Isolation and Characterization of Bioconversion Microbes and Aerobic Conversion of Crude Glycerol to Value-Added Bioproducts

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// Abstract

Renewable forms of energy are of significant interest in today's economies. Biofuel is a renewable fuel produced by biological means. Biofuel is very useful because it does not have a finite supply, and it produces fewer pollutants than fossil fuels. Recently, there is a serious concern about future of fossil-based fuels due to increasing price of crude oil around the world, depletion of its sources, several important environmental issues (greenhouse effect and global warming) and increasing energy demands. Thus, this situation has imposed researchers for further exploring the renewable energy sources to produce environmentally friendly fuel that not cause any pollution to the atmosphere. Additionally, nowadays, supplies like plants, organic wastes, and agricultural products are often used to produce biofuel. Until now, bioethanol and biodiesel are the most widely produced and used as biofuel.

Biodiesel-derived crude glycerol and lignocellulosic (from cell walls of woody plants) biomass are two attractive low-cost renewable resources for biofuel production. Efficient and cost-effective production of biofuel from these sources through biotechnological methods depends on the development of a suitable bioconversion process. The main aims of this study were to i) isolate and characterize the novel as well as highly efficient cellulase or glycerol dehydrogenase (GDH) producing bacterial strains, and ii) optimize the enzyme production parameters during fermentation. These strains could be considered for the biorefinery industry for hydrolysis of lignocellulosic biomass or bioconversion of glycerol so as to accelerate biofuel commercialization.

In this research project, three potential glycerol metabolizing strains of *Serratia* sp. were isolated from paper mill waste of Resolute Forest Products, Thunder Bay, Ontario, Canada. The glycerol bioconversion abilities of the promising strain *S. proteamaculans* SRWQ1 were analyzed

using crude glycerol (byproduct from biodiesel production) as the sole carbon source. During shake flask fermentation under aerobic conditions at an optimal incubation temperature of 25°C, the strain SRWQ1 used 98% of the crude glycerol and produced 18.43 ± 1.55 g/L 2,3-butanediol (BDO) and 8.38 ± 0.76 g/L acetoin, with yields of 0.4 and 0.06 g/g, respectively. When the culture medium (minimal salt medium) was supplemented with 50 g/L of glycerol as the sole carbon source, and 5 g/L yeast extract and 5 g/L peptone as the nitrogen sources, the maximum glycerol dehydrogenase (DGH) activity was 408.69 ± 0.069 U/mg protein. The incubation temperature, pH, glycerol concentration and nitrogen sources are the most important factors ruling the GDH activity. Slightly acidic initial pH (pH 6.0) led to enhanced GDH activity and biomass production. This is the first report that *S. proteamaculans* species can efficiently convert glycerol to produce green products 2,3-BDO and acetoin.

Twenty cellulase-producing bacterial strains were isolated from Kingfisher Lake, Ontario, Canada, and screened for cellulase activity using the carboxymethyl cellulose (CMC) agar plate assay. Isolates showing large halos of depolymerization were further assayed to quantify enzyme production ability and identified by using 16S rDNA sequence analysis. The molecular weight of crude cellulase samples was determined ~50 kDa with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The *Bacillus* strain IM7 showed the highest carboxymethyl cellulase (CMCase) activity (17.7 ± 0.17 IU/mL) after 48 h of incubation at a yeast extract concentration of 15 g/L. A temperature of at 30°C and pH 5.0 were the optimal conditions for cellulase production. The highest activity (24.59 ± 0.09 IU/mL) was recorded when the culture medium was supplemented with 2% mannose as a co-substrate. The increased glucose content by using mannose in the hydrolysate process resulted in the dramatic increase in enzyme activity.

Keywords

Glycerol, Crude glycerol, 2,3-Butanediol, Acetoin, Bioconversion, Biotransformation, *Serratia proteamaculans,* Glycerol dehydrogenase, Value-added products, Optimization, Fermentation, Cellulase, Depolymerization, Characterization, *Bacillus* sp., Hydrolysis

List of Abbreviations

16S – Small subunit of a prokaryotic ribosome

1,3-PDO – 1,3-Propanediol2,3- BDO – 2,3-Butanediol

BLAST – Basic Local Alignment Search Tool

CMC – Carboxymethylcellulose

CMCase – Carboxymethyl cellulase

DHA – Dihydroxyacetone

DNA – Deoxyribonucleic acid

DNS – Dinitrosalicylic acid

EG – Endoglucanases

GDH – Glycerol dehydrogenase

GC-MS – Gas chromatography-mass spectrometry

LB – Luria Bertani (broth)

MS – Minimal salt

NAD – Nicotinamide adenine dinucleotide

NADH – Nicotinamide adenine dinucleotide-hydrogen

NCBI – National Center for Biotechnology Information

PBS – Potassium phosphate buffer

PCR – Polymerase Chain Reaction

rDNA – Ribosomal deoxyribonucleic acid

rRNA – Ribosomal ribonucleic acid rpm – Revolutions per minute SDS – Sodium dodecyl sulfate

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CHAPTER I

Microbial production of value-added chemicals from crude glycerol

Abstract

In large amounts, crude glycerol can have negative effects on the environment. Therefore, crude glycerol generated as a by-product in biodiesel production plants should be effectively used to contribute to the sustainability of the biodiesel industry and the bio-economy. Converting this crude glycerol into value-added bioproducts (e.g., ethanol, biopolymers, unsaturated fatty acids, and hydrogen) using biotechnological techniques would enhance the economic benefits to biodiesel producers. Thus, this study reviews techniques to isolate and characterize microbes and to convert crude glycerol to useful bioproducts.

Keywords: Crude glycerol, Biodiesel, Feedstock, Biotechnology, Characterization

1. Introduction

Carl Wilhelm Scheele first discovered glycerol, and synthesized and characterized several other compounds such as lactic, tartaric, and citric acids (Behr et al., 2008; Thompson et al., 2006). Glycerol contains three hydroxyl groups ($C_3H_8O_3$) (Fig. 1), and thus is termed a polyol, specifically a triol (da Silva et al., 2009). It is the main byproduct of transesterification of vegetable oils and animal fats to produce biodiesel (Rahman et al., 2015; Papanikolaou et al., 2016). Glycerol is highly soluble in water, slightly soluble in dioxin like ethyl acetate, and ether. Glycerol is known for solvent properties similar to water and aliphatic alcohols (Blankschien et al., 2010). Glycerol is a constituent of all natural fats and oils taking the form of fatty acid esters. It is fundamental to the metabolism of living organisms (Nada et al., 2016). It has been extracted in yields up to 90% by splitting triglycerides. Glycerol is a very important byproduct of oleochemistry and its supply has increased concomitant with the increase in oleochemicals production. Glycerol is also a primary waste product from the production of biodiesel, a widely used alternative to conventional fossil fuels (Jeon et al., 2014; Rosenblueth et al., 2004).

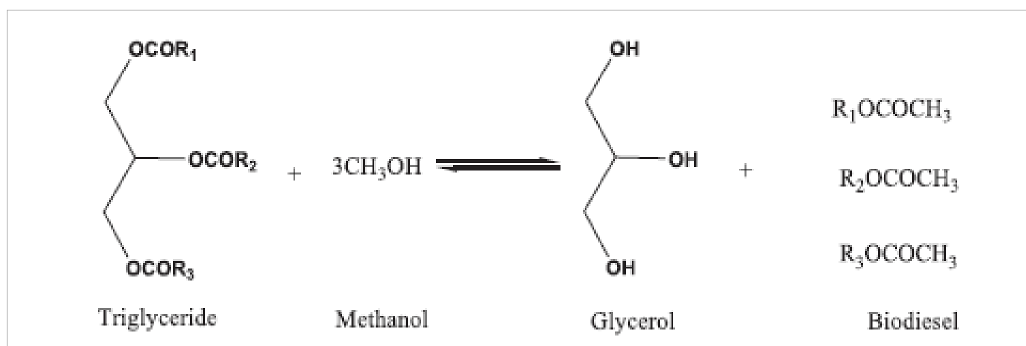

Figure 1. Glycerol as a by-product of biodiesel production (Nada et al., 2016)

As of 2012, the major producers of glycerol were the USA (1.3 billion gallons) and India (0.8 billion gallons) (Nwachukwu et al., 2012) (Fig. 2). It is currently produced from diverse sources through allyl chloride chlorohydrination, ethanolic fermentation of glucose, the acrolein

route, and fat division (Haas et al., 2006). However, glycerol production as a byproduct from the biodiesel industry (Fig. 2) is significantly increasing, lowering production costs and making it a good feedstock for industrial fermentation (Johnson and Taconi, 2007). Glycerol can be converted via bio-conversion to various compounds such as 1,3-propanediol (PDO), which has been used in the production of polymers such as polytrimethylene terephthalate, commercially available as Corterra™ or Sorona® (Xu et al., 2009). 1,3-PDO is one of the most valuable products and is widely used in the chemical industries, for example, in the preparation of laminates, plastic, adhesive materials, ultraviolet-cured the coating, and as a solvent (Zheng et al., 2008).

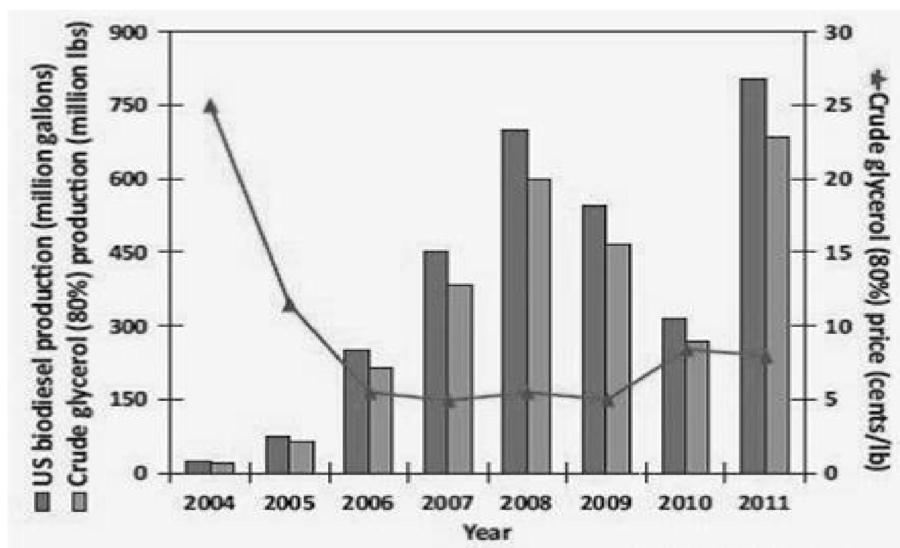

Figure 2. US biodiesel production and crude glycerol price (Gholami et al., 2014)

In the recent years, much attention has been given to biosynthesis of useful products from glycerol, since it uses only renewable feedstock and does not produce harmful byproducts unlike in chemical production (Subramaniam et al., 2010). A few microorganisms can convert glycerol at 30–40°C under atmospheric pressure to various products (Liang et al., 2010). For example, *Escherichia coli*, *Klebsiella pneumonia*, and *Enterobacter aerogenes* utilize glycerol as a source of carbon to produce commercially valuable chemicals (Jarvis et al., 1997) (Fig. 3). Of importance

to the present research, *E. coli*, *Klebsiella*, and filamentous fungi have been isolated that can ferment glycerol to value-added products, primarily 1,3-PDO or 2,3-BDO, along with other chemicals (Almeida et al., 2012). These organisms are considered the most important for the industrial production of value-added bioproducts because of their high yield and productivity (Huang et al., 1999).

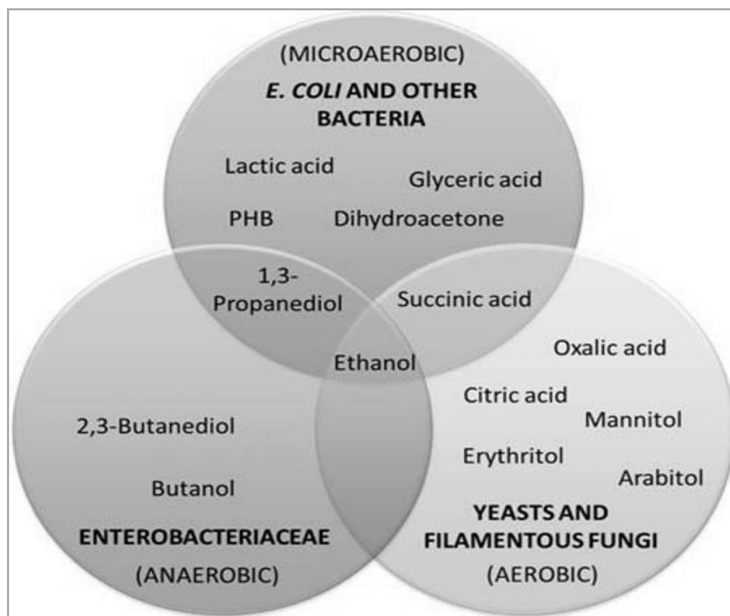

Figure 3. Examples of chemicals produced by microbial fermentation of crude glycerol (Almeida et al., 2012)

Many microorganisms can grow in a medium containing glycerol as the sole carbon and energy source under aerobic or anaerobic conditions. However, anaerobic conditions require costly equipment and supplies. In addition, there is no electronic acceptor during fermentation and bacterial growth is inhibited by low pH in the culture medium. Further, glycerol dehydrogenase (GDH), an important enzyme in oxidative glycerol metabolism, requires aerobic conditions (Viana et al., 2012). As well as aerobic conditions being required for the large-scale production of value-

added products, GDH activity can be enhanced by optimization of fermentation parameters such as nitrogen source, pH, and incubation temperature.

In this thesis, the focus is on the characterization of novel bacterial strains capable of aerobic bioconversion of crude glycerol. It identifies novel microbes capable of producing the enzyme in aerobic conditions, proposes strategies to use microbes to produce high-value chemicals (1,3-PDO, 2,3-BDO, ethanol, n-butanol, organic acids, polyols, and others), and quantifies the efficiency of bioconversion of crude glycerol to bioproducts by these isolates.

1.2. Biodiesel

Although there has been a significant development in the commercialization of biodiesel, several challenges remain to be effectively addressed (Converti et al., 2003; Nada et al., 2014). Surplus production of glycerol from the biodiesel industry from transesterification of vegetable oil and animal fats has led to a decline in the cost of crude glycerol and dumping of crude glycerol as a waste product (Gholami et al., 2014). Consequently, many studies have focused on aerobic and anaerobic conversion of crude glycerol into valuable products for industrial use (Wang et al., 2001).

Until the year 2000, biodiesel represented only 10% of the global biofuel production, with ethanol comprising the rest. Between 2000 and 2005, biodiesel production increased four folds, with the top producers (in descending order) being in France, Germany, USA, Italy, and Austria. In Canada, fuel supplies comprise 32% of coal, 45% of imported crude oil, 16% of natural gas, and 13% of domestic crude oil (Junginger, 2008). Canada is one of top ten carbon-emitting countries globally (Global Carbon Atlas, 2016).

1.3 Glycerol Purification, Breakdown, and Products

The two main impurities present in crude glycerol are methanol and free fatty acids (Ji et al., 2011). The presence of methanol is due to the fact that biodiesel producers use excess methanol to enhance chemical transesterification, and do not recover all of it. Crude glycerol also can contain magnesium, calcium, and sulfur (Choi et al., 2011).

Glycerol can be used for aqueous-phase reforming, chemical synthesis, and bio-conversion (Lin et al., 2011). Currently, it is expensive to chemically break down the triol into useful chemicals for example, via esterification of glycerol with alcohol to form branched oxygen-containing components for use as solvents or fuel (Ji et al, 2011). For example, Karinen and Krause (2005) esterified glycerol with isobutene in a liquid with an acidic ion exchange resin catalyst. The product was five esters and isobutene, which reacted to form other C8–C16 hydrocarbons. The ideal reaction conditions for selectivity towards esters were determined to be an isobutene/glycerol molar ratio of 3 at 80°C (Zhou et al., 2008).

GDH is an intracellular bacterial enzyme that can convert glycerol to dihydroxyacetone (DHA) and other products under aerobic conditions. The bacteria use glycerol aerobically through a complex oxidative pathway (Hao et al., 2008) consisting of dehydrogenating glycerol via NAD^+-dependent GDH to form DHA, phosphorylation of DHA (mediated by DHA kinase), and conversion to succinate, which is subsequently converted to propionate or pyruvate (Deutsch et al., 2007). Reactions that lead to the formation of compounds from pyruvate vary with environmental conditions and with the enzymes that mediate the reactions. They ultimately produce simple compounds such as 2,3-BDO, lactate, butyrate, ethanol, formate, acetate, hydrogen gas, and carbon dioxide (Raj et al., 2010; Liu et al., 2012).

