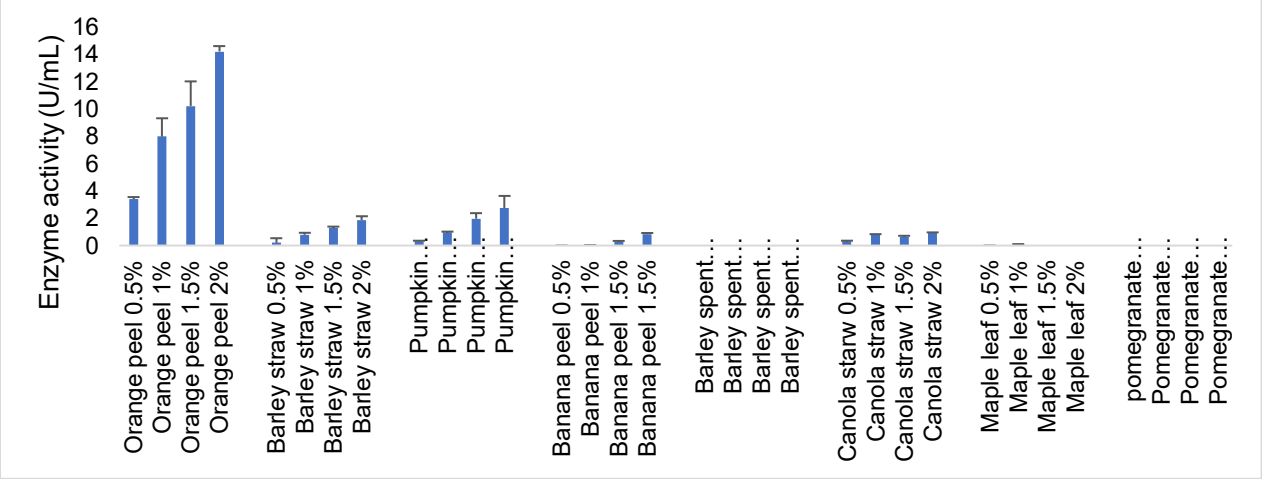
Supplementary Fig. 3a Pectinase activity at different biomass concentrations exhibited by *Bacillus* sp.



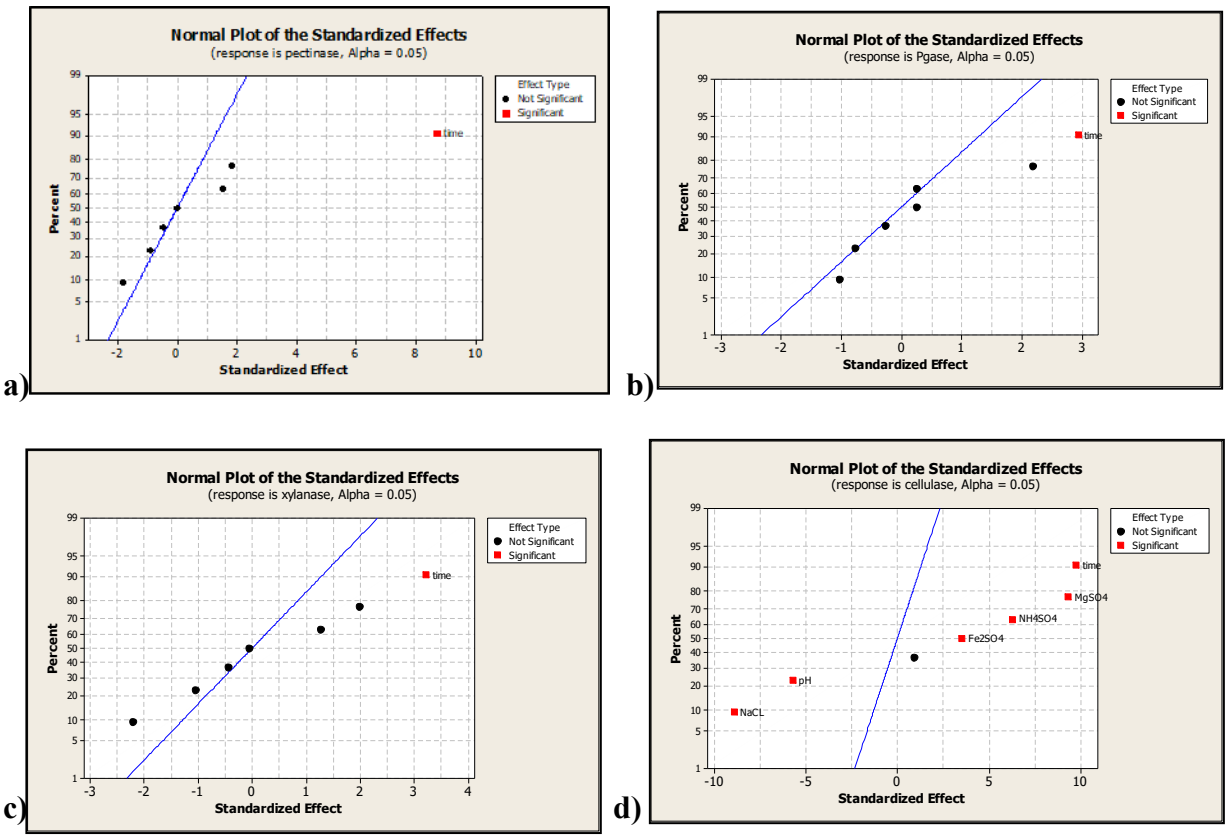
Supplementary Fig. 3b PGase activity exhibited by different biomass concentrations exhibited by *Bacillus* sp.



