Enzymatic hydrolysis of primary paper mill sludge and fermentative production of microbial lipids

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Abstract

Canada is known for its abundant forests and thriving forest products industry. For every ton of paper produced in mills across the country, approximately 30 kg of dry primary sludge is produced. The dry sludge is then either shipped to landfills or incinerated along with dried secondary sludge. However, this waste stream has the potential to be used more efficiently for the production of higher value products. Primary sludge is cellulosic in nature and contain fractions of hemicellulose, lignin and ash. Cellulose in the primary sludge is more accessible to enzymatic hydrolysis as compared to natural lignocellulosic biomass due to the physico-chemical treatment, that wood undergoes during pulping process. This stream was used to produce fermentable reducing sugars using enzymatic hydrolysis. Supplementation of β-glucosidase enzyme (1:0.5) reduced the time required for 35% conversion to 24 hours instead of 96 hours. The hydrolysate obtained for four days contained 41 g/l of glucose based on 81 % conversion of the cellulosic fraction in the primary sludge with β-glucosidase supplementation to a level of 1:2. The obtained hydrolysate was fermented to produce microbial lipids using oleaginous yeasts strains (*Cryptococcus curvatus*). The fatty acid compositions of microbial lipids produced were found to similar to vegetable oil. It thus can be used as a feedstock for the production biodiesel and other value-added products which in turn can add to the revenue of the industry.

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With immense gratitude, today, I submit this thesis, hopeful that it is a beginning of a new journey for me. This, without the support and encouragement of many would not have been possible. Foremost, I would like to thank the Almighty for giving me the goodwill, health and unwavering strength for the successful completion of my thesis.

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Abbreviation

PMS- Paper mill sludge

PPMS- Primary paper mill sludge

PMI- Paper mill industry

HPLC- High pressure liquid chromatography

GC- Gas chromatography

NREL- National renewable energy laboratory

FPU- Filter paper unit

BG- β-glucosidase

PT- Pretreatment

CC- *Cryptococcus curvatus*

CBH- Cellobiohydrolase

TG- Triglyceride

HMF- Hydroxymethylfurfural

DNSA- Dinitrosalicylic acid

ASL- Acid soluble lignin

AIL- Acid insoluble lignin

OD- Oven dry

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Chapter 1 : Introduction

Greenhouse gases such as carbon dioxide and methane have a severe effect on the environment. The resulting effects of global warming and climate change has led to major negotiations between various countries in the world (Balat, 2011). Climate mitigation efforts include substituting fossil resources by renewable bio-based products for the production of fuels and chemicals. At the moment, the energy contribution by the bio-sector is only 10-15 % of the total energy use. The possibility of producing chemicals and plastics from renewable resources is an important option (Chandra et al., 2012).

Biorefineries are being studied to produce bio-fuels and high demand platform chemicals (Succinic acid, levulinic acid, maleic acid, etc) from renewable resource (Dalli et al., 2017). The cost of the raw material to produce bio-based products is one of the important criteria for the feasibility of the project. It is very important to find substrate which are cheap, renewable and not a threat to food security. Hence, biorefineries research is focused on the use of lignocellulosic such as forest residues, agricultural and industrial waste. Using a waste material as a substrate not only improves the economics of the industry but also solves environmental issues related to its disposal.

The pulp and paper industry use wood as its raw material to produce newspapers, specialty paper, market pulp and wood products. For a very long-time paper mill industry has had considerable influence on the Canadian economy and its citizens (Pokhrel et al., 2004). Annually, around 31 million tons of cellulose pulp is produced. This accounted to revenues of $6 billion in 2012 (Pervaiz & Sain, 2015). Subsequently, huge quantity of wastewater and solid waste are generated and can potentially pollute lakes, air and soil (Pervaiz & Sain, 2015).

The second most important resource that is used in the in the paper Mill Industry (PMI) is water. Somewhere between 20,000 and 60,000 gallons of water per ton of product produced is used in the industry (Pokhrel et al., 2004). Kamali et al., (2016) has reported that around 88 % of the water

used in the industry is returned to its source after proper wastewater treatment, adhering to the guidelines set by local authorities. Currently, the activated sludge process is the most common system used by paper mill sludge for effluent treatment (Kamali et al., 2016). While processing the wastewater two different kinds of sludge, primary and secondary are produced (Faubert et al., 2016). Primary sludge is solid in nature and basically consists of short fibers, clay and filler materials (Alkasrawi et al., 2016). Since these solid residues are from pulping industry the fibrous material that is collected in the primary clarifier of the effluent treatment plant is highly cellulosic in nature. The cellulosic material has also undergone mechanical and chemical pretreatment and is relatively free of lignin (Chen et al., 2014a). The cellulose fraction in the sludge, which is as high as 50-80 % (dry basis), can be hydrolyzed using acid or enzymes to produce glucose. The monosaccharides obtained can be fermented using various microbes to produce different value-added product.

Microbial lipids have become a focus of considerable research in the bio-fuel industry. The lipids produced from sugars and wastes using oleaginous microorganism have similar properties to vegetable oil, which is mostly commonly used for the production of bio-diesel (Yu et al., 2011). Using a waste stream to produce sugars, and subsequently microbial oil, will not only end the food versus fuel debate but also make the bio-fuel production economical (Fei et al., 2016).

The overall objective of this work is to utilize the primary sludge from a pulp and paper mill and hydrolyze enzymatically to produce fermentable sugars and subsequently produce microbial lipids that can be converted to biodiesel easily.

The specific objectives of the study were:

i. To optimize conditions using commercial cellulase enzymes for the production of fermentable sugars from primary paper mill sludge.

ii. Determination of the correct ratio of cellulase and β- glucosidase enzyme for high yields and productivity of glucose syrups.

iii. Optimization of conditions for the fermentative production of microbial lipids from the cellulosic glucose syrups.

Chapter 2 : Literature review

2.1. Composition of Lignocellulosic Biomass

Lignocellulosic biomass is an amalgam of cellulose, hemicellulose and lignin, which are bound together, intricately, resulting in a complex structure. Other than these three-major components (Fig 2.1), these resources contain pectin, proteins, ash, salts and minerals (Van Dyk & pletschke, 2012). The complexity of the chemical structure makes the lignocellulosic biomass recalcitrant to depolymerization.