1.3.1 1,3-Propanediol

The primary product obtained through bioconversion of glycerol is 1,3-PDO ($C_3H_8O_2$), which was first observed in 1881 after fermentation of glycerol (Celińska, 2010). In 1914, the production of 1,3-PDO was described. In 1928, the School of Microbiology at Delft University evaluated 1,3-PDO utilizing various Enterobacteriaceae (Vivijs et al., 2014). Since then, the conversion of glycerol to 1,3-PDO under aerobic and anaerobic conditions has been verified in a wide range of bacteria, such as *Citrobacter*, *Klebsiella*, *Lactobacillus*, *Pseudomonas*, and *Clostridium* (Biebl and Marten, 1995) through a two-step process: 1) glycerol is converted to 3-hydroxypropionaldehyde and water, and 2) 3-hydroxypropionaldehyde is reduced to 1,3-PDO through oxidoreductase (Yazdani and Gonzalez, 2007; Hao et al., 2008). Secondary products such as succinate, formate, and ethanol are produced in varying amounts. For example, the bacterium *C. pasteurianum* converts glycerol to butyric acid, n-butanol, and lactic acid. The assimilation of glycerol into these microorganisms are associated with their ability to synthesis 1,3-PDO (Ahrens et al., 1998; Alves et al., 2006).

1,3-PDO has been used in various industries to produce polyether, polyesters, and polyurethanes. It can be formulated into adhesives, moldings, solvents, co-polyesters, and many others products. A recent application 1,3-PDO is in the production of polytrimethylene terephthalate, a new form of polyester (Ringel et al., 2012).

1.3.2 2,3-Butanediol

2,3-BDO ($C_4H_{10}O_2$) can be employed for the chemical synthesis of plastics, anti-freeze solutions, and solvents (Jensen et al., 2012). Additionally, 2,3-BDO has been converted to methyl ethyl ketone and 1,3-butadiene. Traditionally, 2,3-BDO has been obtained from petroleum (Jeon et al., 2014); however, the development of a microbial production route based on renewable

feedstocks is of interest. In one previous studies, glycerol fermentation by several strains of *Klebsiella* sp. yielded 1,3-PDO as a major product and 2,3-BDO as a minor product (Agirre et al., 2011; da Silva et al., 2015). However, various bacterial genera have been identified and reported to metabolize crude glycerol into 2,3-BDO as a major product with very high yields (Xu et al., 2014). These comprise *Citrobacter*, *Klebsiella*, and *Clostridium*. The assimilation of glycerol into these microorganisms are associated with their ability to synthesis 2,3-BDO (Petrov et al., 2009; Ma et al., 2009; Cheng et al., 2010)

1.4 Implications for Research Objectives

Microorganisms have importance for large-scale fermentation of industrially important enzymes such as GDH, proteases, cellulases, and pectinases. To lower the high cost of production of these enzymes, several problems need to be addressed, such as slow growth rates, the length of induction period for enzyme expression, and the low specific activity of the enzymes produced by fungi that have been widely used in the industrial sector (Sun and Cheng, 2002; Balasubramanian et al., 2013). It is therefore important to screen novel enzyme-producing microorganisms, optimize enzyme production, and improve enzyme effectiveness.

Several microorganisms, including fungi and bacteria, produce enzymes to degrade cell wall polysaccharides. However, the selection of a particular strain for commercial enzyme production is tedious. The bacterial strain has received preference over the fungal strains because they are easier and faster to grow during fermentation. Also, the production to increase yields are quicker for bacteria because of their relatively short generation times. The isolation and characterization of bacterial strains are helpful to identify the isolates producing important enzymes such as GDH and cellulase, which might be potential candidates for many industrial

processes (Reczey et al., 1996). Enzyme characterization is important to develop methodologies to increase enzyme stability and help to understand their mechanisms of action.

The overall research objectives are to identify novel GDH-producing bacterial strains. Also, identify novel cellulase-producing bacterial strains and optimize enzyme production parameters during fermentation. More specifically, they are 1) isolate and characterize several novel bacterial strains producing cellulase or GDH 2) optimize fermentation parameters to maximize production of these enzymes and 3) determine the bio-product concentrations from optimized fermentations.

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CHAPTER II

Enzymatic Conversion of Glycerol to 2,3-Butanediol and Acetoin by *Serratia proteamaculans* SRWQ1

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Abstract

Biodiesel, a renewable and environment-friendly biofuel is produced by transesterification process using animal fats and vegetable oils. However, the flourishing of biodiesel industries has led to produce a huge amount (10% v/v) of crude glycerol as a core by-product, created an overflow problem. Therefore, biotransformation of glycerol into biofuel and other value-added products is one of the promising applications of glycerol due to its high availability at low cost. In this study, we report the capability of converting glycerol as a sole carbon source to 2,3-butanediol (2,3-BDO) and acetoin by using a newly isolated *Serratia proteamaculans* SRWQ1 strain in batch biotransformation process under aerobic condition. Strain SRWQ1 displayed a maximum up to 18.43 ± 1.55 g/L of 2,3-BDO, yielding 0.4 g/g using 49.0 g/L glycerol which was 98.0% of glycerol utilization. The strain SRWQ1 also successfully produced a significant amount of acetoin 8.38 ± 0.76 g/L with yields 0.06 g/g. Moreover, the maximum activity of glycerol dehydrogenase (GDH) which is a key enzyme of glycerol metabolisms was 408.69 ± 0.069 units/mg protein. The newly isolated strain *S. proteamaculans* SRWQ1 displayed the best ability to synthesize 2,3-BDO and

acetoin using glycerol as the sole substrate, and it is the first report on biotransformation of glycerol by *S. proteamaculans*. Therefore, this aerobic conversion of glycerol to value-added green products 2,3-BDO and acetoin with potential industrial applications would represent a noteworthy alternative to add value for biodiesel production helping biodiesel industries development.

Statement of Novelty

The worldwide booming of biodiesel industry has led to the generation of a large amount of crude glycerol (10% w/w), and created an overflow problem. Bioconversion of glycerol to value-added green products 2,3-butanediol (2,3-BDO) and acetoin, with potential industrial applications, represents a noteworthy alternative to add values to biodiesel production and promote the development of the biodiesel industry. Consequently, we report a newly isolated efficient glycerol dehydrogenase (GDH) producing bacterial strain *S. proteamaculans* SRWQ1 with high yields of 2,3-BDO and acetoin. In this report, the high GDH activity (408.69 units/mg protein) and 2,3-BDO yield (18.43 g/L) were attained using SRWQ1 strain which is the first time proved that *S. proteamaculans* can efficiently convert glycerol, and until today there is no report on bioconversion of glycerol by *S. proteamaculans*. Therefore, SRWQ1 strain has the potential for 2,3-BDO and acetoin productions from low-cost biomass glycerol in an industrial bioconversion process.

Keywords Glycerol dehydrogenase. Bioconversion. *Serratia proteamaculans.* 2,3-butanediol. Acetoin

2.1 Introduction

Glycerol, a simple polyol, is a major by-product of biodiesel industries [1]. Typically, around 10 % (w/w) of crude glycerol is produced from biodiesel synthesis process [2]. Thus, every hundred pounds of biodiesel produced ten pounds of crude glycerol as a core by-product [3]. Globally, the major producers of glycerol are USA and India, which produced 1.3 and 0.8 billion gallons respectively in 2013 [4]. Pure glycerol is used in various manufacturing industries such as pharmaceuticals, soaps, paint, toothpaste, and cosmetics [5]. However, biodiesel is an eco-friendly source of energy that recently has begun to replace the use of older sources of energy such as diesel, petrol, gasoline, and others [6]. The main reason why biodiesel is being adopted to replace these petroleum products is to promote an eco-friendly way of harnessing energy. The use of petroleum products has had a few drawbacks such as having adverse effects on the environment [7], and have created global ecological disturbances due to harmful effects on the environment in the long run.

In addition, biodiesel is a renewable fuel that can be produced from animal fats, vegetable oil, tallow and waste cooking oil [8]. The transesterification, a biodiesel production process performs a set of processes leading to the production of biodiesel and furthermore, releasing crude glycerol a core by-product [9]. This process involves a string of reactions that are catalyzed by the addition of an acid or base catalyst [10,11]. The transesterification is often performed on large manufacturing fronts for the purpose of mass production [12]. Despite that, the transesterification ends up producing an eco-friendly fuel source called biodiesel, however; this core by-product raw glycerol produced from biodiesel synthesis is not quite the environment-friendly, because of its impurities like methanol, soap, catalysts and matter organic non-glycerol (MONG) which are negatively influence on biodegradation process, creating a substantial environmental concern.

Consequently, crude glycerol in large amounts which poses a threat to the environment and needs high cost for purification. Therefore, there is a need for the conversion of this crude glycerol to value-added products that are not harmful to the environment [13]. Nevertheless, the process of glycerol bioconversion involves biotechnical processes which produce other products that have proven valuable to the industry [14]. Bioconversion of glycerol to producing value-added green products is an economic advantage for adding value to the fuel industry. In fact, the amount of crude glycerol production as a by-product of biodiesel industry is significantly increased by increasing of biodiesel industries; therefore, it has made the purified glycerol as a cheaper product. Moreover, glycerol is regarded as an efficient and low-cost feedstock for industrial fermentation [15]. Ideally, glycerol can be converted by few microorganisms which are able to utilize glycerol as a source of carbon to produce commercially valuable chemicals, and *Serratia proteamaculans* SRWQ1 could be one of the most promising candidates for industrial bioconversion process.

S. proteamaculans, a facultative anaerobic bacterium that can be isolated from the soil. There is only a small fraction of the *Serratia* that has been discovered in the field of medical research [16]. *Serratia* sp from unexplored habitats have gained considerable attention in recent years to characterize various organic acids such as malic acid, lactic acid, and acetic acid through phosphate solubilization process [17]. In this research, a novel bacterial strain *S. proteamaculans* SRWQ1 is attained through the investigation for the production of glycerol dehydrogenase (GDH) enzyme. Additionally, in this research, the bioconversion of crude glycerol to other high-value chemicals by *Serratia proteamaculans* SRWQ1 is discussed in this research article. The focus was given on isolation and characterization of glycerol utilizing microbes, as well as the aerobic conversion of glycerol by a novel strain *Serratia proteamaculans* SRWQ1. Moreover, an aerobic

condition was used for the large-scale production of value-added bioproducts 2,3-BDO and acetoin.

However, two significant platform chemicals acetoin ($C_4H_8O_2$) and 2,3-BDO ($C_4H_{10}O_2$) can be obtained from aerobic pathways of glycerol metabolisms of many bacteria [18],[19]. Furthermore, 2,3-BDO is extensively used as a reagent, liquid fuel or fuel additive, lubricant, antifreeze agent, and in the preparation of polymers, a pharmaceutical carrier, printing ink, fumigants, perfumes, softening and moistening agents [20][21]. Nevertheless, acetoin is a product of fermentation. It is a component of the butanediol cycle in microorganisms and commonly used in food, flavor, cosmetics, and a fragrance [22]. In the recent years, several strains of the species of *Citrobacter, Klebsiella, Clostridium,* and *Enterobacter* are able to ferment glycerol, biodiesel derived raw glycerol and sugars, and the main product was 1,3-PDO, while 2,3-BDO was not reported along with other products [23],[24]. In the oxidative pathway of glycerol metabolisms, glycerol dehydrogenase (GDH) is an intracellular NAD^+-dependent key enzyme, can convert glycerol to dihydroxyacetone (DHA) and other metabolic products (Fig. 1). Therefore, the objective of this present work is to isolate a novel bacterial candidate from environmental samples to efficiently convert glycerol to a green product 2,3-BDO. The biotransformation kinetics of batch culture processes was studied in detail, and the novel bacterial strain providing the gain of increased 2,3-BDO product yield were evaluated.

There were several reasons of this research, namely bacteria can be easily cultured, can be found inhabiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the potential exploitation of hardier bacterial strain for the industrial biotransformation of a large volume of glycerol or crude glycerol to biofuel and other high-value products. This research work led to contribute to the development of biorefineries and reduce

industrial waste disposal. Also, this research work helps in summarizes different strategies employed to produce valuable chemicals (1,3-PDO, 2,3-butanediol, ethanol, n-butanol, organic acids, polyols and others). Moreover, this work can evaluate the efficiency of bioconversion of crude glycerol to bioproducts by novel bacterial strains under the aerobic conditions with potential in future studies for advancement in bioconversion processes. A very little work has been done on the aerobic process of glycerol metabolisms, and it is our first report for biotransformation of glycerol to 2,3-BDO using our newly isolated *S. proteamaculans* SRWQ1. Bioconversion of glycerol to value-added green products 2,3-butanediol (2,3-BDO) and acetoin, with potential industrial applications, represents a noteworthy alternative to add values to biodiesel production and promote the development of the biodiesel industry. Consequently, we report a newly isolated efficient glycerol dehydrogenase (GDH) producing bacterial strain *S. proteamaculans* SRWQ1 with high yields of 2,3-BDO and acetoin. This is the first study that showed 2,3-butanediol and acetoin productions by *S. proteamaculans* until now.

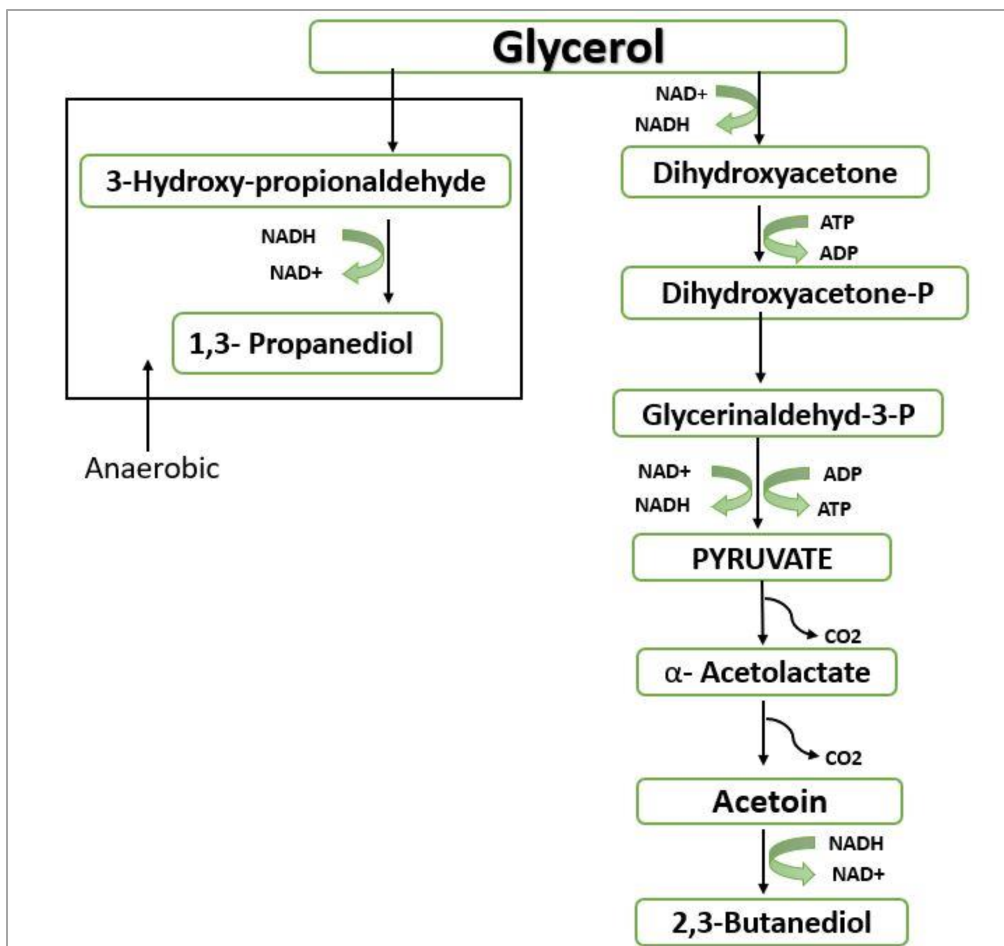

Fig. 1 Glycerol metabolic pathways to produce 1,3-propanediol, acetoin, and 2,3-butanediol

2.2 Material and Methods

2.2.1 Growth media

For isolation of bacterial strain, the minimal salt (MS) medium per liter composed of 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl and 0.05 g $MgSO_4 .7H_2O$ supplemented with glycerol was used. However, for GDH enzyme activity assay and optimization of bioconversion process, MS medium supplemented with glycerol, yeast extract and/ peptone was used. Luria-Bertani (LB) broth medium was used for seed cultures of bacterial strains, and composed per liter: 10.0 g peptone, 5.0 g yeast extract, 5.0 g NaCl [25]. The batch cultivation medium MS-2 used for bioconversion of

glycerol to value-added bioproducts, and composed (per L) of 50 g glycerol as a sole carbon source, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, and 0.05 g $MgSO_4.7H_2O$, 5.0 g yeast extract and 5.0 g peptone.

2.2.2 Isolation of bacterial strains

Organic materials including paper mill waste, soil and rotting wood were collected from the forest in Thunder Bay, Ontario, Canada. Five grams of each sample was added to the flask containing 100 ml of MS medium supplemented with 100 g/L glycerol, homogenized through vortex using a magnetic stirrer, and then incubated at 30°C in a shake flask incubator (200 rpm) for 72h. After incubation, a 100 µl of broth culture was spread on MS agar plates supplemented with 100 g/L glycerol, incubated at 30°C. From the 24h culture plates, different colonies of bacteria were selected based on their morphological features like size, color, and colony morphology. All the isolated bacterial strains were identified and screened for their capability of utilizing glycerol under aerobic condition.