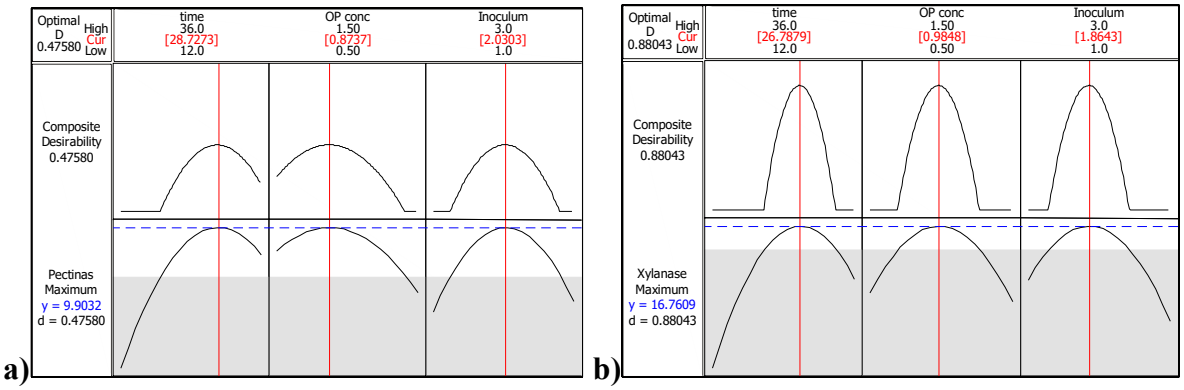
Supplementary Fig. 3c Xylanase activity at different biomass concentrations exhibited by *Bacillus* sp.



Supplementary Fig. 4 Normal plot of different factors in Plackett-Burman design



Supplementary Fig. 5 The optimal conditions from the response optimizer for a) Pectinase and b) Xylanase activities



Supplementary Tables

Supplementary Table 1 Uncorrected genetic distance between the species

S.N	Name of species	1	2	3	4	5	6	7	8
1	*Bacillus*_sp.	0.000							
2	*Bacillus siamensis_(LC556998)*	0.035							

3	*Bacillus velezensis_(LC617083)*	0.035	0.000					
4	*Bacillus amyloliquefaciens (MN749553)*	0.035	0.000	0.000				
5	*Mycobacterium* sp._(*MH699149)*	0.206	0.198	0.198	0.198			
6	*Bacillus megaterium_(MT510154)*	0.088	0.051	0.051	0.051	0.223		
7	*Cellulomonas massiliensis_(NR125601)*	0.214	0.206	0.206	0.206	0.054	0.247	
8	*Streptomyces thermocarboxydus (AB907696)*	0.211	0.202	0.202	0.202	0.081	0.235	0.088	0.000

Supplementary Table 2 Statistical analysis of Plackett-Burman design showing coefficient values, t- and p-values for each variable on enzymes activities