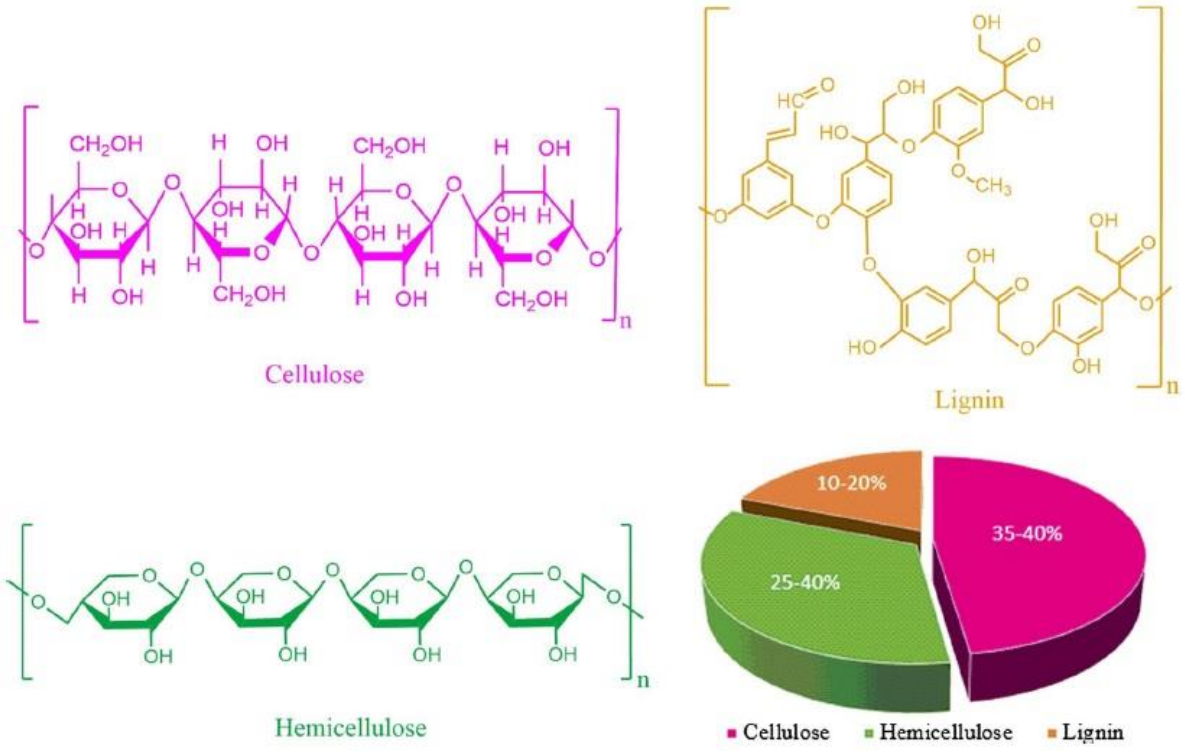

Figure 2.1:Composition of lignocellulosic Biomass (Amin et al., 2017)

Lignocellulose is the primary building block of plant cell walls. Kumar et al,(2009) reported that approximately 90 % of the total plant material(dry weight) consists of cellulose, hemicellulose, lignin and pectin. The composition of individual compounds varies from plant to plant as can be observed in Table 2.1. For example, the cellulose component can be as low as 6 % in swine waste or as high as 99 % in paper waste. However, in naturally occurring substances these distributions fall in a small range but have different chemical properties. Even the same lignocellulosic biomass

harvested in different batches have different compositions (Van Dyk & pletschke, 2012). The covalent and non-covalent linkages formed between the cellulose, hemicellulose and lignin in the plant cell wall, provides plants, strength and rigidity and protects it from microbial degradation (Kumar et al., 2009; Sun et al., 2016).

Table 2.1: Cellulose, hemicellulose and lignin content in common agricultural residue and wastes (Kumar et al.,2009)

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
grasses	25-40	35-50	10-30
paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal bermudagrass	25	35.7	6.4
Switchgrass	45	31.4	12
Swine waste	6.0	28	na

For producing biofuels, the polymeric carbohydrates (cellulose and hemicellulose) in the lignocellulose must be broken down to monomeric sugars, which can be converted to useful products by the microbial cultures by fermentation (Amin et al., 2017). The recalcitrant nature of the lignocellulose makes it difficult to break down and therefore must undergo various pretreatment. The objective of pretreatment is to expose the cellulose, so that it can hydrolyzed into monosaccharides (Kumar et al., 2009). This is one of the major bottlenecks to the use of such resources for biorefinery.

2.1.1 Cellulose

Cellulose is the most abundant organic polymer with a well-organized linear series structure of D-glucose units linked to each other by β-1-4 glycosidic bonds. These cellulose chains are further attached together by hydrogen and van der waals bonds. There are two types of cellulose polymers, crystalline and amorphous (Kumar et al., 2009). In the crystalline region of cellulose, the cellulose chains are bound together in parallel orientation in a compact manner, which makes it difficult to break down chemically or biologically. The amorphous part of cellulose has loosely bound cellulose series which are irregular in shape and are very easily hydrolysable (Sun et al., 2016; Van Dyk & Pletschke, 2012). Irrespective of its bonding with hemicellulose and lignin, the digestibility of cellulose is highly dependent on its crystallinity (Sun et al., 2016), which is not easy to digest.

2.1.2 Hemicellulose

Hemicellulose has a complex structure, consisting of both pentose (xylose and arabinose) and hexoses (mannose, glucose and galactose) sugars (Hendricks & Zeeman, 2009). Hemicellulose are sensitive to thermo-chemical reactions, making it easily hydrolysable at milder conditions(dilute-acid). The components of hemicellulose may vary from plants to plants. For example, xylan is more prominently found in hardwood and agricultural plants, as compared to glucomannan that are found in softwood (Hendricks & Zeeman, 2009). It also plays a role as a binding agent in the plant cell wall, binding the cellulose and lignin, resulting in a rigid structure capable of withstanding extreme weather conditions. Removal of hemicellulose is very important for increasing the porosity of lignocellulose, eventually improving the yield of enzyme hydrolysis of cellulose to sugars (Sun et al., 2016).

2.1.3 Lignin

Lignin is high molecular weight compound and a heterogenous polymer. The basic structure of lignin is made up of three phenyl propionic alcohols: coniferyl alcohol, coumaryl alcohol and sinapyl alcohol (figure 2.2). These phenolic monomers are linked to one another using alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds (Kumar et al., 2009). In the plant cell wall, it usually fills in the space between the carbohydrates and forms a protective layer, providing toughness and impermeability. Characteristics such as insolubility in water and optical inactivity, makes it difficult to degrade (Hendriks & Zeeman, 2009). Removal of lignin from the lignocellulosic biomass reduces the unwanted adsorption of enzymes on the lignin surface, as well as improves the pore size of the biomass (Sun et al., 2016). While providing the structure and resistance to plants in nature, this compound prevents the easy conversion of cellulose like polymers to useful chemicals.

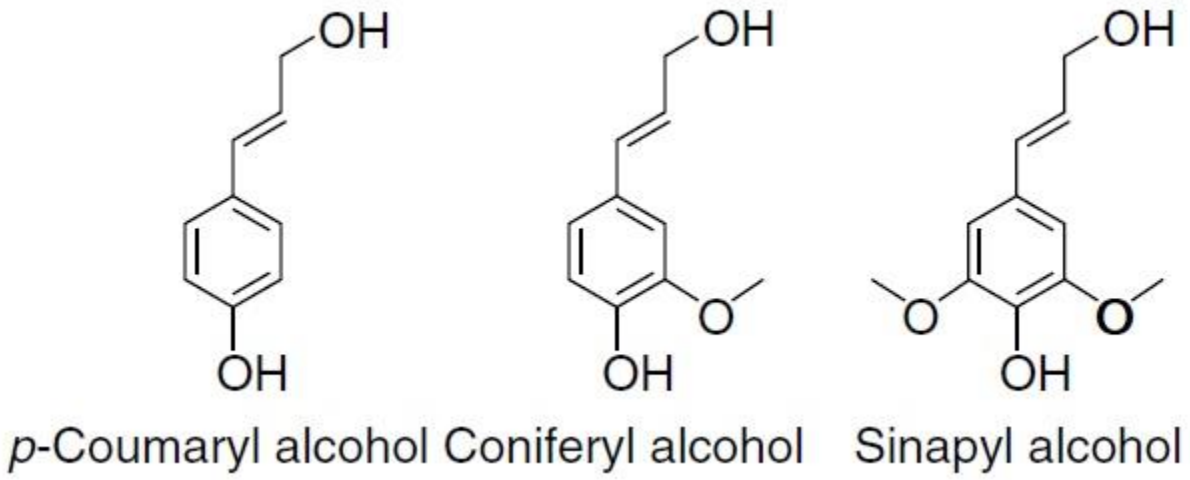

Figure 2.2: Basic three components of lignin (Meng & Ragauskas, 2014)

2.2 Paper mill sludge (PMS)

Paper mill sludge is a solid waste that is produced during the effluent treatment of pulp and paper mill industry. The Activated sludge process is the most common technique followed in the paper mill industry (PMI) for wastewater effluent treatment. As shown in figure 2.3, the wastewater first goes through the primary clarifier, where the larger size particles are retained, and the clear effluent goes to secondary clarifier for further biological treatment. The material that is retained in the primary clarifier is known as primary sludge and the sludge that is obtained after the biological degradation in the secondary clarifier is known as secondary sludge or biological sludge (Likon & trebse, 2012). The other two kinds of sludge that are found in the industry is mixed sludge, which is a mixture of different proportions of primary and secondary sludge, and de-inked sludge, which is produced in paper mill using recycled pull.