2.2.3 Identification of bacterial strains

The bacterial isolates were identified using 16S rRNA gene sequencing. However, for identification, the isolated bacterial strains were inoculated in LB broth medium, and incubated at 30°C in a shaker incubator at 200 rpm for 24 h. The genomic DNA of the strains were isolated using bacterial DNA extraction kit (Presto™ mini genomic DNA Bacteria kit, Geneaid) following the manufacturer's protocol. To amplify the genes from bacterial samples, the PCR products for 16s rDNA sequences were prepared using HDA-1: 5´-GAC TCC TAC GGG AGG CAG CAG T-3´ (forward) and E1115R: 5´-AGG GTT GCG CTC GTT GCG GG-3´ (reverse) primers (Eurofins Scientific, Toronto, Canada). The mixture of PCR reaction composed of 25 µl Taq mix (2x) (FroggaBio, Toronto, Canada), 1 µL HDA-1 primer, 1 µL E1115R primer, 1 µL genomic DNA

template, and 22 μL nuclease-free water. The PCR thermal cycling conditions was followed: primary denaturation for 3 min at 94°C followed by 35 amplification cycles consisting of denaturation at 94°C for 30 seconds, annealing for 30 seconds at 65°C, and extension at 72°C for 1 min, and then concluding with a final extension step at 72°C for 10 min. The PCR products were visualized using a 1% agarose gel electrophoresis to confirm the quantity and purity of size. The DNA bands from the gel were cut, purified using Geneaid PCR/Gel Purification Kit following the manufacturer's protocol [26]. The nucleic acid DNA concentration was measured using Nanodrop spectrophotometer system. Finally, the purified bacterial DNA samples were sent to the Centre for Applied Genomics (Eurofins Genomics, Toronto, Canada) for sequencing. The sequencing results were input into the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov) for the possible identification of the strains. The phylogenetic relationship of isolates was constructed using the ClustalX Omega software (Fig. 2).

2.2.4 Preparation of seed culture

One set of bacterial strains were stored at -80°C with 20% (w/v) glycerol added as a frozen stock. The other set was working culture which was maintained at 4°C by sub-culturing after every 25-30 days [27]. All experiments including GDH and biotransformation assays were performed using seed culture. Briefly, a loopful of 24h bacterial culture from LB agar plate was transferred to a fresh 5 ml of LB broth medium, incubated at 30ºC for 24h. Then 1 ml of the 24h seed culture from LB broth was inoculated into 200 ml Erlenmeyer flask containing 50 ml of culture medium used for enzyme assay or biotransformation process.

2.2.5 Cell extraction and enzyme activity assay

For GDH enzyme activity, cells from 1.5 ml aerobic broth culture were harvested by centrifugation at 12,000xg for 3 minutes, and washed twice with 100 mM potassium phosphate

buffer (pH 8.0). Cells were resuspended in the same buffer containing 50 mM potassium chloride (KCl) at 4ºC, disrupted using sonicator. Briefly, the mixture was vortexed for 30-60 sec, and then sonicated at 4ºC for 2-3 minutes (10 sec at a time until 2-3 min). The lysate was discarded after centrifuged (2-3 min, 15,000xg). The supernatant was kept at low temperature using ice, and 50 µl were taken for GDH enzyme assay [28].

However, to determine the GDH enzyme activity, a 300 µl of the reaction mixture was contained 30 mM ammonium sulfate, 0.2 M glycerol, 1.2 mM NAD^+ and 50 µl of cells supernatant [29]. By following the method described by Tian et al, (2007)[30] and Raj et al, (2008) [31], the GDH activity was determined using reduction of NAD^+ to the substrate-dependent absorbance change of NAD(H) at 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$). In the GDH activity assay, the initial reduction rate of NADH was measured using spectrophotometer (Epoch BioTeck, Gen 5 software) by increasing of absorbance per minute of time. The GDH activity is expressed as µmoles of substrate reduction per minute per milligram of cell protein. As such, one unit of activity is the amount of enzyme required to reduce 1 mmole of substrate per minute under the condition specified [32].

The concentration of the protein (mg/ml) in crude extract was determined by using the standard curve of BSA protein (Fig. S1). Bradford Protein Assay kit purchased from Bio Basic Inc., Canada was used to determine protein concentration following the manufacturer's protocol. The activity of the GDH enzyme was measured as units of enzyme per milligram of protein (U/mg protein) [33].

2.2.6 Effect of different concentrations of glycerol and yeast extract

For maximum enzyme activity of highly efficient bacterial strains, the concentrations of sole substrate glycerol and a nitrogen source yeast extract in MS medium were optimized in a

batch culture at different incubation (24, 48 and 72h) times. The experiment was performed in a shake flask culture was contained 50 ml MS medium supplemented with different concentrations (2%, 5% and 7%) of glycerol as a sole carbon source and various concentrations (0.1%, 0.25% and 0.5%) of yeast extract. The initial pH of the medium was 7.0. In our study, glycerol was only the substrate. Yeast extract was only used as an effective organic nitrogen source stimulating cell growth and enhancing GDH enzyme production by SRWQ1 strain. All experiments were repeated at least three times.

2.2.7 Effect of different temperature and pH

This study was conducted with the consideration of the effects of temperature on the enzyme production process. The culture medium used for optimization of incubation temperature and medium initial pH was MS medium supplemented with 5% glycerol as a sole carbon source and 0.5% yeast extract. Flasks were incubated at different temperatures (20, 25, 30 and 37°C). However, for optimization of pH, culture medium of different initial pH values (4, 5, 6, 7, 8 and 9) were used. The pH for the specific enzyme activity was evaluated using an AB15 pH meter (Fisher Scientific, Ottawa, Canada). Initial pH levels were adjusted from pH 4 to 9 to study the impact of pH on the cell growth and enzyme production. The pH of the culture medium was adjusted by adding 1.0 M KOH solution or 0.2 M HCl solution before autoclaving.

2.2.8 Effect of different nitrogen resources

The study was conducted for inducing the GDH activity as well as bacterial growth using 5.0 g/L of different nitrogen sources including peptone, malt extract, ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4)_2SO_4$), or ammonium nitrate (NH_4NO_3). Nitrogen sources were used in MS medium with 5% glycerol as the supplementary carbon source. The control culture for the experiment was contained 0.5% yeast extract as the nitrogen source. One mL (2% v/v) of overnight

grown bacterial culture was inoculated in each 200 mL Erlenmeyer flask containing 50 mL MS medium.

2.2.9 Fermentation process and sample preparation for bioproduct quantification

The batch fermentation experiment was performed using optimized culture conditions for production of value-added products from glycerol biotransformation process. MS-2 medium was used for biotransformation of glycerol to 2,3-BDO and acetoin in aerobic process. After sterilization of the growth medium at pH 6.0, 1 mL of seed culture was transferred to the medium and incubated at 25°C for 24 h under the aerobic condition. The bacterial culture was shaken at 200 rpm using a rotary shaker. Subsequent incubation, 1.0 mL of the broth sample containing bacterial cell was collected in 1.5 mL centrifuge tube and the tube was centrifuged at 12,000×g for 5 min. After 24, 48, 72, 96, 120, and 144 h incubation, approximately 1.5 mL of culture was collected. The supernatant was filtered through a membrane filter (pore diameter 0.45 µm) and frozen for subsequent GC-FID analysis [34].

2.2.10 Analytical

The cell density was determined by measuring the optical density at 600 nm using an EpochTM microplate spectrophotometer (BioTek Instruments, USA). The pH values of culture broth medium were determined using AB15 pH meter (Fisher Scientific, Canada). The products from fermented broth were identified by GC-MS (Varian 1200 Quadrupole GC/MS) using helium as the carrier gas. Acetoin and 2,3-BDO concentrations in the fermentation broth were quantified periodically using gas chromatograph equipped with a flame ionization detector (GC-FID, Shimadzu/GC-14A) where nitrogen gas was a carrier (Fig. S2). The concentration of glycerol in terms of g/L was also determined by GC-FID equipped with a 30 m length column (DB-WAXETR) (Fig. S2). The column temperature was set to the range from 45 for 2 min to 240°C

for 1 min at the rate of 10°C per minute. The injector and detector temperatures were set to 250°C. Carrier gas was nitrogen, and the sample volume of 1.0 µL cell-free supernatant was used.

2.3 Results

2.3.1 Isolation, identification and phylogenetic relationship of bacterial strains

Several bacterial strains were isolated from paper mill waste, forest soil and rotting wood samples for screening their glycerol utilizing activity under aerobic condition. Under the experimental conditions described in this research article, the strains SRWQ1, SRWQ2 and SRWQ3 isolated from paper mill waste exhibited significant GDH activities, and were identified using 16S rRNA gene sequencing. Genomic DNA were successfully isolated from all three GDH producing isolates using DNA isolation kit, and amplified 16S rRNA genes using PCR (Polymerase chain reaction). Sequencing and sequence analysis results of all 16S rRNA genes from three strains were successfully obtained. The sequences of 16S rRNA of the three strains SRWQ1, SRWQ2 and SRWQ3 were submitted to the GenBank for their accession numbers. Nevertheless, the sequence of the isolated strain SRWQ3 reported in this paper was found 100% similarity to the strain *Serratia* sp. 243 (accession No. KT461863). Moreover, sequence alignments of other two strains SRWQ1 and SRWQ2 in NCBI revealed 99% similarity to the sequence of *Serratia proteamaculans* and *Serratia liquefaciens* respectively. Therefore, the potential two strains SRWQ1 and SRWQ2 have been nominated as the new strains *Serratia proteamaculans* SRWQ1 and *Serratia liquefaciens* SRWQ2 respectively. The gen bank accession numbers of two newly isolated novel bacterial strains *S. proteamaculans* SRWQ1 and *S. liquefaciens* SRWQ2 released by NCBI are KX602658 and KX602659 respectively (Fig. 2).

Nevertheless, a phylogenetic tree was constructed to analyze the evolutionary relationship among the newly isolated strains as well as other *Serratia* strains retrieved from gene bank using

their 16S rRNA sequences. As shown in Figure 2, the phylogenetic tree was constructed using ClustalX Omega software. Thus, the identities of our new isolated strains were confirmed by the result of a phylogenetic relationship through the distance between all the newly isolated and other strains (Fig. 2).

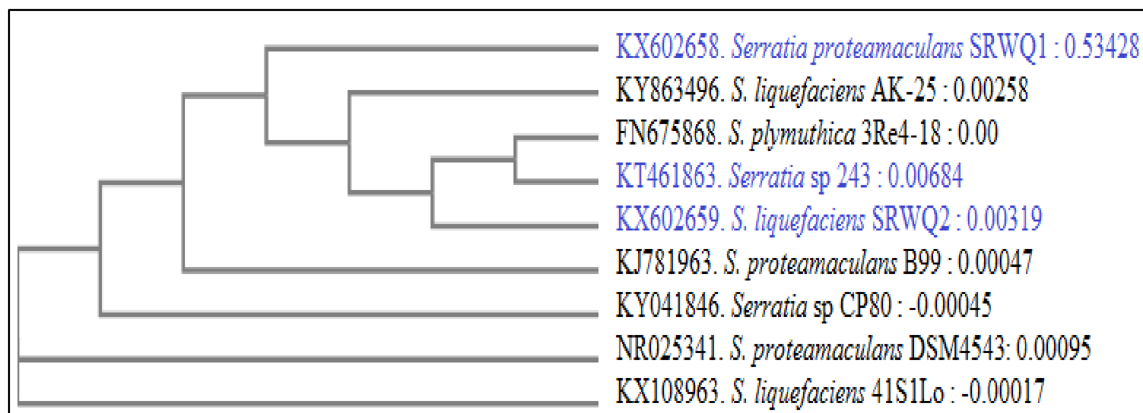

Fig. 2 Evolutionary relationships of the strains: Phylogenetic tree drawn from sequence alignment program using ClustalX Omega software. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are accession numbers and bootstraps of published sequences

2.3.2 Primary screening for GDH activity

Three bacterial strains *Serratia* sp 243, SRWQ1 and SRWQ2 were screened for their GDH enzyme activity and biomass production using MS medium supplemented with 20 g/L (2%) glycerol as the sole carbon source (Fig. 3). All three isolates (SRWQ1, SRWQ2, and *Serratia* sp. 243) were able to utilize glycerol as a sole substrate to produce significant amount (78.31 – 108.72 units/mg protein) of GDH enzyme in 24h incubation at 30°C (Fig 3a). The use of these bacterial strains can increase attention for the bioconversion of glycerol to produce value-added products. The maximum enzyme activities of all the strains were attained after 24 hours of incubation; however, strain SRWQ1 exhibited the greatest enzyme activity (108.72 ± 0.773 U/ mg protein)

after 24h incubation compared with the rest of strains (Fig. 3a). However, as shown in Figure 3b, the biomass productions of all three strains were increased dramatically until 24h of incubation, and then increased slowly up to 96h. The maximum biomass productions obtained after 96h of incubation were almost same (OD_{600} 2.03 – 2.07) with all the three bacterial strains (Fig. 3b). Overall strain SRWQ1 was found as an efficient strain for capable of utilizing glycerol to produce GDH enzyme. Therefore, the strain SRWQ1 was selected as the best candidate for the further study of glycerol biotransformation process. Also, the culture conditions of SRWQ1 strain were optimized for maximum yields of bioproducts from glycerol bioconversion process.

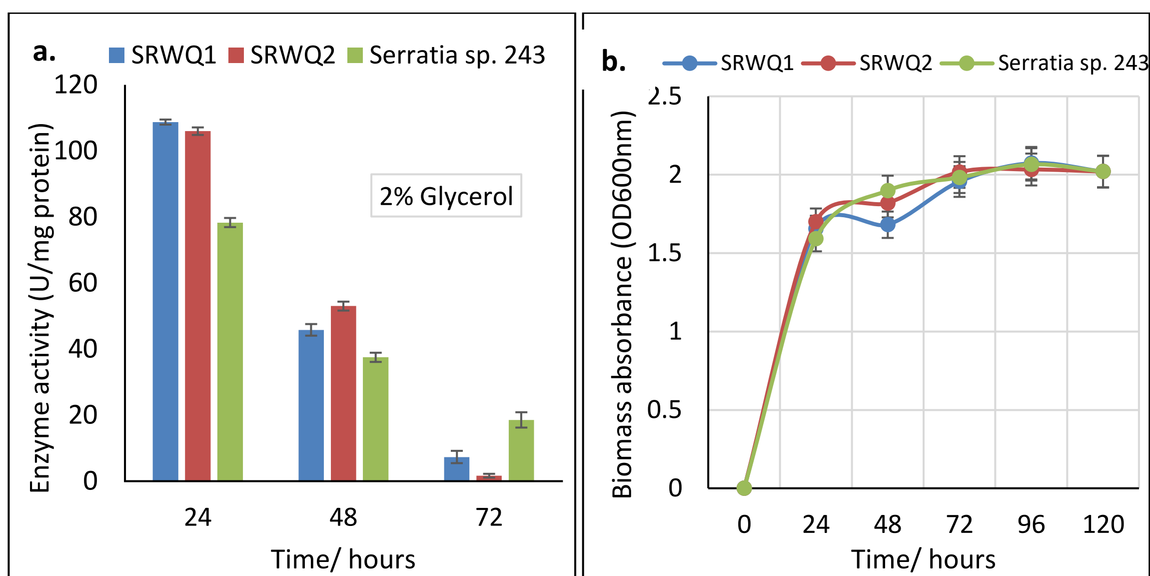

Fig. 3 a) Specific activity of GDH and b) bacterial growth curve by SRWQ1, SRWQ2, and *Serratia* sp. 243

2.3.3 Effect of glycerol and yeast extract concentrations on GDH activity

In this study, glycerol was only the substrate, and the yeast extract was the nitrogen source for GDH enzyme production. Yeast extract is a water-soluble extract of selected autolyzed yeast cells. It is rich in amino acids, growth factors, and vitamins, particularly B-complexes. It is widely

used as a growth source in many culture media formulations [35]. However, for optimization of biotransformation process, MS medium supplemented with three different concentrations of both glycerol (20, 50 and 75 g/L) and yeast extract (1.0, 2.5 and 5.0 g/L) were used. The experiment results showed that 50 g/L of glycerol and 5 g/L of yeast extract were the favorable carbon and nitrogen sources respectively for maximum enzyme activity at 30°C after 24, 48 and 72h of incubations (Fig. 4). The maximum enzyme activity 242.24 ± 2.6 U/mg protein was attained in 24h of incubation with 5.0% (50 g/L) of glycerol and 0.5% (5 g/L) of yeast extract respectively.