Variable		Effect	Coefficient	t-value	p-value
Constant	A		3.83	19.14	0.00*
	B		4.05	10.42	0.00*
	C		3.19	5.78	0.00*
	D		-0.73	-2.98	0.02*
Temperature	A	-0.18	-0.09	-0.45	0.67
	B	0.19	0.09	0.25	0.81
	C	-2.43	-1.22	-2.20	0.07
	D	0.45	0.22	0.92	0.39
pH	A	-0.35	-0.18	-0.89	0.41
	B	-0.59	-0.29	0.25	0.47
	C	-0.49	-0.24	-0.44	0.67
	D	-2.79	-1.39	-5.72	0.00*
Time	A	3.49	1.75	8.73	0.00*
	B	2.28	1.14	2.94	0.03*
	C	3.56	1.78	3.22	0.02*
	D	4.79	2.39	9.79	0.00*
$MgSO_4$	A	0.61	0.31	1.53	0.18
	B	1.70	0.85	2.19	0.07
	C	2.19	1.09	1.98	0.09
	D	4.58	2.29	9.36	0.00*
NaCl	A	-0.72	-0.36	-1.80	0.12
	B	-0.79	-0.39	-1.02	0.34
	C	-1.16	-0.58	-1.05	0.33
	D	-4.36	-2.18	-8.92	0.00*
Fe_2SO_4	A	0.00	0.00	0.00	0.99
	B	-0.20	-0.10	-0.26	0.80
	C	-0.06	-0.03	-0.05	0.96
	D	1.72	0.86	3.25	0.01*

NH_4SO_4	A	0.73	0.37	1.83	0.12
	B	0.19	0.09	0.26	0.81
	C	1.41	0.70	1.27	0.25
	D	3.08	1.54	6.30	0.00*
Lack of fit	A		0.69		
	B		0.19		
	C		0.34		
	D		0.42		

Notes: [A] Pectinase activity, [B] PGase activity, [C] Xylanase activity, [D] Cellulase activity

*Significant values at $p \leq 0.05$

Compositions of Media

Pectinase screening agar

Components	Percentage
Ammonium sulphate	0.2
Yeast extract	0.1
Na_2HPO_4	0.6
KH_2PO_4	0.3
Citrus pectin	0.5
Agar	2

Nutrient agar

Components	Percentage
Peptone	0.5
Beef extract/ yeast extract	0.3
NaCl	0.5
Agar	1.5

Xylanase screening agar

Components	Percentage
Xylan	0.5
Yeast extract	0.5
Peptone	0.5
$MgSO_4.7H_2O$	0.02
K_2HPO_4	0.1
Agar	2

Amylase screening agar

Components	Percentage
Starch	1
$(NH4)_2SO_4$	0.2
Peptone	0.5
$MgCl_2$	0.01
K_2HPO_4	0.2
KH_2PO_4	0.1
Agar	2

Cellulase screening agar

Components	Percentage
CMC	0.5
Yeast extract	0.5
Peptone	0.5
$MgSO_4.7H_2O$	0.02
K_2HPO_4	0.1
Agar	2

Lipase screening agar

Components	Percentage
NaCl	0.5
$CaCl_2$	0.1
Peptone	1.5
Tween 80	1
Agar	2

Tween sterilized separately and added later aseptically (pH)6.8

Pectinase production media

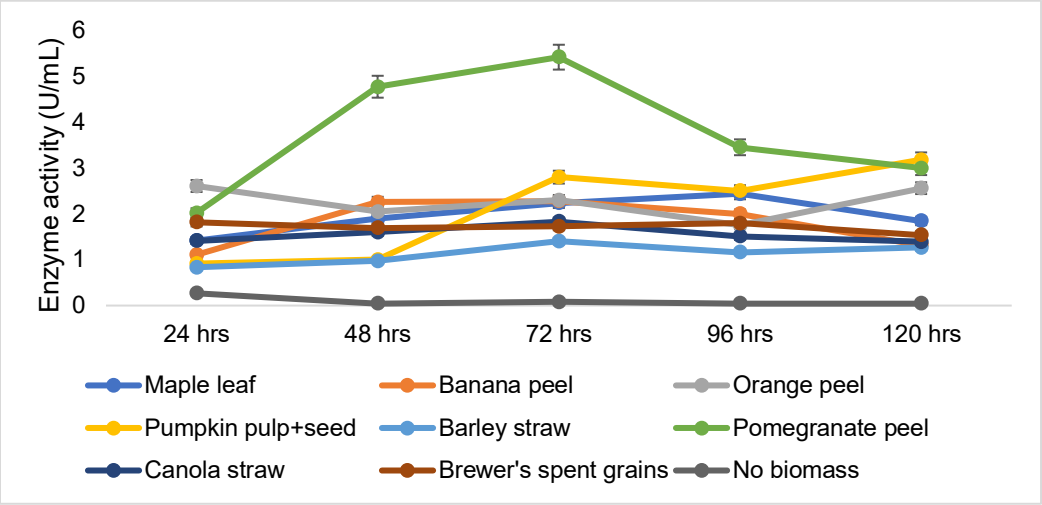
Components	Percentage
Citrus Pectin	1
Yeast extract	0.3
K_2NO_3	0.2
KH_2PO_4	0.2
K_2HPO_4	0.2