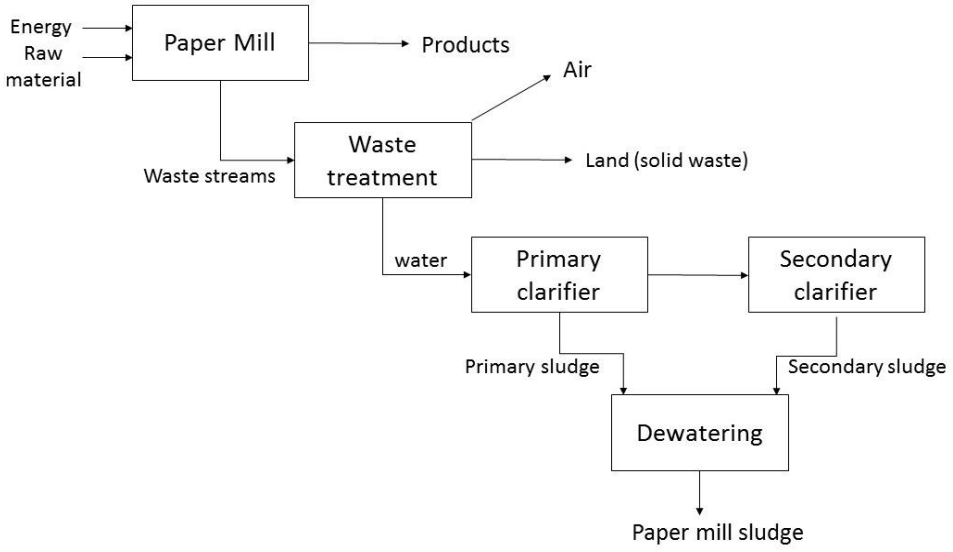

Figure 2.3: Schematic diagram of paper mill sludge

The Pulp and Paper Mill Sludge (PPMS) mainly consist of short fibers, clays and filler material, which adds on to the effluent stream throughout the paper making process (Chen et al.,2014b). The characteristics of the sludge is highly dependent on the raw materials and the technology used for processing of wood into pulp in the paper mill industry (Pervaiz & Sain, 2015; Kamali et al., 2016). The difference in their properties can be seen in Table 2.2. Paper mills which uses recycled pulp, tend to produce two to four times of sludge as compared to Virgin pulp mills. The ash content of sludge from recycled pulp is also high, due to the deinking step involved in the pulping process (Likon & Trebse, 2012).

Table 2.2 :Physical and chemical properties of a typical sludge (Faubert et al., 2016)

Parameter	Primary PPMS	Secondary PPMS	Mixed PPMS	De-inking PPMS
Dry matter (% FM[a])	15–57	1–47	19–60	32–63
Ash content (% dry solids)	10–15	10–20	20	40–60
Nitrogen (% DM[b])	0.045–0.28	1.1–7.7	0.7–3.6	0.15–1.0
Phosphorus (% DM)	0.01–0.06	0.25–2.8	0.22–0.74	0.0012–0.16
Potassium (% DM)	0.02–0.09	0.078–0.7	0.03–0.33	0.0029–0.2
C:N ratio	111:1–943:1	8:1–50:1	13:1–31:1	34:1–344:1
pH	5.0–11.0	6.0–8.5	3.8–8.1	7.2–9.2
Heating value (MJ kg^{-1} DM)	5.5	0–25	14–19	1.5–5.7

[a] Fresh matter.
[b] Dry matter.

The important factors that make PMS pivotal for the production of bio-fuels and other value-added product is its zero cost and absence of the need for pretreatment before further processing. Since the sludge is a waste stream of the paper mill industry, there is no cost associated with it. While dealing with hydrolysis of lignocellulosic biomass, pretreatment is a very crucial step, which separates the hemicellulose and cellulose from the lignin fraction, thereby making the carbohydrates more susceptible to enzymatic digestion (Mendes et. al., 2016). In case of PMS, during the paper making process the sludge undergoes various physical & chemical process, like cooking, bleaching and refining, resulting in fine fibrous material with high surface area. Hence,

pretreatment of this material is not necessary, reducing the overall cost of sludge hydrolysis process (Chen et al., 2014a).

The presence of high ash content and other filler material can be disadvantageous as it leads to inefficient enzymatic hydrolysis. Chen et al, (2014a) also reported that acid soluble ash like $CaCO_3$, not only act as a buffering agent but also adsorbs the cellulase enzyme with higher affinity as compared to cellulose fibers and helps in reducing enzyme loading capacity.

2.2.1 Primary Sludge

The sludge which is produced by sedimentation or dissolved air floatation in a primary clarifier of paper mill effluent treatment plant is known as primary sludge. They are highly fibrous in nature and contain 1.5 to 6.5 % solids depending on the methodologies followed in the paper mill. The carbon to nitrogen ratio is very high in primary sludge, as most of the nitrogen material is passed on to the secondary tank for biological treatment (Pervaiz et al., 2015). The average ratio of primary sludge to secondary sludge in a Canadian paper mill is 70:30 and could differ in different paper mills (Faubert et al., 2016). The high fibrous nature of primary sludge is attributed to the inefficient separation of cellulose pulp from water during the pulping process (Chen et al., 2014b). PPMS is a potential feedstock for the biorefining industry because of two reasons: (1) Huge quantity of sludge produced (2) Low lignin content, no pretreatment of lignocellulose required.

Table 2.3 :Comparison of composition analysis of primary paper mill sludge.

	Components (%)				
Sr. no	Cellulose	Hemicellulose	Lignin	Ash	Reference
1	52.17	7.02	20.92	6.53	Li et al., 2015
2	60.8	14.2	8.4	10.7	Peng & chen., 2011
3	32.8	13.9	14.6	NM	Deeba et al., 2016
4	44	11	NM	NM	Prasetyo et al., 2011
5	23.6	4.9	5.36	38.3	Zhu et al., 2011
6	58.6		2.2	39.2	Carvalho et al., 2011
7	60.4		4.8	34.8	Mendes et al., 2016

2.2.2 Secondary Sludge

After the primary treatment, clear effluent with dissolved solids enters the secondary tank for biological degradation. In the secondary tank, the microorganisms digest the suspended and dissolved solids present, thereby reducing the biological oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) of the wastewater effluent. Microbial biomass and cell debris produced during this process settles in the secondary clarifier and is known as secondary sludge (Kamali et al., 2016). Dewatering of secondary sludge is a important issue that is faced by paper mill industry. Using the primary sludge as a thickening agent for secondary sludge is the most common practice that is followed in the industry before combustion to produce energy. The quantity of secondary sludge produced is lower compared to primary sludge as all the fibrous cellulosic material is separated in the primary clarifier (Faubert et al., 2016).

2.2.3 De-inked sludge

Paper mills that use recycled paper produces de-inked sludge. It is a complex mixture of organic and inorganic solids, as well as the chemical additives which gets included during the de-inking process (Abdullah et al.,2015). As compared to primary and secondary sludge, the de-inked sludge is less fibrous and contains high quantities of heavy metal, making it less desirable in fields of land application (Likon & Trebse, 2012). A further treatment of de-inked sludge is required due to its high ash and heavy metal content before disposal, which adds as an economical burden on the PMI (Pervaiz et al., 2015). Generally, pretreatment is not required for PPMS for enzymatic hydrolysis, but, de-inked sludge requires an additional step of deashing for efficient hydrolysis (Kang et al., 2011).