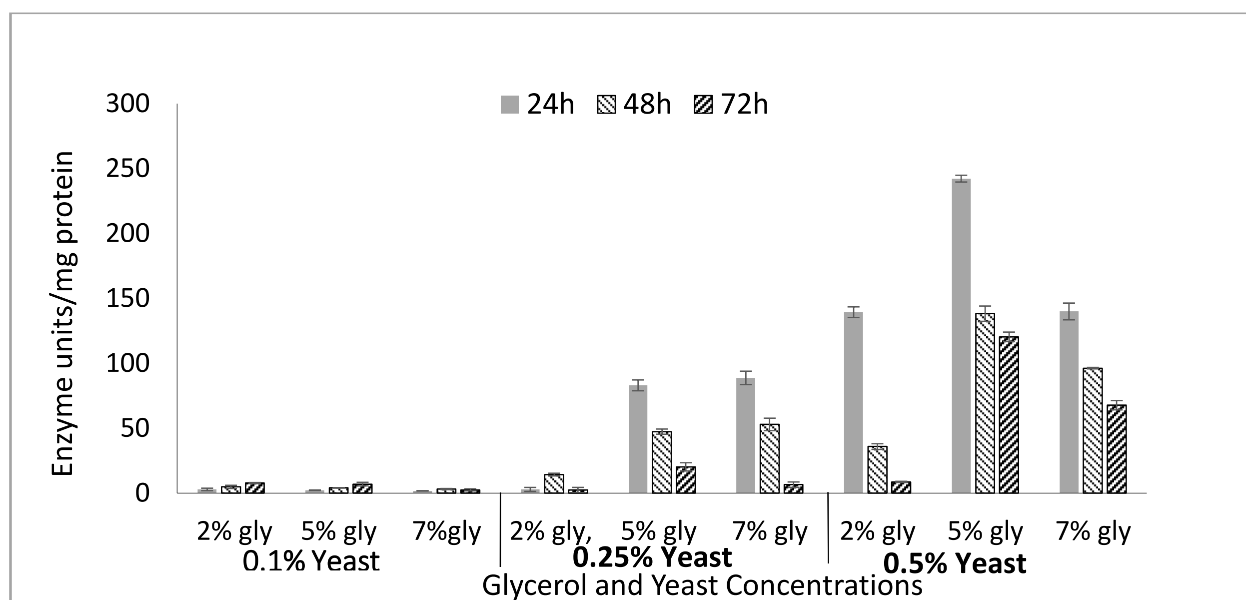

Fig. 4 Effect of different glycerol and yeast extract concentrations on enzyme activity of SRWQ1 strain

2.3.4 Effect of initial pH and incubation temperature on enzyme activity and biomass production

To determine the effects of different levels of incubation temperature and time for maximum enzyme activity, the experiments were performed in batch fermentation processes under the incubator temperatures 20, 25, 30, and 37°C respectively. Results of the effect of incubation

temperature and initial pH of culture on GDH activity using SRWQ1 strain are presented in Figure 5. The MS medium containing glycerol (50.0 g/L) and yeast extract (5.0 g/L) was used for growth of the bacterium. The strain SRWQ1 displayed the maximum enzyme activity 343.12 ± 0.52 U/mg protein at 25°C and pH 7.0 in 24h of incubation (Fig. 5a). The influence of pH on GDH enzyme and cell growth in the batch bioprocess without pH control was significant. However, the results of enzyme activity in shake-flask fermentation with various pH ranging from 4.0 to 9.0 are presented in Figure 5b. As shown in Figure 5b, the maximum enzyme activity 370.94 ± 0.88 U/mg protein was obtained at pH 6.0.

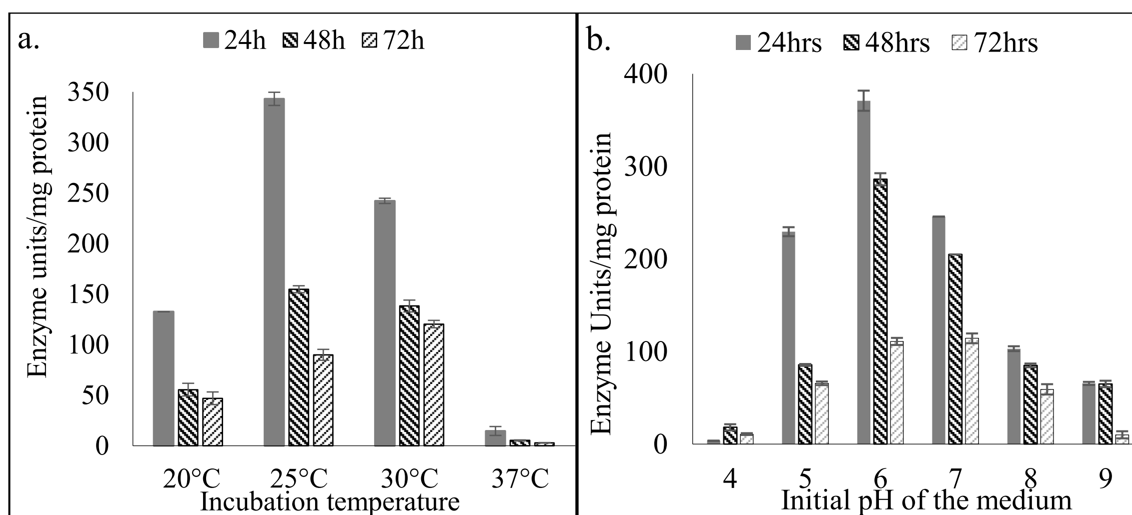

Fig. 5 a) Effect of different temperatures and incubation times on enzyme activity by SRWQ1strain. b) Effect of different pH levels on enzyme activity by SRWQ1

Moreover, the time profiles of pH changes and the production of cell biomass with different starting pH are shown in Table 1. Low and higher pH inhibited cell growth and GDH enzyme production (Table 1 and Fig. 5b). However, a pH higher than 6.0, a similar phenomenon was observed. As shown in Table 1, the maximum cell growths were obtained at initial pH 6.0 and 7.0. Interestingly, the pH of the medium sharply dropped from the initial pH after 24 h of incubation

for pH 6–9, whereas it increased slightly for cultures with an initial pH of 4 and 5 (Table 1). The first 24 h of the incubation period is the exponential growth phase of the batch culture and the pH changes can be attributed to the production of metabolites in the culture medium. After 24 h, the initial pH 6–9 cultures generally continued to show lower pH values until they plateaued at approximately pH 5.0.

Table 1 Effect of different pH levels on cell growth after 24, 48, 72 and 96 hours of incubation at 25°C of the bacterial strain SRWQ1

Initial pH	*Biomass (OD600nm) of the culture broth				*Final pH of the fermented broth			
	24h	48h	72h	96h	24h	48h	72h	96h
4.00	0.3423	0.6953	0.6656	0.649	4.52	4.73	4.85	4.92
5.00	1.2903	1.3736	1.43233	1.48633	5.16	5.39	5.84	5.77
6.00	1.3653	1.42466	1.524	1.61766	5.17	5.12	4.89	4.91
7.00	1.404	1.454	1.4906	1.59733	5.15	5.13	5.06	5.03
8.00	1.392	1.445	1.551	1.59733	5.21	5.16	5.06	5.08
9.00	1.35	1.382	1.46533	1.58366	5.34	5.31	5.11	5.09

*Mean values are presented of three replications

2.3.5 Effect of different nitrogen resources

The effects of five nitrogen sources other than yeast extract viz., peptone, malt extract, $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 on GDH enzyme activity were also investigated. MS medium supplemented with 50 g/L glycerol, 5.0 g/L yeast extract and 5.0 g/L of different nitrogen sources were used throughout the experiments. As shown in Figure 6, peptone showed the highest enzyme activity compared to that of other nitrogen sources. In the case of peptone, the maximum enzyme activity was obtained 408.69 ± 0.069 U/mg protein at a concentration of 5 g/L peptone after 24h

(Fig. 6). Peptones are the most widely used organic source of nitrogen made from animal products like milk or meat with trypsin, pepsin or other proteolytic enzymes to digest the protein to a mixture of amino acids, peptides, and polypeptides [36]. A similar result was also reported by *Klebsiella variicola* SRP3 [37].

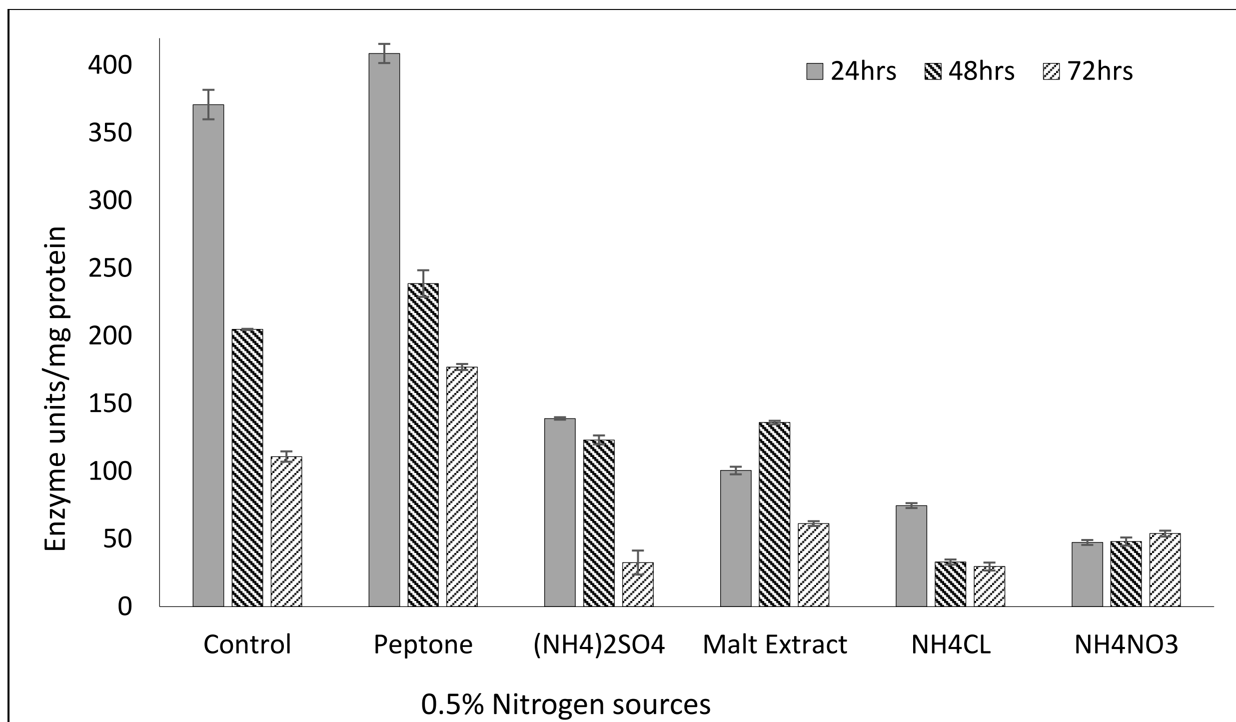

Fig. 6 Effect of different nitrogen sources on enzyme activity of the strain SRWQ1 after 24h incubation. Minimal salt (MS) medium supplemented with 5.0% of glycerol and 0.5% yeast extract was used. Initial (stating) pH and incubation temperatures were 6.0 and 25°C respectively

2.3.6 Production kinetics of major products of the strain SRWQ1

Combining the results from above optimization experiments, an optimum system for maximum enzyme activity was developed using glycerol as the sole substrate. However, to evaluate glycerol utilization, biomass and value-added bioproduct productions using the efficient strain *Serratia proteamaculans* SRWQ1, experiment was carried out under aerobic biotransformation process with glycerol as the sole substrate. Biotransformation of glycerol to

bacterial metabolic products was conducted at 25°C in 200 ml flasks containing 50 ml MS-2 medium at initial pH 6.0. Shake flask batch culture was carried out under optimized conditions, and two major bio-products 2,3-BDO and acetoin were revealed. The concentrations of the substrate (glycerol), as well as metabolic products (2,3-BDO and acetoin) as a function of incubation time in a batch culture process of pure glycerol biotransformation by the SRWQ1, are presented in the Fig. 7a. There is no 1,3-PDO as we expected to get it from the culture broth based on the literature review [37]. Moreover, cell biomass production and GDH activity were also determined, and results are presented in Fig. 7b. The GDH enzyme activity was gradually decreased after 24h incubation by increasing incubation time. After 144h of the incubation, glycerol was totally consumed by SRWQ1 strain with yielding 7.01 g/L of 2,3-BDO and 8.11 g/L of acetoin (Fig. 7a). The maximum product yields of 2,3-BDO obtained from the broth culture in 96h and 120h incubations were 13.913 ± 1.24 g/L and 18.43 ± 1.55 g/L respectively. Acetoin is another major metabolic product that was detected from the fermentation medium. However, the maximum concentration of acetoin obtained after 144h of the incubation was 8.38 ± 0.76 g/L using SRQW1 strain.

In addition to our novel strain SRWQ1, the other two strains *Serratia liquefaciens* SRWQ2 and *Serratia* sp 243 were also screened for their glycerol assimilation and metabolic products accumulation. These strains SRWQ2 and *Serratia* sp 243 were grown under the same optimized condition of strain SRWQ1. Comparison the final values of products, GDH and biomass obtained from metabolized of glycerol by three strains is presented in Table 2. In case of strain SRWQ2, the maximum product concentrations of 2,3-BDO after 96h and 120h incubations were 15.4 ± 0.6 g/L and 12.3 ± 0.83 g/L respectively (Table 2, Fig. S3). However, for strain *Serratia* sp 243, the

maximum yields of 2,3-BDO obtained from the culture medium after 96h and 120h incubation were 13.8 ± 0.22 g/L and 11.41 ± 0.5 g/L respectively (Table 2, Fig. S4).

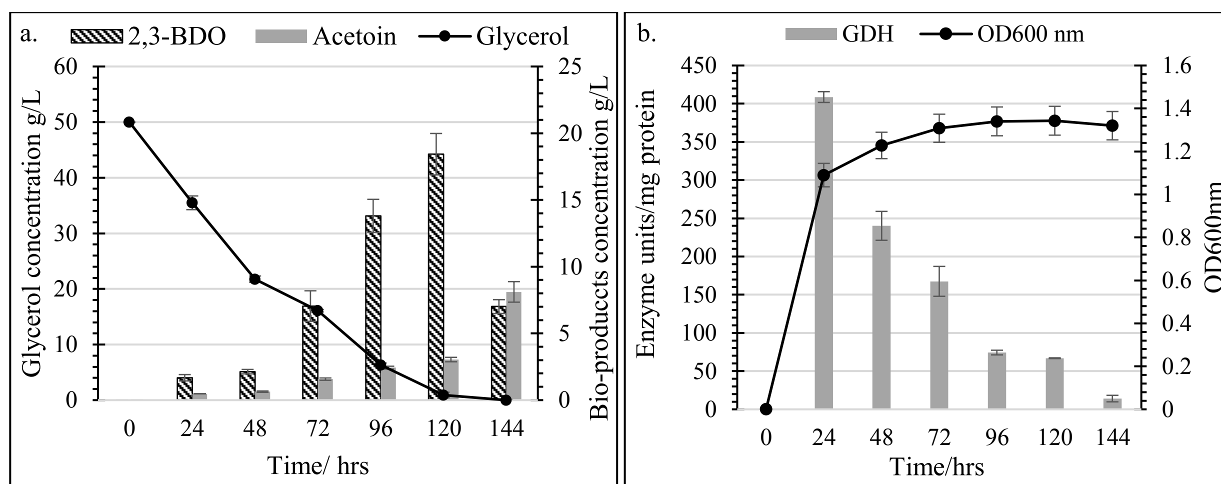

Fig. 7 a) Different concentration of bioproducts produced by *Serratia proteamaculans* SRWQ1. b) biomass production and GDH enzyme activity of SRWQ1. The MS-2 medium was used for growth of bacterium, and culture pH and incubation temperature were maintained at 6.0 and 25°C respectively.

Table 2 Comparison the final values of metabolic products, GDH and biomass obtained from metabolized glycerol by three strains at 25°C incubation temperature

Strain	Glycerol Utilized (%)		Biomass (OD600)		GDH (U/mg protein)		2,3-BDO (g/L)		Acetoin (g/L)	
	96h	**120h**	**96h**	**120h**	**96h**	**120h**	**96h**	**120h**	**96h**	**120h**
S. *proteamaculans* SRWQ1	87.4	98.14	1.34	1.4	74.3	66.89	13.91	18.43	2.4	3.04
S. liquefaciens SRWQ2	82.32	100.0	1.7	1.8	196.4	140.5	15.4	12.3	3.1	9.0
Serratia sp 243	74	100.0	1.6	1.8	106.0	88.8	13.8	11.4	2.9	6.6

2.4 Discussion

Biodiesel is being adopted to replace petroleum products in order to promote an eco-friendly way of harnessing energy [38]. The use of fossil fuel has had a few drawbacks such as having adverse effects on the environment, created a global ecological disturbance and harmful effect on the environment in the long run [39]. Nonetheless, biodiesel is an environmentally friendly renewable source of energy. With the flourishing of the biodiesel industry, a global surplus of glycerol is increasing [37]. Consequently, biotransformation of glycerol to value-added products including platform chemicals and liquid fuel or fuel additive from different perspectives through cost-effective processes with the main focus on novel bacterial strain would lead to both environmental and economic dividends of biodiesel plant [40]. Microbial conversion of glycerol to valuable chemicals is a subject of interest in the last few years [37][40]. There were several reasons of this research, namely bacteria can be easily cultured, can be found inhabiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the potential exploitation of hardier bacterial strain for the industrial biotransformation of a large volume of glycerol to biofuel and other high-value products.

First of all, our focus was given on isolation and characterization of novel glycerol utilizing microbes, as well as the aerobic conversion of glycerol (a core by-product of biodiesel production). In this research work, our three newly isolated strains SRWQ1, SRWQ2, and *Serratia* sp. 243 isolated from paper mill waste were found to have the greatest glycerol dehydrogenase (GDH) activity. However, until now, in a biotechnological application like the production of high-value products from glycerol looking for efficient GDH enzymes producing bacteria, traditional microbiological isolation techniques are still important [40],[41]. The conversion of glycerol to 2,3-BDO is an oxidation process reported in many previous studies [42].