Luria-Bertani (LB) broth

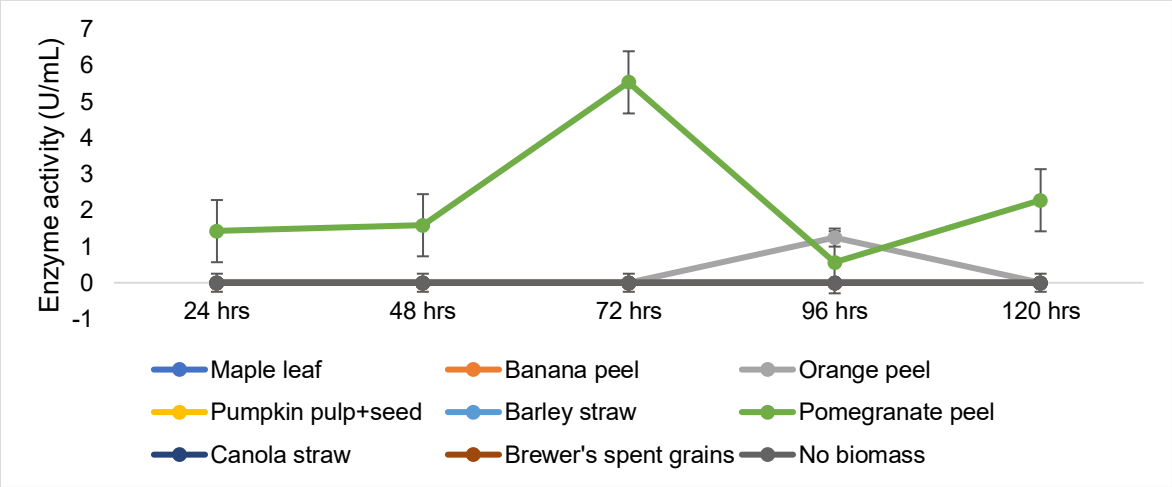
Components	Percentage
Yeast extract	1
Peptone	1
NaCl	0.5