2.3 Hydrolysis of lignocellulosic material

The main purpose of introducing a pretreatment in a biorefinery industry, is to distort the complex and recalcitrant structure of lignocellulose. A good pretreatment technique will not only change the physical and chemical structure, but also efficiently separate all three fractions (Cellulose, hemicellulose & lignin) of lignocellulose, as shown in the Figure 2.4. This separation helps, enzyme or acids, get more access to the long-chain oligosaccharide for further fractionation into monosaccharides, during the hydrolysis (Kumar et al., 2009). A pretreatment step enhances the conversion of cellulose to glucose and some studies have also reported conversions to 90% of theoretical yield (Mosier et al., 2005). There are four different categories of pretreatment that is followed in research, each has its own effect and is applied on the basis of structural and compositional analysis of the lignocellulosic biomass (Sun & Cheng, 2002). The four categories of pretreatment are: (1) Physical, (2) Physio-chemical, (3) chemical and (4) Biological (Fig. 2.5). Each of these methods have their own advantages and dis-advantages (Table 2.4).

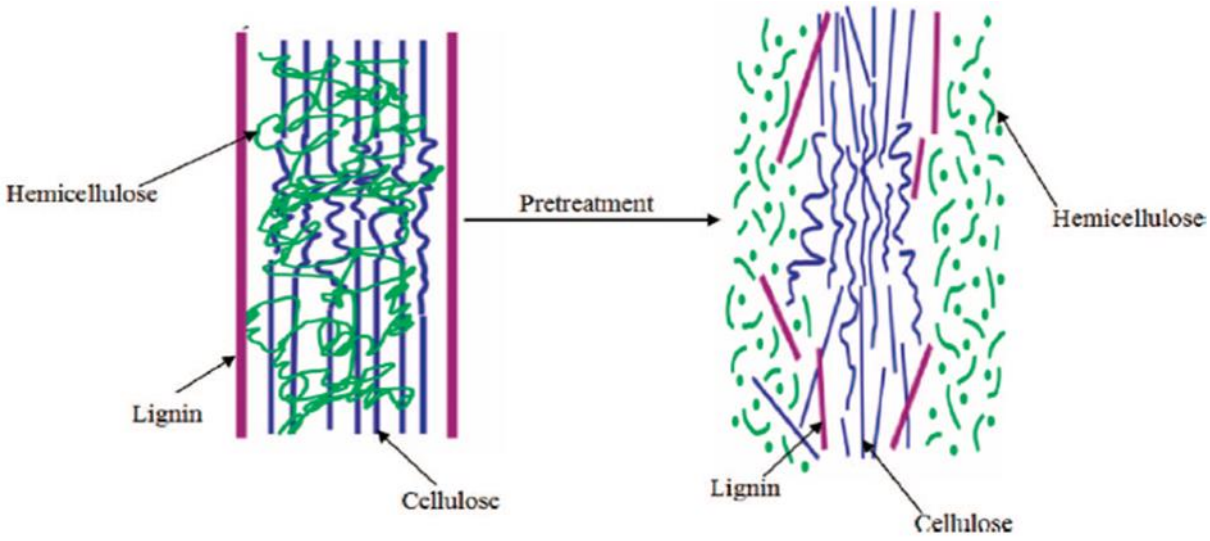

Figure 2.4: Effect of pretreatment on lignocellulosic biomass (Kumar et al., 2009)

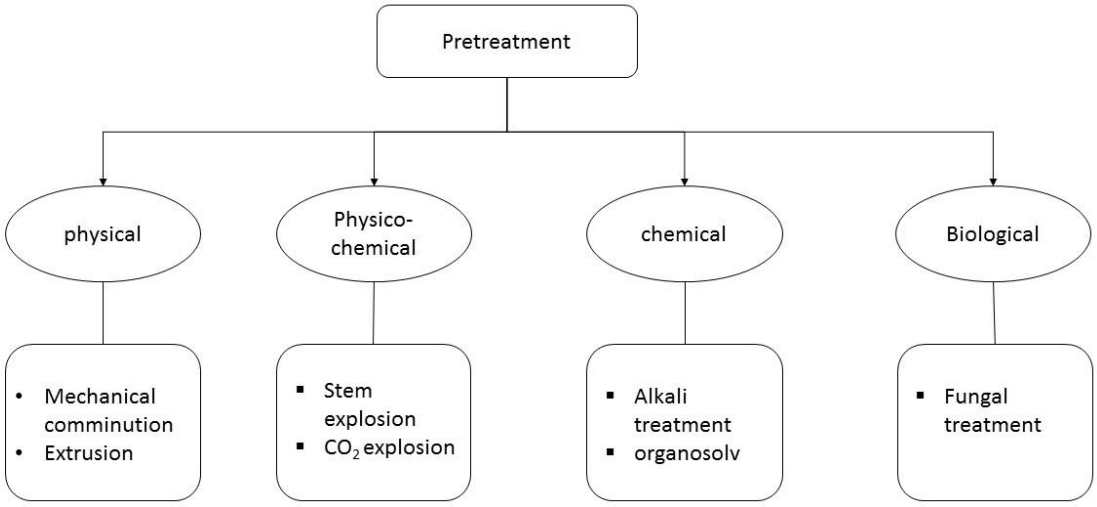

Figure 2.5 : Types of pretreatment techniques used for lignocellulosic material

Table 2.4: Advantages and disadvantages of various pretreatment process (Kumar et al.,2009; Alvira et al., 2010; Sun et al., 2016)

Pretreatment methods	Advantage	Disadvantage
Mechanical comminution	Reduces cellulose crystallinity	High energy input
Extrusion	High continuous throughput	Expensive equipment's
Steam explosion	Causes hemicellulose degradation and lignin transformation	Generation of toxic compounds
CO_2 explosion	Increases accessible surface area	High pressure requirement
Alkali treatment	Simple procedure	Irrecoverable salts formed and incorporated into biomass
Organosolv	Separation and recovery of high quality lignin	High price of organic solvent
Biological treatment	Degrades lignin and hemicellulose Low energy requirement	Rate of hydrolysis is very low

2.3.1 Pretreatment techniques for lignocellulosic biomass

2.3.1.1 Physical pretreatment

2.3.1.1.1 Mechanical comminution

The objective behind using mechanical comminution is to fractionate the lignocellulose and reduce its size. The shredding of lignocellulosic biomass not only reduces the crystallinity of cellulose but also increases the specific surface area. The degree of polymerization is also reduced significantly by fractionation. The reduction in size improves the enzyme digestibility as the rate of enzyme substrate binding increases. There are combination of steps, such as chipping, grinding or milling, which are incorporated as pretreatment for downsizing of long chain polymers. The selection of above steps are based on the final particle size required. The particle size usually observed after chipping is around 10-30 mm and 0.2-2 mm after milling or grinding (Alvira et al., 2010). Taherzadeh & Karimi, (2008) also reported that, using different milling techniques, such as ball, hammer, colloid and vibro energy milling, improved the sugars produced after the enzymatic hydrolysis. Even though the production of inhibitory compounds is less as compared to other

pretreatment methods, the energy input is really high making it economically not feasible (Alvira et al., 2010).

2.3.1.1.2 Extrusion

In the extrusion process the raw materials are injected into the extruder with a screw driven setup, compression in the middle and expansion at the end (Alvira et al., 2010). The compression, expansion and the heat generated due to friction, all these lead to depolymerization of lignocellulosic material. The parameters that are considered using extrusion are reaction time, pressure and biomass dry matter. Excessive heat generated during the extrusion process can also have a negative effect such as degradation of sugars and amino acid (Zheng et al., 2014). Chang & El-Dash (2003) reported 6.8 % higher ethanol production, while using extrusion as pretreatment for cassava starch.