2,3-BDO is a valuable building blocks chemical, and it can be used for the synthesis of 2-3-butanone [43]. The aerobic condition is often employed for 2,3-BDO production in which oxygen can be used as an electron acceptor. In anaerobic condition, the lake of external electron acceptor causes the hampering in microbial growth [39],[40]. Therefore, very little works have been done on the aerobic process [37],[40]. In our study under aerobic condition, both of the high enzyme activity and low pH of the growth culture boosted 2,3-BDO product yield, while low enzyme reaction and higher pH can enhance the accumulate of 1,3-propanediol (1,3-PDO) [44]. In our study, strain *S. proteamaculans* SRWQ1 with optimal pH 6.0 of the growth culture was enhanced GDH activity up to 408.7 ± 0.06 U/mg protein, in which the SRWQ1 strain blocked the synthesis of 1,3-PDO and produced a high concentration of 2,3-BDO using glycerol as a sole carbon source under the optimized fermentation condition. The maximum 2,3-BDO product yields produced by SRWQ1 were 18.43 ± 1.05 g/L and 0.4 g/g respectively, where 98.2% (49.1 g/L) of glycerol was utilized. This is the first study that showed 2,3-butanediol and acetoin productions by *S. proteamaculans* SRWQ1 and until today there is no any report on bioconversion of glycerol by *S. proteamaculans*.

Glycerol has been converted to value-added products by several microorganisms only in cultures controlled at pH 7.0 [45]. Similarly, glycerol bioconversion has been reported by *Serratia* sp only in a culture controlled by pH 8.00 and incubation temperature 40°C [46]; however, our new isolate *S. proteamaculans* SRWQ1 able to convert glycerol and produce 2,3-butanediol and acetoin at the optimal pH 6.0 and below and optimum temperature 25°C. Some microorganisms have developed a strategy to escape progressive of pH decrease by switching over to the production of less toxic products such as alcohol or glycols [47].

In glycerol fermentation, 1,3-PDO is the characteristic bio-product formed through the reduction step of pyruvate pathway [48][49]. In our research, 1,3 PDO was expected to produce as reported earlier using different microorganisms through glycerol bioconversion [50]. However, our new isolate SRWQ1 was not able to produce 1,3-PDO. The GDH enzyme can produce 1,3-PDO, 2,3-BDO and acetoin, but this does not mean all the bacteria which can produce GDH enzyme are able to produce all these three bio-products [51]. Some of the bacteria could produce a high amount of 2,3-BDO, little 1,3-PDO and quite amount of acetoin. Also, sometimes there is no cell wall pathway to produce 1,3-PDO [52]. According to the preliminary experiment results at slightly acidic pH 6.0, 1,3-PDO formation is reduced in the presence of oxygen, probably because of the oxygen sensitivity of the two enzymes of the propanediol route [53]. Those, it seems possible that, under aerobic conditions and low pH, glycerol is converted to 2,3- butanediol and acetoin only.

2.5 Conclusion

In this study, we have demonstrated that a novel bacterial strain *S. proteamaculans* SRWQ1 represents a foundation for the exploration of GDH producing microorganisms which might be more efficient in the industrial environment. It showed that *S. proteamaculans* SRWQ1 has a great ability to produce high concentrations of 2,3-BDO and acetoin without producing any co-product at higher glycerol concentrations. Therefore, the newly isolated strain SRWQ1 could be a better organism for aerobic conversion of glycerol to 2,3-BDO. Our work would lead to contribute to the development of biodiesel industries and reduce industrial waste disposal. Moreover, this work can evaluate the efficiency of bioconversion of glycerol to bioproducts by novel bacterial strains under the aerobic condition with potential in future studies for advancement in bioconversion processes.

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3.7 Supplementary documents

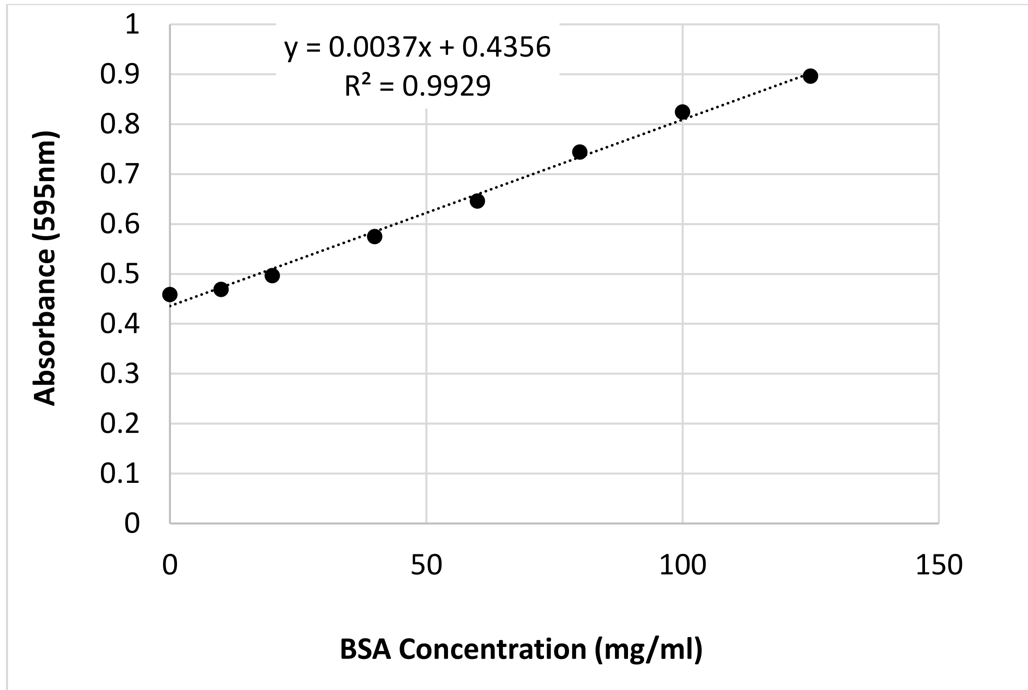

Figure 1. A standard curve of BSA concentration using Bradford assay (mg/mL)

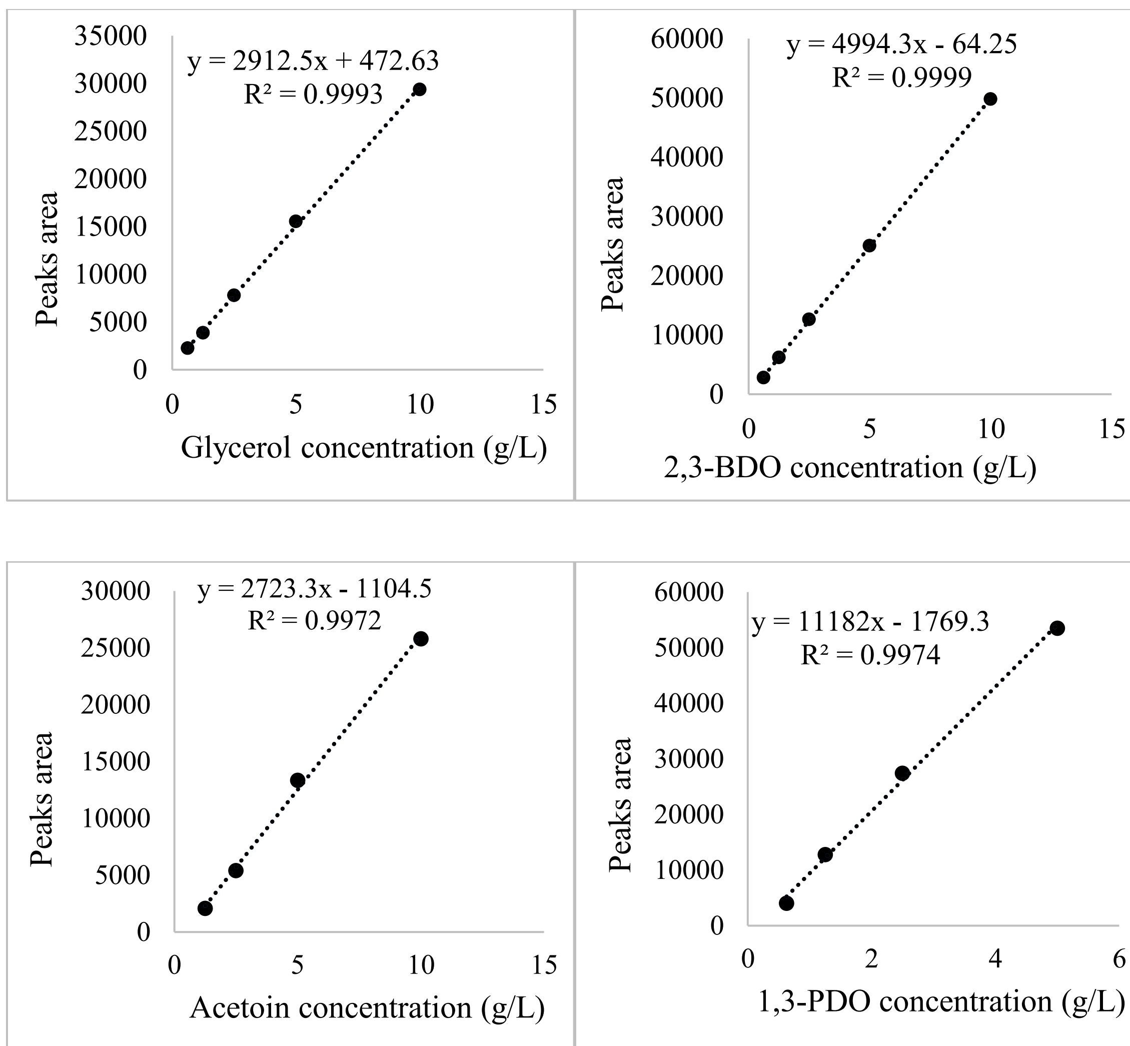

Figure 2. Standard samples of four bioproducts

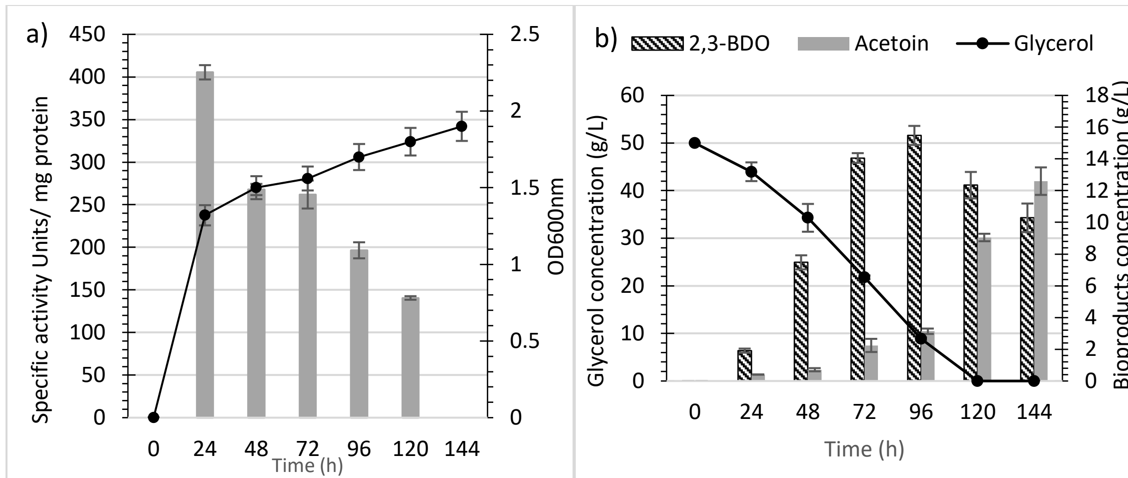

Figure 3. a) Biomass growth and GDH enzyme activity and b) bioproducts generated by *S. liquefaciens* SRWQ2. The minimal salt medium contained 5.0% glycerol and 0.5% each peptone and yeast extract with an initial pH 6.0 and temperature was 25°C

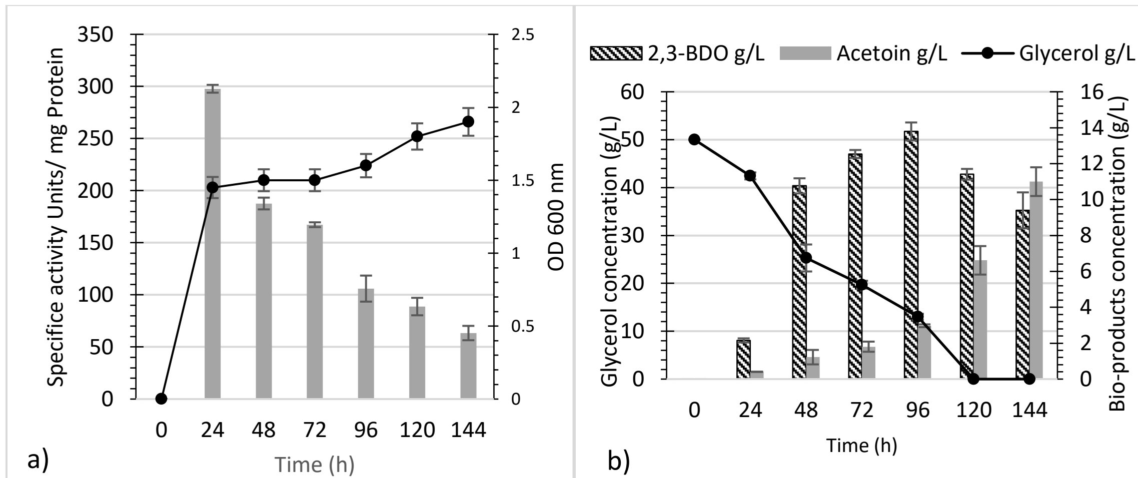

Figure 4. a) Biomass growth and GDH enzyme activity and b) bioproducts generated by *Serratia* sp. 243 The minimal salt medium contained 5.0% glycerol and 0.5% each peptone and yeast extract with an initial pH 6.0 and temperature 25°C

CHAPTER III

High production of cellulase by a newly isolated strain *Bacillus* sp. IM7

Abstract

Lignocellulosic biomass produced in agriculture and forest-based industries is the most abundant renewable resource to convert biofuels and value-added chemicals. Twenty bacterial strains isolated from soil and rotted wood samples from Kingfisher Lake, Thunder Bay, Canada were screened for their cellulolytic activity using carboxymethyl cellulose (CMC) as the sole carbon source. The cellulose degrading potential of the isolates was qualitatively estimated as carboxymethyl cellulase (CMCase) activity by calculating hydrolysis capacity via diameter of the zone of clearance. Five isolates among the twenty strains showed higher CMCase activity, and isolate IM7 exhibited the highest CMCase activity. The strain IM7 was identified as *Bacillus* sp. on the basis of 16S rRNA gene sequence analysis. In this study, the optimal conditions for high production of CMCase were defined in a completely aerobic process. The newly isolated *Bacillus* sp. IM7 strain showed the highest CMCase activity up to 17.7 ± 0.17 IU/mL using 1.5% yeast extract in the culture medium as a nitrogen source. Moreover, when the culture medium supplemented with 2% mannose as an inducer the CMCase activity was markedly boosted up to 24.59 ± 0.09 IU/mL. The optimum pH and temperature of the culture for CMCase production by the strain IM7 were 5.0 and 30°C respectively, and the significant enzyme production was obtained at a wide temperature range of 25–55ºC. Therefore, this *Bacillus* IM7 strain could be a potential candidate for CMCase production in an industrial bioconversion process.

Keywords: Lignocellulose, CMCase, *Bacillus* sp. IM7, Hydrolysis, reducing sugar

3.1 Introduction

With decades of studies on lignocellulose bioconversion, cellulases have been playing an important role in producing fermentable reducing sugars from cellulosic biomass. Typically, cellulases are primarily composed endoglucanases that hydrolyze the exposed cellulose chains of the cellulose polymer, exoglucanases that act to release cellobiose from the reducing and nonreducing ends, and β-glucosidases that help to cleave the cellobiose and short-chain cello-oligosaccharide into glucose (Kim et al., 2006). Bioethanol, a renewable biofuel produced from fermentation of sugar is most commonly used as an additive to gasoline for motor vehicles. It is a form of renewable energy obtained from fermentation of sugars in agricultural feedstocks (Mussatto et al., 2010). Among the many advantages of using bioethanol as a fuel are its low toxicity and susceptibility to biodegradation, making it "eco-friendly" (Balat et al., 2009). However, to achieve high production levels, fertile land and ample water supplies are required to grow agricultural feedstocks (Smith, 2013). To produce bioethanol from the fibrous part of a plant cellulose, a cellulolytic process has been developed that consists of hydrolysis of pretreated lignocellulosic material, with the help of cellulase enzymes including to break complex cellulose into simple sugars (Pereira, 2014). As an alternative form of energy, cellulose has gained importance recently, and it represents millions of dollars to countries with the means to obtain energy from it (Yang et al., 2014). Plant cellulose is convenient because it can be easily stored and moved, and is non-toxic. Cellulose is a linear biopolymer of glucose molecules. The enzymatic hydrolysis of cellulose requires mixtures of hydrolytic enzymes including endo-β-glucanases (EC 3.2.1.4), exo-β-glucanases (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), all acting in synergy (Bhat, 2000). Mechanistically, the endo-β-glucanase randomly breaks the internal glycosidic bonds which result in formation of glucan chains of different lengths; the exo-β-gluconase acts on

the ends of the cellulose chains resulting the formation of β-cellobiose as end products and the β-glycosidase finally break the glycosidic bond of β-cellobiose or small polysaccharides to produce glucose molecules (Lamed et al.,1983). These cellulases have been used for both academic research and industrial production. Cellulase enzymes produced by microorganisms could be used for bioconversion of cellulosic biomass to renewable biofuels because they can depolymerize the β-(1–4) linkages in the cellulose molecule into cellobiose and glucose (Subramaniyan and Prema, 2000). Most of the cellulases currently used in industries and laboratories are produced by fungi because of their high yields. Indeed, both bacteria and fungi are able to produce cellulases, but bacterial cellulase production is higher due to their high growth rate compared to fungi (Pérez et al., 2002). However, unlike fungi, bacteria have a limited capacity to produce a large number of extracellular enzymes. For several years, spectrophotometric methods have been used to detect cellulase activity based on reducing sugars using 3,5-dinitrosalicylic acid (DNS) (Maki et al., 2011). In the recent years, enzymatic bioconversion of lignocellulosic wastes to biofuels and value-added chemicals is attracting because of the rapid depletion of fossil fuels and increasing demand for renewable energy. It is a complicated process for the production of cellulases by microorganisms because different strains induced by different substrates might produce different cellulases which are more suitable for acting on the specific substrates (Fontes et al., 2010). Consequently, even though there are a number of cellulase-producing microbial strains commercially available, it is still necessary to isolate new effectual strains for efficient degradation of specific lignocellulosic biomass. Therefore, the aim of our research work is enzymatic cellulose degradation by efficient cellulase producing bacterial strain to produce sugar, which can be further converted to value-added chemicals. In our research work, a novel bacterial strain *Bacillus* sp. IM7 was isolated and characterized, which can efficiently produce cellulase (CMCase). Thus, this

research has a significant contribution to the development of biofuel industries other than fossil fuels and other synthetic fuels.