Appendix 4: Supplementary materials for Chapter 5A

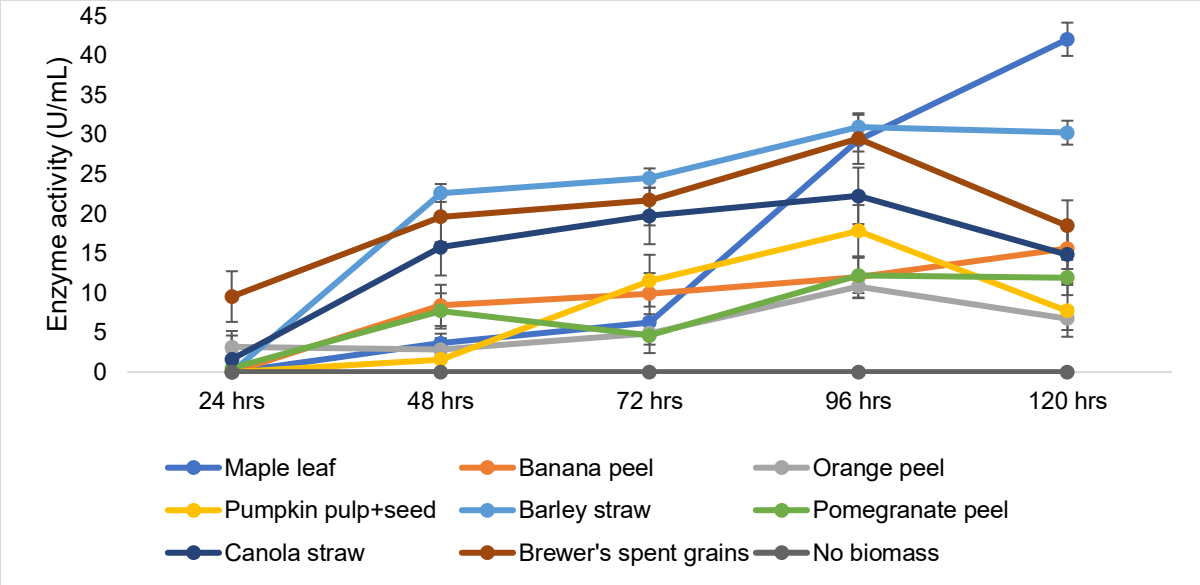
Supplementary figures



Supplementary Fig. 1 Pectinase activity by *S. thermocarboxydus* using different concentrations of agro-wastes/biomasses



Supplementary Fig. 2 Cellulase activity by *S. thermocarboxydus* using different concentrations of agro-wastes/biomasses



Supplementary Fig. 3 Xylanase activity by *S. thermocarboxydus* using different concentrations of agro-wastes/biomasses

Appendix 5: Supplementary materials for Chapter 5E

Supplementary Table 1 Reducing sugar from formulation of four agro-waste (orange peel, brewer's spent grains, canola straw, and barley straw) mixture

Run	Brewer's spent grains (%)	Orange peel (%)	Canola straw (%)	Barley straw (%)	Reducing sugar (mg/g dry weight)
1	20	20	20	40	11.21
2	40	20	20	20	11.26
3	20	40	20	20	12.03
4	20	20	40	20	12.27
5	25	25	25	25	11.20
6	22.5	22.5	22.5	32.5	11.48
7	32.5	22.5	22.5	22.5	11.18
8	22.5	32.5	22.5	22.5	11.29
9	22.5	22.5	32.5	22.5	11.61

Supplementary Table 2 Reducing sugar from formulation of nine agro-waste (orange peel (OP), brewer's spent grains (BSG), canola straw (CS), algae, banana peel (BP), maple leaf (ML), pomegranate peel (PP), pumpkin pulp+seeds (PPS) and barley straw (BS)) mixture

Run	Algae (%)	BP (%)	BS (%)	CS (%)	ML (%)	OP (%)	PP (%)	PPS (%)	BSG (%)	Reducing sugar (mg/gdry weight)
1	0	0	0	0	0	0	0	0	100	11.61
2	100	0	0	0	0	0	0	0	0	10.32
3	0	100	0	0	0	0	0	0	0	10.72
4	0	0	100	0	0	0	0	0	0	11.37
5	0	0	0	100	0	0	0	0	0	11.88
6	0	0	0	0	100	0	0	0	0	10.77
7	0	0	0	0	0	100	0	0	0	13.69
8	0	0	0	0	0	0	100	0	0	12.86
9	0	0	0	0	0	0	0	100	0	11.97
10	11.11	11.11	11.11	11.11	11.11	11.11	11.11	11.11	11.11	10.11
11	5.56	5.56	5.56	5.56	5.56	5.56	5.56	5.56	55.56	12.06
12	55.56	5.56	5.56	5.56	5.56	5.56	5.56	5.56	5.56	10.26
13	5.56	55.56	5.56	5.56	5.56	5.56	5.56	5.56	5.56	9.41
14	5.56	5.56	55.56	5.56	5.56	5.56	5.56	5.556	5.56	10.26
15	5.56	5.56	5.56	55.56	5.56	5.56	5.56	5.56	5.56	8.85
16	5.56	5.56	5.56	5.56	55.56	5.56	5.56	5.56	5.56	8.53
17	5.56	5.56	5.56	5.56	5.56	55.56	5.56	5.56	5.56	11.88
18	5.56	5.56	5.56	5.56	5.56	5.56	55.56	5.56	5.56	15.79
19	5.56	5.56	5.56	5.56	5.56	5.56	5.56	55.56	5.56	11.24