2.3.1.2 Physico-chemical treatment

2.3.1.2.1 Steam explosion

Steam explosion is a proven technique that has been widely used for the ethanol production from various agricultural residues (poplar, corn stover, etc) (Alvira et al., 2010). Basic procedure for steam explosion is to steam the biomass in a huge vessel at high temperature (upto 240 ^{o}C) and pressure (Hendriks & Zeeman, 2009). High temperature enables the removal of hemicellulose and degrade the polymeric lignin. There are some acids which are formed during the steam explosion process, Acetic acid, from the acetyl groups that are attached to the hemicellulose. Levulinic and formic acid, derivatives of HMF and furfural, are also formed in the hydrolysate due to the excess heat in the reactor (Alvira et al., 2010). Hendriks & Zeeman, (2009), reported few steps to avoid formation of inhibitory products: (1) removal of condensate during pretreatment, (2) adjusting the pH between 5 and 7, (3) using a two-step pretreatment. There are two types of steam explosion, catalyzed and catalyzed (Zheng et al., 2014; Mosier et al., 2005). In catalyzed steam explosion,

acids or base are used to enhance the carbohydrate separation and to reduce the severity of reaction conditions (temperature and time). The most common catalyst that are used for these kind of reactions are H_2SO_4 , SO_2, H_3PO_4 and NaOH (Zheng et al., 2014). In uncatalyzed steam explosion, the hydrolysis takes place in absence of any chemical (Mosier et al., 2005).

2.3.1.2.2 CO_2 explosion

The idea behind CO_2 explosion is same as the steam explosion, only CO_2 is used instead of water. The idea behind using a supercritical fluid (CO_2) was to reduce the temperature, so that there is no degradation of monomeric sugars (Kumar et al., 2009). It is also reported in few studies that CO_2 when dissolved in water form carbonic acid, which acts as a catalyst and enhances the hydrolysis rate (Sun & cheng, 2002). Though Sugar yields are less as compared to other explosion techniques, it requires less operation cost and the sugar yields obtained are better than the sample without pretreatment (Alvira et al., 2010).

2.3.1.3 Chemical pretreatment

2.3.1.3.1 Alkali treatment

The objective behind Alkali treatment is to remove the lignin and the hemicellulose from the lignocellulosic biomass. The ester linkages between the hemicellulose and lignin gets saponified, which increases the porosity and digestibility of the lignocellulosic biomass (Sun et al., 2016). There is minimal solubilization of sugar and production of inhibitory compounds, which is advantageous as compared to other pretreatment methodologies (Alvira et al., 2010). Sodium hydroxide (NaOH), potassium hydroxide (KOH) and calcium hydroxide ($Ca(OH)_2$) are most commonly used agents for alkaline treatment (Singh et al., 2014). It is cost effective and also uses less severe conditions as compared to other pretreatment methods. NaOH and KOH are frequently used alkaline agents, despite of their limitations associated with its reusability and effect on the environmental (Amin et al., 2017). Using lime instead, have found to be an efficient approach, as

calcium carbonate, solid state, can be recovered and reused by neutralizing the hydrolysate using cheap CO_2 (Badiei st al., 2013).

2.3.1.3.2 Organosolv

Organosolv pretreatment works on the principle of using a organic solvent with inorganic acid catalyst (H_2SO_4 or HCL), for delinking of hemicellulose and lignin bonds (Kumar et al., 2009). Addition of acid catalyst is optional, depending on the yield of xylose required. For delignification addition of catalyst is unnecessary, beyond high temperatures (> 185 ^oC) (Sun & cheng, 2002). The most frequently used solvent for organosolvation is ethanol, methanol, acetone, ethylene glycol and tetrahydrofurfuryl (Alvira et al., 2010), ethanol being the most favored one because of its low toxicity and easy recovery (Sun et al., 2016). The recovery of organic solvents are necessary for two reasons; the presence of organic solvent can inhibit microbial fermentation and recovery and reuse of solvents would make the process cost effective (Badiei et al., 2013). A generally preferred organosolvation conditions are as follows: temperature of 180-190 ^oC, Cooking time of 30-90 min, ethanol concentration of 35-70 %(w/w), a liquid to solid ratio of 4:1 to 10:1 (w/w) and pH between 2.0 to 3.8 (Kumar et al., 2009).

2.3.1.4 Biological treatment

Biological pretreatment is safe as compared to other pretreatment techniques. It is ecofriendly and also uses less energy, thereby making it highly suggested 'cost effective' method for delignification (Kumar et al., 2009). However, the long reaction time and huge space requirement has limited its use in the industry. It uses various microorganisms, mostly fungi (white-, brown- and soft-rot), for the degradation of lignin and hemicellulose from the lignocellulosic biomass, thereby making more accessible space for enzymes to depolymerize cellulose. White-rot fungi are predominantly used because of its ability to produce lignin- degrading enzymes such as peroxidase and laccase (Kumar et al., 2009). *Phanerochaete crysosporium*, *Ceriporia lacerata*, *Cyathus*

stercoletus, Ceriporiopsis subvermispora, *pycnoporus cinnabarinus* and *pleurotus ostreatus* are the various white-rot fungi that are used for the lignocellulosic biomass (Sun et al., 2016).

2.3.2 Acid Hydrolysis

Acid hydrolysis can be used as a pretreatment or the only hydrolysis technique to produce fermentable sugars from complex lignocellulosic biomass. Acid hydrolysis is considered to be advantageous because of its ability to penetrate through lignin and attack the holocellulose (Cellulose and hemicellulose) to produce monosaccharides (Verardi et al., 2012). There are few studies that have reported using Sulfuric acid, hydrochloric acid, phosphoric acid and nitric acid for the hydrolysis of lignocellulosic biomass (Singh et al., 2014). Organic acid such as acetic, formic, fumaric, propionic, maleic and oxalic acids have also been used by itself or in combination with inorganic acid for acid hydrolysis (Sun et al., 2016).

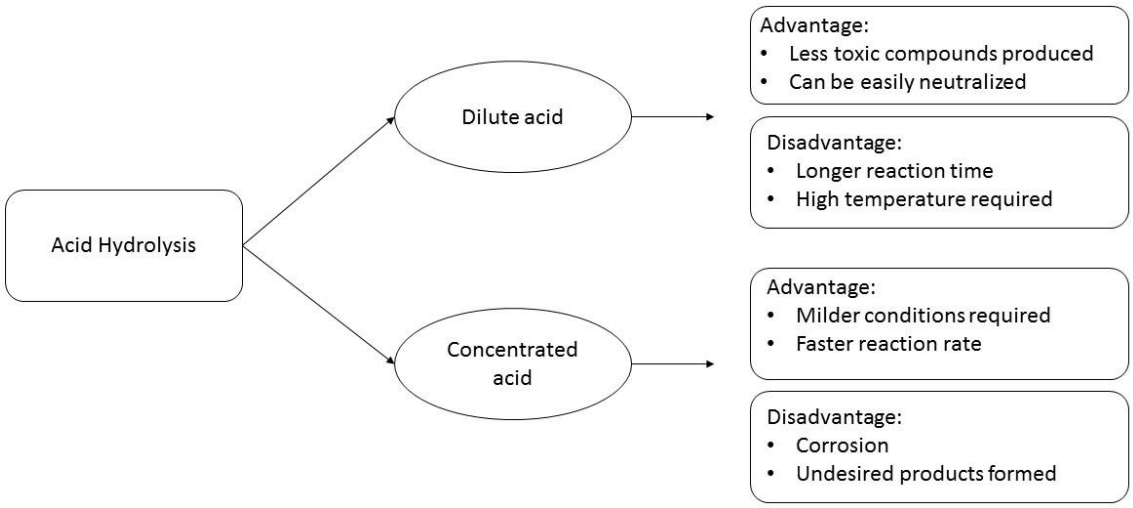

Figure 2.6: Advantages and disadvantages of acid hydrolysis

There are two types of acid hydrolysis; (1) Dilute acid, (2) Concentrated acid. Selection of these hydrolysis techniques depends on the composition of lignocellulosic biomass and the yield of sugar required. For example, dilute acid hydrolysis is used for the extraction of xylose, as xylans are

more prone to depolymerize to xylose at milder conditions as compared to cellulose or lignin (Kumar et al., 2009). For cellulose to glucose conversion, a two-step acid hydrolysis process is followed. In the first step, milder conditions are used to hydrolyze hemicellulose and in the following step, more severe conditions are used to convert rigid cellulose to glucose (Verardi et al., 2012). By following two step hydrolysis technique, the degradation of sugar to toxic inhibitory compounds are avoided, thereby improving the overall sugar yield. Dilute acid hydrolysis uses high temperatures, 160-230 ^{o}C, as compared to concentrated acid hydrolysis (< 50 ^{o}C). The acid concentration also varies from 2-5 % for dilute acid hydrolysis to 30 % for concentrated acid hydrolysis (Kumar et al., 2009). Even though it has many advantages, there are few disadvantages which also needs to dealt with. Even though concentrated acid is highly efficient in converting cellulose to glucose, it dangerous, corrosive and produce toxic inhibitory compounds during hydrolysis. To avoid corrosion issues, expensive non-metallic materials or alloys needs to be used, which makes the pretreatment process high-cost (Zheng et al., 2014).