3.2 Material and methods

3.2.1 Isolation of bacterial strain

Soil samples were collected from Kingfisher Lake, Thunder Bay, Ontario, Canada. One gram of sample was added to an Erlenmeyer flask containing 100 mL of 1% peptone water. The sample was mixed thoroughly using the sterilized magnetic stirrer, then 100 µL of sample was streaked on Luria-Bertani (LB) agar (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) plate using sterilized glass spreader (Paudel et al., 2015). Plates were incubated for 48 h at 30°C. From these plates, bacterial colonies were selected based on morphological features such as colony morphology, color, and size. Pure bacterial isolates were screened for their CMCase activity.

3.2.2 Screening for CMCase activity

Isolates were grown in tubes containing 5.0 mL LB broth (10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl) (Rahman et al., 2015) for 24h at 30ºC, with shaking at 200 rpm. Subsequent incubation, 5.0 µL of culture broth was dropped on the center of CMC agar (5 g/L CMC, 1 g/L $NaNO_3$, 1 g/L K_2HPO_4, 1 g/L KCl, 0.5 g/L $MgSO_4$, 0.5 g/L yeast extract and 15 g/L) agar plates, incubated for 48 h at 30°C, then stained with Gram's iodine solution (per 300 mL: 2 g KI and 1 g I) to determine the visible hydrolysed zone of CMC formed by bacterial CMCase (Maki et al., 2014). The formation of clear zones after approximately 30 min in the areas around colonies indicate an absence of CMC. These "halos" are signs that the colony exhibits cellulase activity. By using a standard ruler, the halos were measured in centimeters.

3.2.3 Identification of bacterial isolates by 16s rRNA gene sequencing

16s rRNA gene sequencing is often used for bacterial identification and discovery of novel bacteria strains. In our study, the most promising five bacterial isolates isolated newly were identified using 16s rRNA sequencing. All five cellulase positive isolates were cultured in LB broth for 24h where the incubation temperature was maintained at 30ºC. Following incubation, cells from 1000 µL of broth culture were collected by centrifugation at 1200xg. The genomic DNA was isolated from these bacterial calls using DNA extraction kit (Ultra-clean Presto™ mini genomic DNA Bacteria kit) following manufacturer protocols. The extracted DNA was amplified by PCR (polymerase chain reaction) using universal 16s primers HDA-1 (5′-GAC TCC TAC GGG AGG CAG CAG T-3′) and E1115R (5′-AGG GTT GCG CTC GTT GCG GG-3′). The PCR reaction mixtures (25 µL) was contained 1.0 µL of each primer, 1.0 µL PCR master mixture (Taq Mix), 22 µL nuclease-free water and 1.0 µL DNA template. The PCR thermal cycling procedure was as follows: primary denaturation at 94°C for 3 min; 35 amplification cycles consisting of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. The amplified gene product was separated by agarose gel electrophoresis and purified using the Geneaid PCR/Gel Purification Kit following manufacturer protocols. The concentration of purified DNA sample was measured using Nanodrop spectrophotometer. Finally, the purified DNA sample was sent to the Centre for Applied Genomics (Eurofins Genomics, Toronto, Canada) for sequencing, and the sequence was analyzed using nucleotide BLAST program (http://www.ncbi.nlm.nih.gov/).

3.2.4 Phylogenetic relationship of bacterial isolates

Phylogenetic relationship of microorganisms helps to provide a better understanding of diversity, systematics, and nomenclature of microbes. The sequencing results were input into the

National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov) to identify possible strains. The phylogenetic tree was drawn using the ClustalX Omega software program.

3.2.5 Quantitative CMCase assay

Caboxylmethylcellulase (CMCase) activity was measured at 50°C and the released reducing sugar was analyzed using a dinitrosalicylic acid (DNS) method. A modified microplate-based assay method was used for measuring reducing sugars. Briefly, 20 µL of cell-free supernatant (enzyme) was mixed with 80 µL of 0.5% CMC and 50 mM citrate buffer at pH 6.0, and incubated for 30 min at 50°C. The reaction was terminated by adding 200 µL DNS solution (3.15 g/L DNS, 10.48 g/L NaOH, 91.0 g/L Na-K tartrate, 2.5 g/L phenol, and 2.5 g/L sodium metabisulfite). The mixture was boiled for 5 min. The absorbance was determined at $OD540_{nm}$ using microplate reader spectrophotometer (Epoch^{TM} microplate spectrophotometer, BioTek instrument, USA). All experiments were repeated at least three times.

3.2.6 CMcase activity of the isolates

Bacterial isolates were grown in LB broth medium and transferred 1.0 mL of an overnight culture into 200 mL Erlenmeyer flask containing 50 mL of Minimal Salt (MS) medium supplemented with 0.5% CMC as a sole carbon source. The MS medium per Litre contained: 1.0 g K_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 0.5 g $NaNO_3$ and 0.01 g $FeSO_4$). The flasks were incubated in a shaker incubator at 30°C and 200 rpm for 144h. Bacterial growth was observed each 24h intervals. CMCase activity was determined by measuring the release of reducing sugars from CMC as a substrate.

3.2.7 Optimization of fermentation process and medium components

Cells from slant culture was inoculated into a LB broth medium to prepare the seed culture. After 24h incubation at 30°C under aerobic condition, the seed culture was inoculated into fermentation medium for optimization of fermentation parameters including incubation temperature and time, medium initial pH, concentrations of carbon and nitrogen sources. In case of CMCase assay, batch fermentations were carried out in 200 ml Erlenmeyer flasks containing 50 mL fermentation medium (MS medium supplemented with 0.5% CMC) with 100 µl of 24 hours seed culture, incubated under aerobic condition at 200 rpm using rotary shaker (New Brunswick Scientific, C25 incubator shaker, NJ, USA). When indicated, the medium was supplemented with a specified concentration of different nitrogen and carbon sources. The pH of the medium was adjusted with 1 M NaOH or 1 M HCl, depending on the experiment. The cell-free supernatant was collected by centrifugation at 12000xg, and used for the CMCase activity.

To optimize the incubation time for maximum enzyme activity, experiments were performed without pH control at 30ºC for up to 144h. However, for extracellular enzyme assay, 1.0 mL of sample (culture broth) was taken each 24h interval.

The effect of initial pH ranging from 4.0 to 8.0 and temperature ranging from 25 to 50°C was evaluated on CMCase activity. Moreover, different nitrogen sources (malt extract, yeast extract, peptone, sodium nitrate, urea, ammonium sulfate and sodium chloride) and carbon sources (fructose, mannose, sorbitol, glucose, lactose, sucrose, CMC, and galactose) were also used in fermentation medium to optimize the culture for maximum enzyme activity. All experiments were performed in triplicate and the results are expressed as mean values of three replicates.

3.2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the molecular weight of the enzyme, the zymogram analysis was performed by SDS- PAGE using 10 % (w/v) separating gel (0.5 mL of 1 % CMC, 3.3 mL water, 3.4 mL of 30 % acrylamide with 0.8% bis-acrylamide, 2.6 mL 1.5 M Tris- pH 8.8, 0.1 mL of 10 % SDS, 100µL of 10 % ammonium persulfate and 10 µL TEMED) and 5.0 % (w/v) of stacking gel (2.975 mL water, 1.25 mL 0.5 M Tris-HCl with pH 6.8, 0.05 mL 10 % SDS, 0.67 ml of 30% acrylamide with 0.8% bis-acrylamide, 0.05 mL of 10 % ammonium persulfate and 0.005 mL TEMED). TEMED and ammonium persulfate were added immediately before each use (Laemmli, 1970). The acrylamide percentage in SDS-PAGE gel depends on the size of the target protein in the sample.

For SDS-PAGE analysis 100 µL of the broth culture was taken from optimized culture condition, centrifuged at 12000xg for 3 min, and 30 µL of cell-free supernatant was mixed with 10 µL 4X protein loading buffer. From the above loading mixture, 10 µL was loaded into wells along with standard protein marker in the first lane. The gel was run using the Mini-Protean system (Bio-Rad) following manufacturer protocols with some modifications. One part of the gel comprised the samples and molecular markers, and was stained with Coomassie brilliant blue R-250 for 1h, further de-stained with decolorizing buffer for proteins and marker band. The other part was treated with 1 % Triton X-100 for 30 min to remove the SDS and allowed refolding of the proteins in the gel. The CMC-Na gel was incubated at 50ºC for 1h in 50 mM citrate buffer containing 0.5% CMC at pH 6.0 to detect the cellulase activity. Then, the gel was submerged in 0.1 % (w/v) Congo red solution for 30 min, and then decolorized the protein band using 1M NaCl until pale-red hydrolysis zones appeared against a red background for zymogram analysis (O'Farrell, 1975).

3.3. Results and discussion

3.3.1 Screening for cellulase activity

A total of 20 cellulose-producing aerobic bacterial isolates were isolated and screened for their CMCase activities. However, five isolates were exhibited high CMCase activity where they produced hydrolyzing zones on CMC agar plate after treated with Gram's iodine stain. Results of the CMCase activities of these five bacterial strains are shown in Figure 2 along with negative control *E. coli* JM109 strain. This protocol for enzyme screening is a simple method for preliminary screening of cellulolytic microorganisms (Maki et al., 2012). As shown in Figure 2, the cellulase-producing isolate IM7 exhibited the largest halo (clear) zone of 2.8 cm in diameter after 48h incubation at 30 ºC, and this isolate IM7 was chosen for further study. The diameters of the halo zones produced by all bacterial isolates are shown in Figure 2 in the photograph of each CMC agar plates. The morphological features of the colony formed by the strain IM7 on CMC agar plate were observed as small, circular, rough, opaque and white.

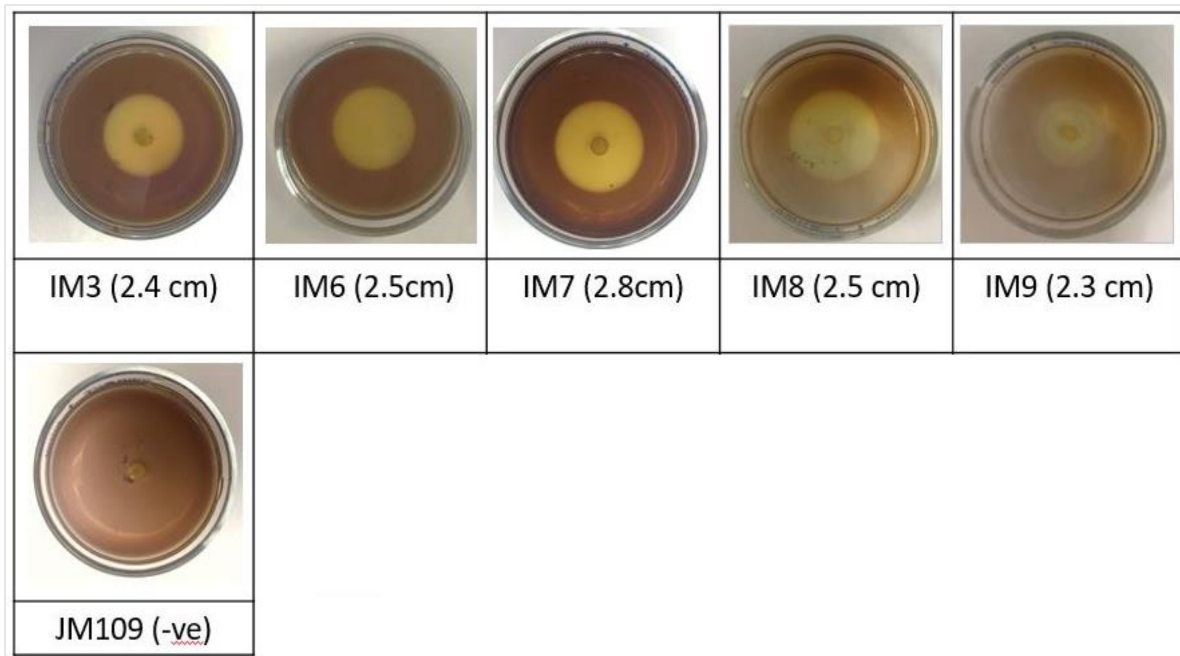

Fig. 1. Five cellulose-producing isolates using carboxymethyl cellulose agar plates. Plates were tested via the gram's iodine method. *E. coli* JM109 (negative control- non-cellulase producer)

3.3.2 Identification and evolutionary relationship of the isolates

16S rRNA gene sequencings of the five selected bacterial isolates (IM3, IM6, IM7, IM8B, and IM9) were used for their identification and discovery of novel bacterial strains. 16S rRNA genes amplified from genomic DNA of all five CMCase producing isolates were successfully obtained using PCR. Moreover, the sequences and sequence analysis results of amplified rRNA genes of five different bacterial isolates were successfully obtained, and the sequences of 16S rRNA genes for the isolates IM3, IM6, IM7, IM8B, and IM9 were submitted to the gene bank for their accession numbers. The sequencing results were also inputted into the nucleotide BLAST of the NCBI database to get possible identities of the isolates based on homology. Nevertheless, the isolates IM3 and IM6 reported in this study were identified as *Paenibacillus* sp IM3 and IM6 strains respectively which have 99% similarity to the genus *Paenibacillus*. Similarly, the isolates

IM7, IM8B, and IM9 have been nominated as *Bacillus* sp IM7, IM8B, and IM9 respectively, the new strains of *Bacillus* sp. The 16s RNA gene fragments were validated and shown as the bands on 1% of agarose gel with demonstrating the unmistakable expected size of around 750 bp.

The 16s rRNA gene sequences of the identified bacterial strains were used for the construction of the phylogenetic tree to show the evolutionary relationship. The phylogenetic tree was successfully constructed using gene sequences of different *Bacillus* sp. and *Paenibacillus* sp retrieved from NCBI GenBank by the Neighbor-Joining method (Fig. 3).

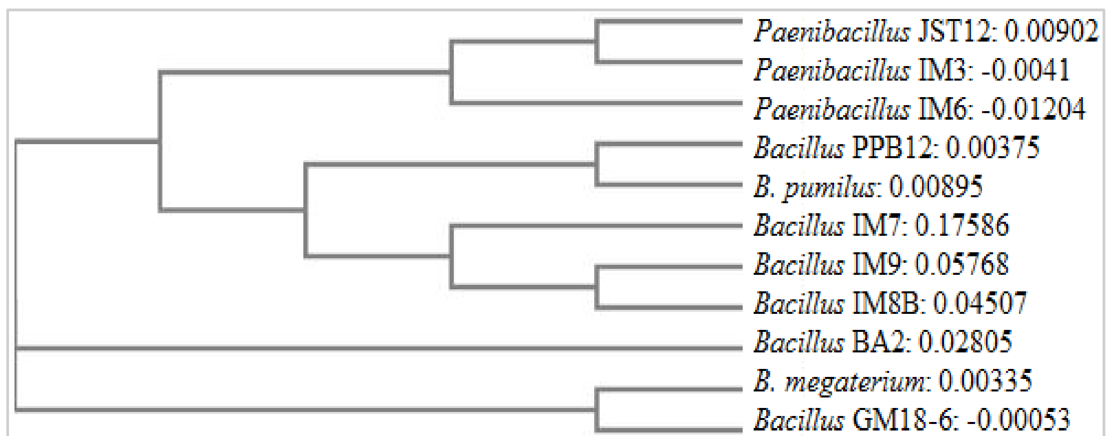

Fig. 2. Evolutionary relationships of the strains: Phylogenetic tree (neighbor-joining tree) drawn from sequence alignment program using ClustalX Omega software. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are bootstraps

3.3.4 Effect of incubation time on biomass and CMCase production

The effect of incubation time on the production of bacterial biomass and CMCase was performed in batch culture at 30°C using MS medium supplemented with 0.5% CMC. Bacterial growths were observed at different time intervals. The enzyme (CMCase) activity was determined by measuring the release of reducing sugars from CMC as a sole substrate. The time course of the

bacterial growth and enzyme activity were performed over a 144h incubation time. The time courses data of the production of biomass, as well as CMCase by the strains IM3, IM6, IM7, IM8B, and IM9, are presented in Fig. 4a, 4b, 4c, 4d and 4e respectively. The strain IM3 and IM7 showed highest enzyme activity after 48h and 72h incubation respectively compared to that of other strains (Fig 4). The maximum CMCase production by strain IM7 *Bacillus* sp. was 6.88 ± 0.047 IU/mL after 48h of incubation (Fig. 4c). Furthermore, the strain IM3 (*Paenibacillus* sp) exhibited the highest enzyme activity 6.93±0.0289 IU/mL after 72h of incubation (Fig. 4a). The maximum enzyme activity of the strain *Bacillus* sp IM6 was 6.47 ± 0.11 IU/mL after 72h incubation. Moreover, the strains *Bacillus* sp IM8B and *Bacillus* sp IM9 produced maximum CMCase 5.36 ± 0.012 IU/mL and 5.05 ± 0.0.04 IU/mL enzyme respectively after 120h incubation. However, the strains IM3, IM6 and IM7 exhibited maximum biomass (OD_{600}) productions 1.14, 1.18 and 1.53 respectively after 72h incubation. The maximum biomass production with the strain IM8B obtained after 48h incubation was 1.26. Moreover, the biomass production of the strain IM9 was increased up to 144h incubation (Fig. 4e).