An additional step of detoxifications needs to be added, to eliminate the inhibitory compounds to make the hydrolysate amenable for fermentation. Overliming and adsorption using activated carbon are the most common types of detoxification that is followed by various researchers. Using calcium hydroxide ($Ca(OH)_2$) for overliming (pH 9-10), have been reported to improve the fermentability of the hydrolysate by reducing the amount of toxins present (Martinez et al., 2001). Low cost of lime makes overliming preferred choice, as it reduces the over-all cost of the process. Activated carbon is well known for its uses as an adsorbent because of its large surface area. When it is mixed in a hydrolysate containing undesired phenolics, it binds together and later separated using various filtration techniques (Seo et al., 2009). The selection of detoxification technique

depends on the type of inhibitory compound present in the hydrolysate, as each pretreatment has different effect on the inhibitory compound (Mussatto & Roberto, 2004).

2.3.3 Enzymatic Hydrolysis

Cellulose is a complex polymer containing 1000 to 1 million D- glucose units, linked by β-1,4 glycosidic bonds. Using enzymes to break cellulose is reported to be more effective than inorganic acids because of its specific nature and less milder conditions (pH & temperature) required for hydrolysis (Verardi et al., 2012). There are few parameters, such as pH, temperature, solid loading and enzyme loading, that needs to consider before running an enzymatic hydrolysis reaction. The activity of enzyme depends on its purity and could vary by huge margin. The difference in enzyme activity of cellulase enzyme could be observed from table 2.5. Cellulase is the enzyme which is used for enzymatic breakdown of cellulose(insoluble) into soluble glucose molecules which can be further used for fermentation. Cellulase enzyme is itself a multi-enzyme, containing different enzymes carrying out different activities. Economic feasibility of a biofuel producing plant depends on high sugar yield from enzymatic hydrolysis of biomass (Zhang et al.,2013). Despite all its advantage, high cost of enzyme and low reaction rate are few challenges which are faced by the scientists.

2.3.3.1 Cellulase enzyme

Cellulase is a complex enzyme consisting of two or more enzymes, working together synergistically. It mainly consists of three types of enzymes, endogluconase, exogluconase also known as cellobiohydrolase and β-glucosidase. Each enzyme has different role to play in the enzyme reaction.

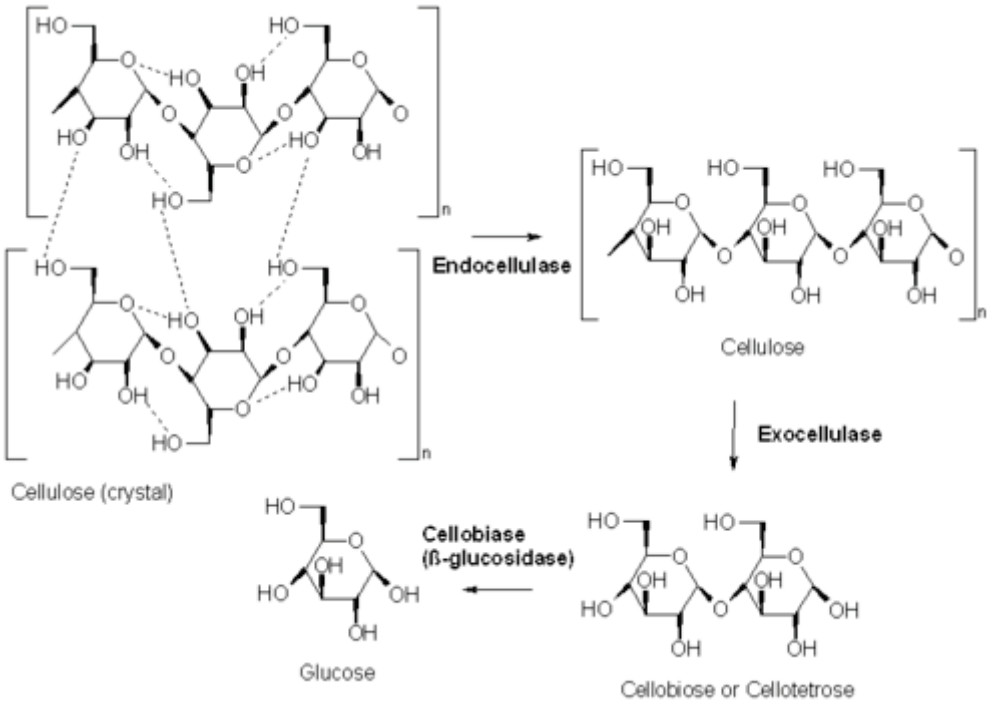

Figure 2.7: Schematic representation of cellulose breakdown by endocellulase, exocellulase or cellobiohydrolase (CBH) and β- glucosidase. (Wikipedia, 2018)

For example, endoglucanase randomly cleaves along the length and attacks the low crystalline region of a cellulose chain, which leads to rapid depolymerization. Endogluconase attack reduces the strength of fibrous material (Teeri, 1997). Exogluconase or cellobiohydrolase cleaves from the ends of cellulose chain. It attacks the crystalline region of cellulose and breaks the β-1,4 glycosidic bonds forming cellobiose in the hydrolysate. β- glucosidase converts the cellobiose to glucose residues by cleaving the β-bonds (Verardi et al., 2012; teeri, 1997). Other than these three common enzymes, there few enzymes present such as xylanase, acetylesterase, β- xylosidase, galactomannase and glucomannase, which acts on the hemicellulose (verardi et al., 2012)

2.3.3.2 Factors affecting enzymatic hydrolysis

The digestibility of biomass depends on the combination of factors such as composition of substrate, enzyme dosage, pretreatment applied and the efficiency of enzyme (Alvira et al., 2010). The factors that affect the enzymatic hydrolysis of cellulose can be classified into two type,

substrate related, and enzyme related (Leu & Zhu, 2013). The parameters that are associated with the substrate (Cellulose) are degree of polymerization, crystallinity, porosity, particle size or available surface area. The presence of lignin and hemicellulose could also reduce the efficiency of enzyme reaction by producing unwanted binding sites (Alvira et al., 2010).

The inhibitors, as shown in figure 2.8 produced during pretreatment process also has a severe effect on the hydrolysis and the subsequent fermentation process (Palmqvist et al., 1996). The enzyme could also undergo product inhibition, i.e. the production of cellobiose and glucose during the enzyme hydrolysis inhibits the activity of cellulase enzyme, thereby reducing the rate of reaction. Furthermore, enzymes are sensitive to pH and temperature of the reaction, which influences its ability to bind carbohydrates.

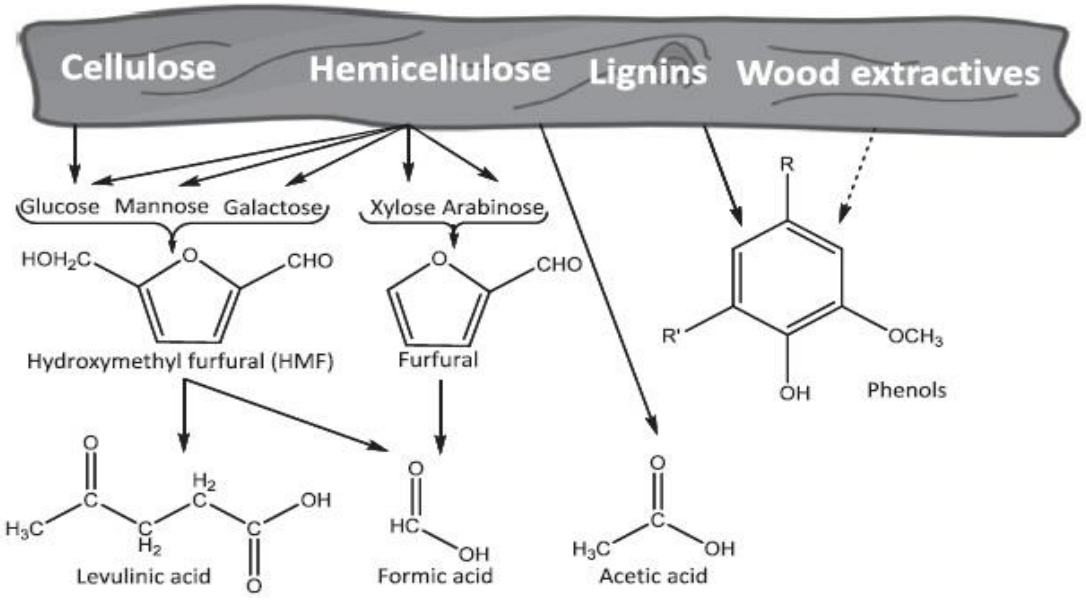

Figure 2.8 : Formation of inhibitors (Jonsson et al., 2013)

2.4 Hydrolysis of paper mill sludge

For the utilization of the carbohydrates present in the primary paper mill sludge, it has to undergo a hydrolysis step. For any bio-based product formation, enzymatic hydrolysis is considered better compared to acidic corrosive hydrolysis, as it can be used simultaneously with fermentations process. The other advantages of using enzymes is its specific action for sugar production and its ability to produce toxin free hydrolysate. Acid hydrolysis is only used for the composition analysis of the biomass as proposed by the National renewable energy laboratory (NREL).

Most of the research work that has been done on enzymatic hydrolysis of PMS is for the production of second generation ethanol (Yamashita et al., 2010; Fan et al., 2003; Chen et al., 2014b; Gurram et al., 2015). Few studies have also been done on the enzymatic production of lactic acid and butanol (Guan et al., 2016; Shi et al., 2015; Marques et al., 2008).

The cellulosic nature of paper mill sludge makes cellulase enzymes the most commonly used biocatalyst for enzymatic breakdown. The cellulolytic enzymes that are used in the industry include Cellic cTec2, Cellic HTec2, Accelerase 1500, Celluclast and Meicelase. These enzymes also have a small fraction of β-glucosidase and hemicellulase, which works synergistically to produce sugars. High sensitivity to parameters such as temperature and pH makes it very important to optimize the enzymatic hydrolysis conditions. As can be observed from the table 2.5, the optimum pH and temperature range for cellulase enzymes lie between 4-5 and 40-50 ^{o}C respectively. Other important parameters that are involved are solid loading and enzyme loading. The enzyme loading of an experiment is highly dependent on the activity of the enzyme and substrate concentration used in the reaction.

The lignocellulosic composition of the paper mill sludge is highly dependent on the processes followed in the paper mill. As can be observed from table 2.5, the cellulose fraction of the paper

mill sludge can vary from 25 to 75 % on a dry weight basis (Zhu et al., 2011). In general, the PMS does not require any pretreatment for its enzymatic hydrolysis, as it undergoes a rigorous treatment in the pulping processes. However, ash content present in the paper mill sludge is highly variable and comes from the filler material used in the pulping process. It also has a huge impact on the enzymatic hydrolysis and so a pretreatment to reduce the ash content is required for an efficient enzymatic hydrolysis (Kang et al., 2011).

Table 2.5: Comparison of enzymatic hydrolysis conditions for paper mill sludge

Sr no	Enzyme name	Solid loading	Enzyme loading	Temp (^{o}C)	pH	Time (hrs)	Glucose	Reference
1	Cellic cTec2 (113.8 FPU)	20 %	3.4%	50	5	72	51-77 g/l	Gurram et al., 2015
2	Cellic cTec2 (136 FPU) Cellic HTec2 (Mxied 9:1)	5 %	2,4,8 FPU/OD g sludge	50	4.8	48	40-50 % Conversion	Chen et al., 2014b
3	Celluclast Cellobiose enzyme	5 %	18 FPU/ g cellulose	50	4.8	96	82.57 % Conversion	Li et al., 2015
4	Accelerase 1500 (77 FPU/ml) GC220 (166FPU)	5 %	5-90 µL/ g	50	5.5	48	Control- 40% PT – 80 %	Alkasrawi et al., 2016
5	Ctec2 200.7 FPU/ml	8.3% consistency	35 FPU/ CH	50	5	NM	50 g/l CH	Mendes et al., 2016
6	Ctec(84FPU) & optimase CX (46 FPU)	5 %	Ctec (0.1%) OCX (0.18%)	50	4.8	50	12000 ppm	Banerjee., 2011
7	Spezyme Cp (59 FPU) Novozyme 188 (750CBU/ml)	3&6 % Glucan	Scp (15FPu) Novo 30 CBU/ g glucan	37	4.8	72	2.5 g/l	Kang et al., 2010
8	Celluclast 1.5L 12.54 FPU/ml Novozyme™ 188 26.42 CBU/ml	2 % CH	Celluclast (15FPU/g cellulose) Novozyme (30 CBU/ g cellulose)	40	5	72	15% sugars	Zhu et al., 2011
9	meicelase	5 %	Meicelase 20FPU/g	45	5	48	146 mg sugars /g sludge WO PT 445 mg/g	Yamashita et al., 2009

The adsorption of enzyme onto the ash can be averted by introducing a de-ashing method. The most common procedure to reduce the ash is by floating and screening, in which the sludge is first suspended in distilled water and then passed through a 100-screen mesh (Kang et al., 2011; Alkasrawi et al., 2016). Kang et al., (2011) also reported a 30 % reduction in enzyme dosage after

the de-ashing of the sludge. Treating sludge with 1 M HCL overnight for removal of calcium carbonate, a major contributor to ash content, is another technique that has been used for deashing, (Gurram et al.,2015). Fractionation of sludge before enzymatic hydrolysis has also found positive results as it improves the enzyme accessibility to cellulose fibers (Chen et al.,2014a; Chen et al., 2014b; Kang et al., 2010). Pretreating PMS with hydrogen peroxide has also been found effective, improving the hydrolysis yield by 25 % (g glucose/ g cellulose) (Gurram et al., 2015).

For the enzymatic hydrolysis of cellulose to be economically feasible, it is necessary to conduct the hydrolysis with high solid concentration. However, higher solid concentration leads to high concentration of products such as glucose and cellobiose. Product inhibitions is an important challenge that is faced by researcher dealing with cellulase enzyme. Adding β-glucosidase to the cellulase enzyme mixture is also being actively studied to overcome the feedback inhibition caused by accumulation of cellobiose, and also to improve the efficiency of cellulase enzyme hydrolysis (Teugias & Valjamae, 2013).