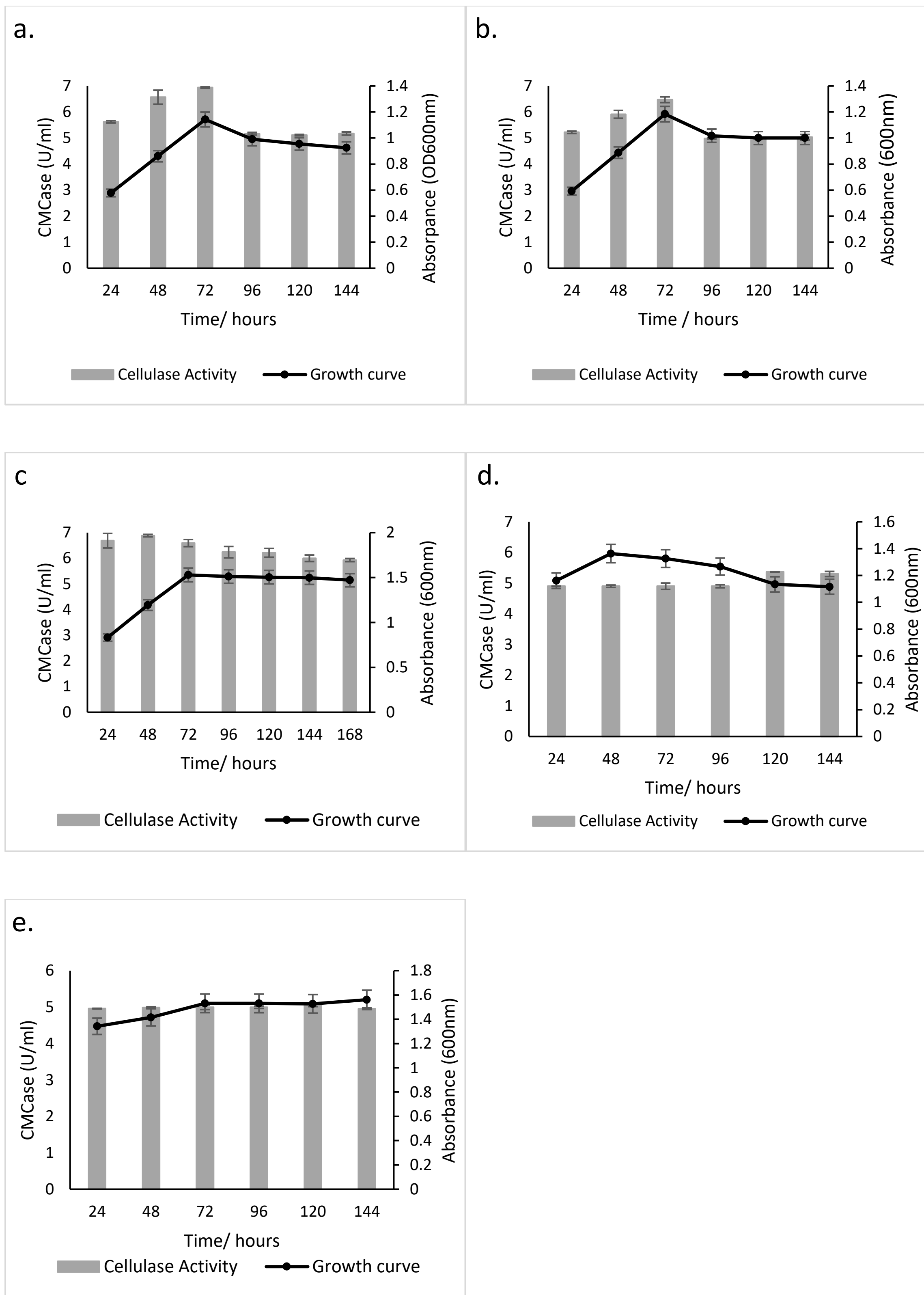

Fig. 3. Effect of Incubation time on bacterial growth and their enzyme production (IU/mL)

3.3.5 Effect of pH and temperature on cellulase activity

CMCase activities have been influenced by the effect of incubation temperature, and the results are illustrated in figure 5a. The cellulase producing isolate *Bacillus* sp. IM7 showed cellulase activity at a wide range of culture temperatures (25 - 50ºC). The highest enzyme activity 7.015 ± 0.096 IU/mL was obtained at 30ºC with the strain IM7, and this optimal temperature is similar to the findings of many researchers who reported using other *Bacillus* sp. (Chellapandi et al., 2008). The CMCase activity was significantly decreased at 25, 35, 40 and 50ºC incubation temperatures. At 25ºC culture temperature, the enzyme activity was very low (61.7 % of the relative activity) (Fig. 5a).

Initial pH of the culture medium was played a significant role on CMCase activity, and results are shown in Fig. 5b. It was observed that the maximum cellulase activity 7.22±0.13 IU/mL obtained at pH 5.0 which was significantly higher compared to that of other pHs (Fig 5b). The cellulase activity was significantly decreased at pH 6.0 which was about 74.53 % of relative activity (Fig. 5b). In the literature, the cellulase enzyme activity (U/mL) under the effects of initial pH and temperature during fermentation with 1% CMC as a substrate, the *Bacillus pumilus* EB3 strain showed the optimum pH 7.0 for maximum CMcase activity (0.058 U/mL) at incubation temperature 37ºC (Ariffin et al., 2008). A similar result was also reported in *Bacillus* sp. K1 when the optimum pH was 6.0 and the maximum enzyme activity was reported as 5.21 ± 0.2 U/mL (Paudel et al., 2015). Moreover, for the strain *Bacillus* sp. K1, the CMCase activity was significantly decreased at pH 5.0, and 60 % of the relative activity was attained (Paudel et al., 2015). On the other hand, the strain *B. subtilis subsp. Subtilis* A-53 showed a stable cellulase activity at pH 6.5, and CMCase activity of the purified enzyme was 40% of the maximal level under a specific condition between pH 5.0 and 7.5 (Kim et al., 2009). Thus, our results indicate

that the enzyme activities of the newly isolated strain *Bacillus* sp. IM7 has much wider temperature and pH stability than those reported in the literature and therefore, has potential application in the related industry.

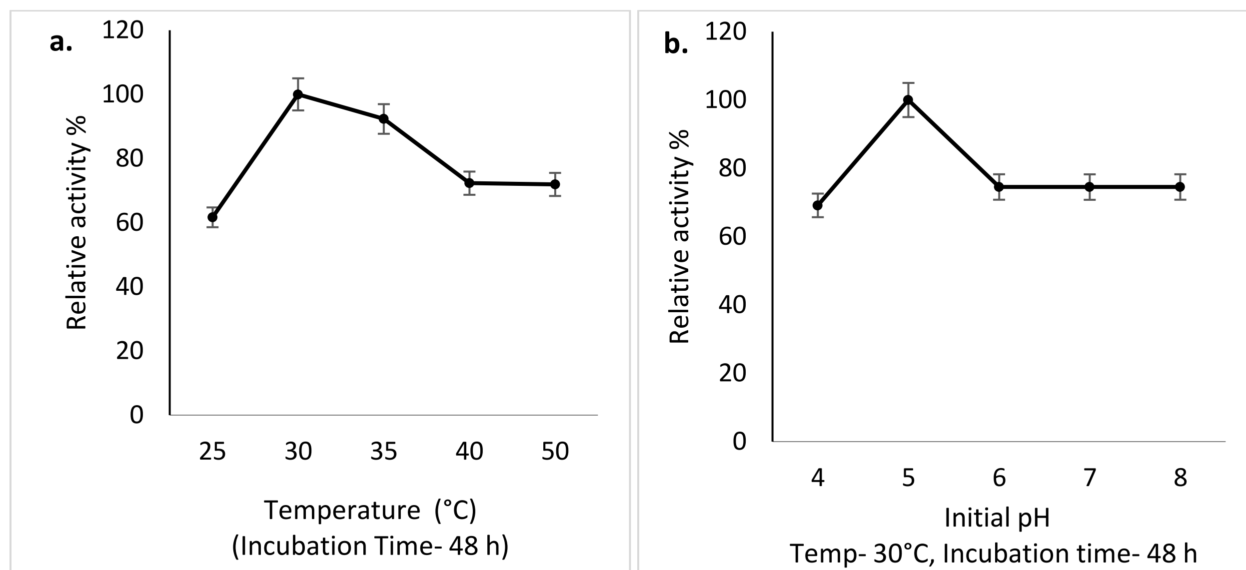

Fig. 4. a) Effect of different incubation temperature, b) effect of pH levels on carboxymethyl cellulase production by strain IM7

3.3.6 Effect of different nitrogen sources on cellulase production

The impact of seven different nitrogen sources on the production of cellulase enzyme was investigated. The results from our experiment showed a clear variation of the relative activity (%) of CMCase using different nitrogen sources viz., peptone, yeast extract, malt extract, sodium nitrate, sodium phosphate, urea, and sodium chloride. The maximum CMCase activity was found by using malt and yeast extracts respectively as a source of nitrogen (Fig. 6a). Our results showed that the strain *Bacillus* sp. IM7 can utilize organic nitrogen sources yeast and malt extracts efficiently, and the maximum CMCase activities were increased (Fig 6a). When 1.5% of yeast

extract was used as a nitrogen source in the culture medium the CMCase activity was boosted up to 17.7 ± 0.17 IU/mL. The cellulase production was increased due to decreased of acetic effect in the medium by nitrogen sources (Lee et al., 2008). Moreover, the relative activity was decreased about 28.6 % and more when inorganic nitrogen sources were used as the nitrogen sources (Fig. 6a). The use of 1.5 % of yeast extract enhanced 21.1 % of the relative activity of cellulase compared with the control. When the concentration of yeast extract was increased from 1.5%, the relative activity of CMCase was significantly decreased (Fig. 5b).

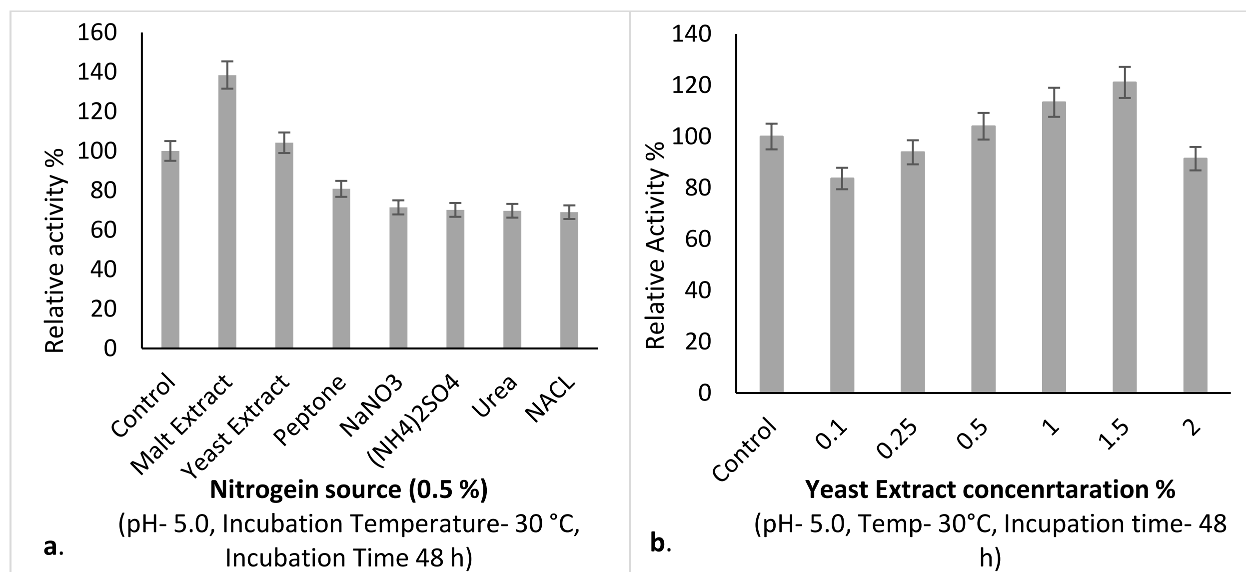

Fig. 5. a) Effect of different nitrogen sources, b) Different yeast extract concentrations on carboxymethyl cellulase production by *Bacillus* sp (IM7)

3.3.7 Effect of different carbon sources on cellulase production

In the present study, 1% of different carbon sources were used in the culture medium to evaluate their effect on cellulase production under optimum condition (culture temperature 30ºC, incubation time 48h and pH 5.0). The results showed that the maximum CMCase activity obtained

after 48h incubation using IM7 strain was 17.8 ± 0.07 IU/mL when 1.0 % of mannose was used in culture medium (Fig. 7a). Furthermore, the concentration of mannose was optimized using different concentrations of mannose in the culture medium. As shown in Figure 7b, the CMCase activity was expressively increased to 24.59 ± 0.09 IU/mL when 2.0% of mannose was used in the culture medium, and the relative activity was increased 35.9 % over control (Fig. 7b). Therefore, the low-cost mannose was finally selected as an inducing carbon source of CMCase enzyme for IM7 strain. This result indicated that the carbon source mannose induced CMCase enzyme activity through the cell membrane. Thus, mannose in the culture medium as a co-substrate with CMC has a potential application in large-scale production processes besides a significant advantage over glucose (Berrios et al., 2011).

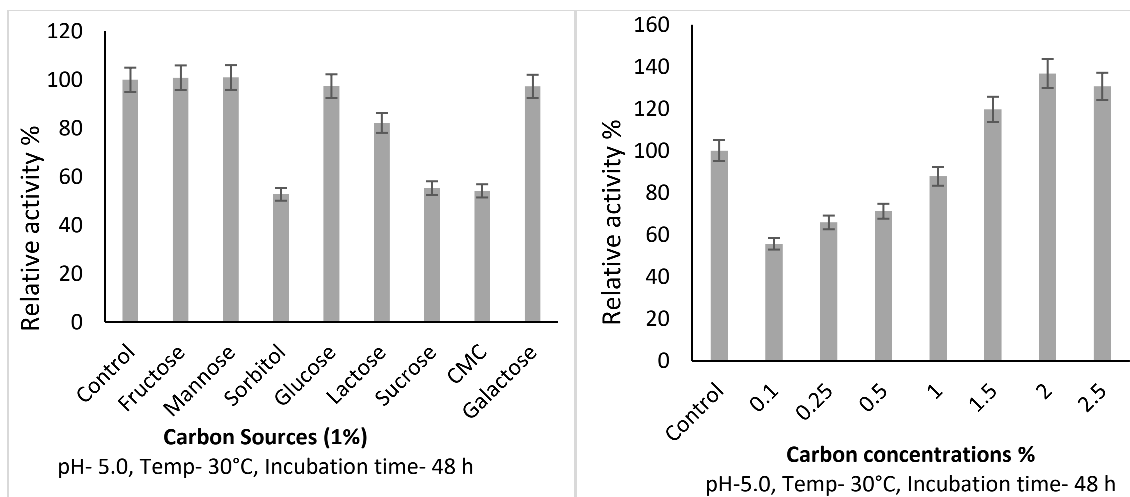

Fig. 6. a) Effect of the carbon sources (1%) on CMCase production by *Bacillus* sp. IM7 b) Effect of various concentration of carbon sources

3.3.8 SDS-PAGE and zymogram analysis:

The CMCase enzyme protein from IM7 strain was confirmed by SDS-PAGE and zymogram analysis. The molecular weight of the protein (CMCase) was estimated as ~50 kDa

and the protein bands including CMCase of IM7 strain are sowed in Fig. 8. The zymogram using CMC as a substrate showed the targeted active bands. However, this molecular weight 50 KDa of the cellulase was similar to the other species of *Bacillus* reported by Bajaj et al (2009).

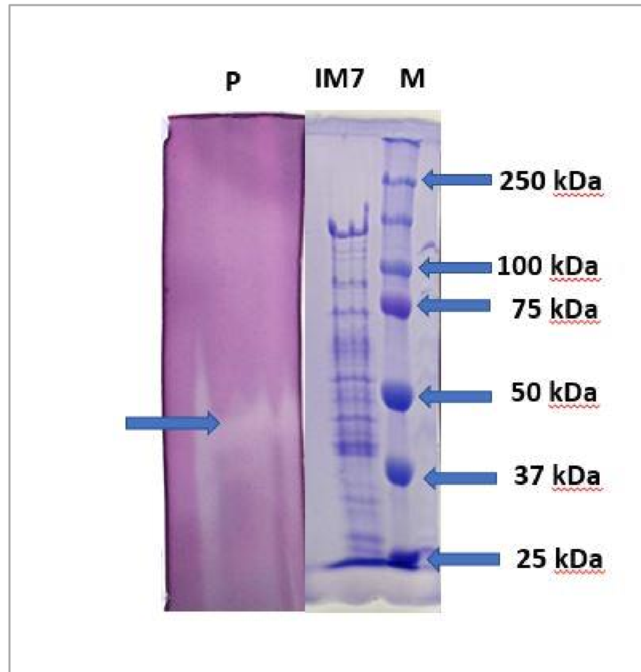

Fig. 7. SDS-PAGE analysis of location and existing state of CMCase protein. Lane M – protein marker; lane IM7- the cellulase zymogram and Lane P- protein in the supernatant

However, this study described the cellulase activity of a new cellulase producing bacterium *Bacillus* sp. IM7 isolated from Kingfisher Lake, Thunder Bay, Ontario, Canada. According to the experiments conducted by Sheng (2012) *Pseudomonas* sp. HP207 had maximum endoglucanase production among the optimized fermentation conditions. In this experiment, results showed that the HP207 strain proved to be at the higher enzyme activity as 1.432 U/mL. The endoglucanase enzyme produced in this case proved to be thermostable as well. In fact, *Bacillus* sp. could utilize both the inorganic and organic nitrogen sources for cellulase production (Acharya et al., 2011). However, in our report, the production of cellulase in presence of different nutrition factors by

Bacillus sp. IM7 was 24.59 ± 0.09 IU/mL which is much higher than previously reported many reports (Pudel et at., 2015 and Maki et at., 2014). Also, the enzyme activity of our new isolate *Bacillus* sp. IM7 was found higher than those widely studied previously by other workers, which have received wide attention for commercial production of cellulase (Kang et al., 2004; Gao et al., 2008).

Nevertheless, cellulases have a potential application for hydrolyzing polysaccharides in biorefining industries which are based on agro-industrial wastes. The bacterial strain IM7 reported in this study was competently able to degrade lignocellulose substrates. This work acts as a step towards a further study on bio-refinery feedstocks that are composed of biomass (Fitzpatrick, 2006). Also, the future studied can be done in identification and cloning of the genes β-1,4 endoglucanase in these cellulase producing isolates. The strain improvement for enhancing cellulase production can be achieved by using different techniques such as mutagenesis and metabolic engineering (Lin et al., 2014).

3.4. Conclusion

Five cellulase-producing bacterial isolates were obtained from different soil and rotting wood samples, and the novel strain *Bacillus* sp. IM7 exhibited a remarkable cellulase activity. Therefore, we have successfully identified an efficient cellulase producing bacterial strain *Bacillus* sp. IM7 with exceptional potential for industrial use in the bioconversion of lignocellulosic biomass to biofuels and value-added products via reducing sugar. Moreover, many of our isolates characterized here also have potential for industrial use. The most promising strain IM7 reported in this research paper could be a foundation for current exploitation of cellulase enzyme by further investigation. Also, the strain IM7 may have great potential for developing metabolically

engineered strain to increase the biodegradation of lignocellulosic biomass and help overcome expensive hurdles being faced for industrial production of biofuels.

3.5 Acknowledgement

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CHAPTER IV

Discussion and Future work

4.1 Discussion

Biodiesel is being adopted to replace petroleum products in order to promote an eco-friendly way of harnessing energy (Polburee et al., 2015). The use of petroleum products has had a few drawbacks such as having adverse effects on the environment. They have created global ecological disturbances and as such prove harmful to the environment in the long run (Rodriguez et al., 2016). Biodiesel is an environmentally friendly source of energy. However, its production is a long cumbersome process which all in all must still be done for the sake of its production (Oh & Park., 2015). Also, biodiesel is a fuel that can be produced from animal oil or fats, vegetable oil, tallow and waste cooking oil. These fats and oils undergo a process called transesterification. This process performs a set of processes leading to the production of biodiesel and furthermore the release of crude glycerol (Uprety et al., 2017). Due to producing crude glycerol in large amounts as a threat to the environment, conversion of crude glycerol to a product friendly to the environment is necessary (Spier et al., 2015). The process of conversion involves biotechnical processes which produce other products like 1,3-propanediol, 2,3-butanediol, and acetoin that have proven valuable to the industry (Pradima et al., 2017). All of the works presented here in this research, approach challenges in the current production of value-added products including platform chemicals and liquid fuel or fuel additive from different perspectives through cost-effective processes with the main focus on novel bacteria strain. There were several reasons for this research, namely bacteria can be easily cultured, can be found inhabiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the

potential exploitation of hardier bacterial strain for the industrial biotransformation of a large volume of glycerol or crude glycerol to biofuel and other high-value products. This MSc research work led to contribute to the development of biorefineries and reduce industrial waste disposal. Also, this research work help in summarizes different strategies employed to produce chemicals (1,3-propanediol, 2,3-butanediol, ethanol, n-butanol, organic acids, polyols and others). Moreover, this work can evaluate the efficiency of bioconversion of crude glycerol to bioproducts by novel bacterial strains under the aerobic conditions with potential in future studies for advancement in bioconversion processes.

In this study, pure and crude glycerol was used as a source of carbon and energy in shake-flask fermentation to produce 2,3-BDO efficiently using a new isolate *S. proteamaculans* SRWQ1. Likewise, the effect of biotransformation process parameters was investigated, and also optimized the fermentation process for a maximum product yield of 2,3-BDO. The major products produced from biotransformation of glycerol were discussed.

First of all, the focus was given on isolation and characterization of glycerol utilizing bacteria, as well as the aerobic conversion of crude glycerol by a novel strain *S. proteamaculans* SRWQ1. The aerobic condition was used for the large-scale production of value-added bioproducts especially 2,3-BDO and acetoin using glycerol as the sole substrate. In this research work, three newly isolated strains *S. proteamaculans* SRWQ1, *S. liquefaciens* SRWQ2, and *Serratia* sp. 243. However, the strain SRWQ1 exhibited the better GDH activity compared to that of other two strains, and therefore, the strain SRWQ1 was selected as a promising candidate for biotransformation process. Until now, in a biotechnological application like the production of high-value products from glycerol looking for efficient GDH enzymes producing bacteria, traditional microbiological isolation techniques are still important. The conversion of glycerol to

2,3-BDO is an oxidative process of microorganisms reported in many previous studies (Jeon et al., 2014). However, 2,3-BDO is a biofuel and building block chemical and it can be used for the synthesis of 2-3-butanone (Vivijs et al., 2014). The aerobic condition is often employed for 2,3-BDO production in which oxygen can be used as an electron acceptor (Chen and Liu., 2016). In anaerobic condition, the lake of external electron acceptor causes the hampering in microbial growth (Ito et al., 2005 and Jensen et al., 2012). A very little work has been done on the aerobic process of glycerol metabolisms, and it is our first report for biotransformation of glycerol to 2,3-BDO using our newly isolated *S. proteamaculans* SRWQ1. In my study, under aerobic condition, both high enzyme activity and low pH of the growth culture were beneficial to 2,3-BDO production and the similar result has been reported by (Cho et al., 2015). Moreover, in my case of study, strain *S. proteamaculans* SRWQ1 with optimal pH 6.0 of the growth culture was efficiently produced a high amount of 408.7 ± 0.06 U/mg protein GDH enzyme, resulting in a high production of 2,3-BDO crude glycerol as a sole of carbon source and did not produce 1,3-PDO. The maximum 2,3-BDO product concentration by SRWQ1 was 18.43 ± 1.05 g/L and the yield was 0.4 g/g. On the other hand, the maximum acetoin concentration was 8.38 ± 0.76 g/L with an obtained yield of 0.06 g/g after utilized 98.2 % glycerol.

This is the first study that showed 2,3-butanediol and acetoin productions by *S. proteamaculans* SRWQ1 and until now there is no any report on bioconversion of glycerol by *S. proteamaculans*. Glycerol has been converted to value-added products by several microorganisms only in cultures controlled at pH 7.00 (Rahman et al, 2017). Similarly, glycerol bioconversion has been reported by *Serratia sp* only in a culture controlled by pH 8.0 and incubation temperature 40°C (Taneja et al., 2017); however, our new isolate *S. proteamaculans* SRWQ1 able to convert glycerol and produce 2,3-butanediol and acetoin at the optimal pH 6.0 and temperature 30 °C.

Some microorganisms have developed a strategy to escape progressive of pH decrease by switching over to the production of less toxic products such as alcohol or glycols (Tchakouteu et al., 2015).

In the glycerol fermentation, 1,3-propanediol is a characteristic bio-product formed by dehydration through reduction step of the pyruvate pathway (Biebl et al.,1998; Saxena et al., 2009). In our research, 1,3 PDO was expected to produce as reported in the literature using different microorganisms through glycerol bioconversion (Xu et al, 2009). However, our new isolate SRWQ1 was not able to produce 1,3-PDO. The GDH enzyme can produce 1,3- PDO, 2,3- BDO and acetoin, but this does not mean all the bacteria which can produce GDH enzyme are able to produce all these three bio-products (Sarchami et al., 2016). Therefore, some of the bacteria produce a significant amount of 2,3-BDO, little 1,3-PDO and quite amount of acetoin. That does not mean all the bacteria has to produce all three value products together or with the same amounts (Garlapati et al., 2016). Moreover, sometimes a bacterium cannot be considered as a candidate for bioconversion process due to its lack of ability for producing a detectable amount of end product. Also, it may produce but the yield is very low, so it considers this bacterium as not able to produce the specific end product like 1,3-PDO or there is a possibility that there is no good inducer in the growth medium. Moreover, sometimes there is no cell wall pathway to produce 1,3-PDO (Vieira et al., 2015). Therefore, for the future, there is needs to find out which chemicals or compounds can induce 1,3-PDO production by SRWQ1 such as adding xylose or other inducers in the growth media. But our conclusion from the study is that bacterial strain SRWQ1 are not able to produce 1,3 PDO.

In addition, this aerobic process by *S. proteamaculans* SRWQ1 has not been investigated for glycerol conversion before. According to the preliminary experiment results at slightly alkaline

pH 6.0, the 1,3-propanediol formation is inhibited in the presence of oxygen probably because of the oxygen sensitivity of the two enzymes of the propanediol route (Nakamura et al., 2003). Thus, it seems possible that, under aerobic condition and low pH, glycerol is converted to 2,3- butanediol and acetoin only.

However, in this research work, we used pure and biodiesel-derived crude glycerol as the sole carbon source in the batch flask cultures to efficiently produce 2,3-BDO and acetoin in bioconversion process. When crude glycerol from biodiesel industry was used as a sole of carbon, ethanol was the most compound might be presented; however, ethanol can be removed from this crude glycerol by pretreatment or purification. The purification process needs special techniques, time and equipment. In our study, it was really surprising that the combined action of low pH 6.0 and a high excess of glycerol in culture broth give the highest acetoin yields (0.101g/g) which attending in the optimal culture broth containing 7.5% of crude glycerol borrowed from University of Guelph. If ethanol formation is repressed by slight glycerol excess and lowered incubation temperature 25°C, there are practically no by-products (Da Silva et al., 2009). Therefore, in our study, the optimal conditions for the highest GDH enzyme activity and high production of a value-added green product 2,3-BDO were defined, and a high yield of product was obtained using a newly isolated novel strain *S. proteamaculans* SRWQ1, which is the 1st time report on bioconversion process by this bacterium *S. proteamaculans* using glycerol as a sole substrate until today.

On the other hand, my second study described the cellulase activity of a new cellulase-producing bacterium *Bacillus* sp IM7 isolated from soil of Kingfisher Lake, Thunder Bay, Ontario, Canada. Due to the ability of several microbes to produce cellulase enzyme, a specific test was conducted to identify the most active strain in the production of cellulase enzyme. This enzyme

test using carboxymethyl agar plate (CMCNa- Salt) was performed (Hankin & Anagnostakis, 1977). The appearance of a bright halo surrounded bacterial colony is conclusive evidence of producing cellulase enzyme by bacterial degradation of this CMC substance transformation of the complex cellulose into simple sugar (Oke et al., 2017). Our newly isolated *Bacillus* sp IM7 showed the largest hallo (2.8cm) comparing with other strains isolated from different locations of Thunder Bay area. The wild-type bacterial strain IM7 demonstrated the biggest halo of diameter and was chosen for facilitating catalyst examine. As there are wide assortments of cellulase-delivering microscopic organisms in nature, their morphological highlights make the segregation of microorganisms simpler from various sources (Ratnavathi et al., 2011).

Our research was showed that DNS method able to detect D-glucose in the culture solution using dinitrosalicylic acid. The DNS method provides advantages that it does not use as potential carcinogens or corrosive chemicals and the experiment does not require using fume hood for conduct the assay because of using a free-cells substrate (Miller et al., 1959). CMC is widely used as a substrate to cellulase assay in many research. This method considered as accurate assay using CMC as a substrate with very low sample variability. The final advantage of using DNS method that can easily detect other enzyme activities by simply changing the substrate and buffer (Lin et al., 2015).

Additionally, In the present study, our new isolate IM7 was capable of detecting D-glucose liberated from CMC over the time. The strain *Bacillus* sp IM7 showed the highest enzyme production after 48 hours of the incubation time and produced a high amount of the enzyme 6.88 ± 0.047 IU/ml (Fig. 4). This optimum incubation time was different from other researchers who reported the maximum enzyme activity after 8 days in wild *Bacillus* sp. MTCC10046 (Sadhu et al., 2014) and 24 h in *Pseudomonas* sp. HP207 (Sheng et al., 2012). Furthermore, the maximum

enzyme was 7.22 ± 0.13 IU/ml achieved by our novel isolate wild strain *Bacillus* sp IM7 at the optimum temperature 30°C and pH 5.0. In 2009, Kim et al have been reported that CMCase produced by the bacterium remains stable over various temperature ranges (20-40). A purified cellulase produced in this case was treated with many chemicals and it has shown the ability to be suitable for use in industrial processes. Similarly, Rastogi et al. (2010) identified 8 isolates which can degrade cellulose. In this report, the optimum pH for thermostable cellulase production in was 5.0; however, the maximum cellulase activity by strain *Bacillus* sp DUSELR13 was only 0.12 U/ml which was less than our isolate under the pH 5.0 (Rastogi et al,. 2010). One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1 micromole of reducing sugar per minute per milliliter. According to the experiments conducted by Sheng (2012) *Pseudomonas* sp. HP207 had highest endoglucanase productivity in the optimized fermentation condition. In this experiment, the result showed that the HP207 strain proved to be at the higher enzyme activity rate 1.432 U ml^{-1}. The endoglucanase enzyme produced in this case proved to be thermostable as well. In fact, *Bacillus* sp could utilize both the inorganic and organic nitrogen sources for cellulase production (Acharya et al., 2011). However, in our report, the production of cellulase in presence of different nutrition factors by *Bacillus* sp IM7 was 24.59 ± 0.09 IU/ml when 1.5% of yeast extract as a nitrogen source and 2% of mannose as a carbon source were used. The enzyme activity of our new isolate *Bacillus* sp IM7 was found higher than those were widely studied previously by other workers, which have received wide attention for commercial production of cellulase (Kang et al., 2004; Gao et al., 2008).

4.2 Future work

In this study, we have demonstrated a total three bacterial strains isolated from soil represent a foundation for the exploitation of GDH producing microorganisms which might be

more efficient in the industrial environment. Consequently**,** the future work is required to characterize as well as development those isolates exhibiting greatest GDH activity. Considering for doing such work, using recombinant strains, it is possible to block the 2,3-butanediol dehydrogenase pathway to end up with the formation of acetoin in very high capacity. The high production of acetoin could convert at a later stage to diacetyl, which constitutes a valuable flavoring. Also, the opposite is likely to happen, which is to promote the production of 2,3-BDO and acetoin in very high yield using genetic manipulation for expressing targeted genes in our highly efficient isolate SRWQ1 which showed the greatest GDH activity.

Nevertheless, cellulases have a potential application for hydrolyzing polysaccharides in biorefining industries which are based on agro-industrial wastes. The bacterial strain IM7 reported in this study was competently able to degrade lignocellulose substrates. It is one of many examples of analysis of a lignocellulose that is a stable structure and capable of decomposing efficiently together through its enzyme system. This work acts as a step towards a further study on bio-refinery feedstocks that are composed of biomass (Fitzpatrick, 2006). Also, the future studied can be done in identification and cloning of the genes β-1,4 endoglucanase in these cellulase producing isolates. The strain improvement for enhancing cellulase production can be achieved by using different techniques such as mutagenesis and metabolic engineering (Lin et al., 2014).

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