The optimum ratio of the cellulase enzyme complex components endoglucanase and β-glucosidase has been found to be sub-optimal. Kang et al, (2010) used β-glucosidase as a supplement in their study conducted on paper mill sludge to ethanol using simultaneous saccharification and fermentation cellulase enzyme. Even though the effect of β-glucosidase was not studied separately, addition of enzymes enhanced the yields and mitigated the inhibitions caused by cellobiose. Immobilized β-glucosidase has also been used as a supplement for hydrolysis of lignocellulosic biomass. Immobilization of the enzyme not only improved its thermal properties but also enhanced the yields 2-fold (Borges et al.,2014; Tu et al., 2006).

2.5 Microbial oil fermentation

Extensive research is being carried out on the production of microbial lipids due to its functional value and use as a substitute lipid feedstock for biodiesel production (Huang et al., 2013). Various microorganisms belonging to genera of algae, bacteria, yeast and fungi have to ability to produce lipids in appropriate conditions. The ability to grow at faster rate and to accumulate high lipid content makes it advantageous to use oleaginous yeast for lipid production. Few yeast species such as *Cryptococcus sp., Lipomyces sp., Rhodosporidium sp*. and *Rhodotorula sp*., has ability to produce lipids more than 60 %, when glucose is used as its main carbon source. However, only 5 % of yeast sp. have been reported to produce lipids over 25 % (Leiva-Candia et al., 2014). The other advantages of using a yeast are, independent on climate conditions, unlike microalgae, their ability to use diverse sugars as carbon source, ability to withstand high concentration of metals ions and low oxygen demand. Moreover, yeast cells could be easily harvested as compared to bacteria due to their large cell size (Qin et al., 2017). Santos & Reis (2014) also reported about the potential of yeast to be easily modified genetically as compared to another microorganism.

The presence of nitrogen in the fermentation media, plays an important role in production of microbial lipids. Oleaginous microorganism has the tendency to produce lipids under nitrogen limited conditions. Usually the nitrogen sources are used for producing the proteins and nucleic acid, while the sugars are used to provide energy and anabolic activities, producing carbohydrates, proteins, nucleic acid and lipids. In absence of nitrogen, the growth rate of microorganism slows down, and the protein and nucleic acid are no longer produced. The carbohydrates are then directed towards lipid synthesis, thereby accumulating triglycerides within the cells (Amaretti et al., 2010).

The economic analysis study conducted by Koutinas et al, (2014) found production of microbial oil from pure glucose as an expensive process. It was also reported in the same study that, for microbial oil to be used in the biodiesel production, the cost of the microbial oil had to be reduced by 50 % in the next decade. Thus, making it very important to find cheap or 'no cost' substrates for the production for microbial oil. Reducing the cost of substrate will significantly reduce the cost of biodiesel.

The search for a cheap substrate has led to ardent research for utilizing waste lignocellulosic material. The zero-cost associated with agricultural or industrial lignocellulosic waste makes it advantageous for using these kinds of material. The lignocellulosic biomass undergoes acid or enzymatic hydrolysis for breaking into simple monomeric sugars, so that it could be easily utilized by the oleaginous microorganism. The hydrolysate mainly contains hexose and pentose sugars, which are utilized by the oleaginous microorganism to produce lipids. Most microorganism prefer glucose over any other kind of sugar present in the hydrolysate. Concurrent utilization of pentose sugars during fermentation is rarely reported (Patel et al., 2016). The carbon catabolite repressions mechanism present in microorganisms, makes them utilize the sugars in a sequential manner (glucose being the first and other sugars later). However, few studies conducted on oleaginous yeast strains, *Rhodosporidium toruloides* and *R. glutinis* have reported on simultaneous utilization of pentose and hexose sugar from the lignocellulosic hydrolysate (Yen et al., 2015).

Cryptococcus curvatus, deposited under the name Candida curvata D at the American type culture collection (ATCC 20509) is also published by names such as *Apriotrichum curvatum*, *Trichosporon cutaneum* and *Trichosporon oleaginosus* (Bracharz et al., 2017).

Table 2.6: Lipid production by oleaginous microorganism using 'low cost' waste substrate

Microbial strain	Substrate	Biomass (g/l)	Hydrolysate Sugar concentration (g/l)	Lipid content (%)	Lipid concentration (g/l)	Lipid Yield (g/g)	Reference
C. curvatus	Wheat straw hydrolysate	15.6	29.2	27.1	4.2	0.14	Yu et al., 2011
R. toruloides	Wheat straw hydrolysate	9.9	29.2	24.6	2.4	0.08	Yu et al., 2011
C. curvatus	Office paper hydrolysate	17.3	49.2	52.5	9.1	0.18	Zhou et al., 2017
T. dermatis	Corncorb	24.4	60.1	40.1	9.8	0.16	Huang et al., 2012
Y. lipolytica	Industrial fats	8.7	10	44.0	3.8	0.38	Papanikolaou et al.,2001
L. starkeyi	Sewage sludge	9.4	NM	68	6.4	NM	Angerbauer et al., 2008
C. curvatus	Corncorb	9.4	40	63.5	6.0	0.15	Chang et al., 2013
T. cutaneum	Elephant grass	22.7	34.6	24.0	5.46	0.15	Chen et al., 2016

It has been known to grow on variety of complex substrate consisting of both pentose and hexose sugars. Moreover, it also possesses the ability to accumulate lipids (> 60 %) in presence of fermentation inhibitors. Lipid production and resistance towards inhibitors such hydroxymethylfurfural (HMF) and furfural present in the non-detoxified hydrolysate have been reported by Yu et al,(2011). At concentration of 1 g/l of HMF and furfural is has shown to impair its growth (Yu et al., 2014a). This study was conducted using *Cryptococcus curvatus* on primary paper mill sludge hydrolysate produced by enzymatic hydrolysis.

Various waste sources containing organic and inorganic material from industrial and agricultural sources have been investigated for production of lipids (Fig 2.9). The solid resides from the industrial or agricultural procedures have been reported to have a good amount of cellulose and hemicellulose content, with a simple introduction of acid or enzymatic hydrolysis, monomeric sugars can be produced which can be further utilized for lipid production.

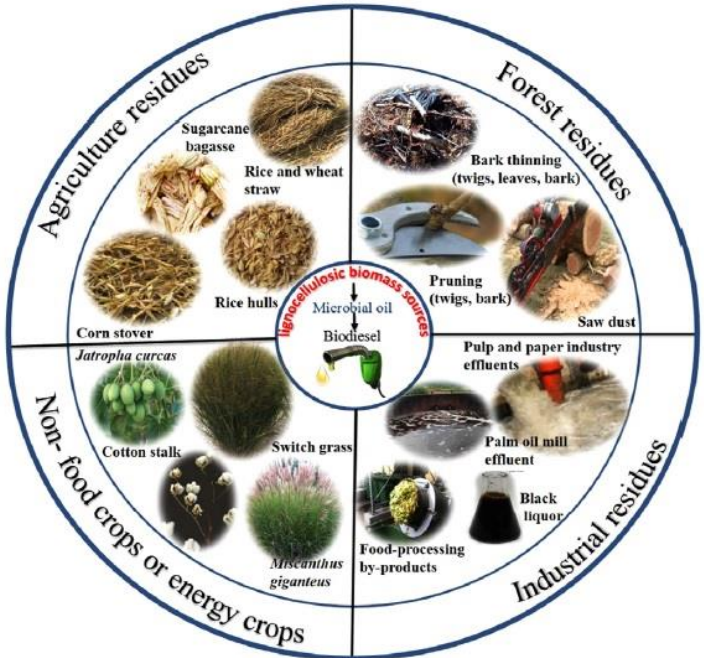

Figure 2.9: Types of Lignocellulosic biomass used for the cultivation of microbial lipids (Patel et al., 2016).

. Generally, waste treatment and disposal are costly. This can be reduced by utilizing these streams as a substrate for microbial oil production. The common types of agricultural resides that have been studied for lipid production include corn stover, sugarcane bagasse, wheat and rice straws (Demirbas, 2008). Other woody biomass such as switch and elephant grass has also been reported (Patel et al., 2016; Chen et al., 2016). Crude glycerol (containing 80 % glycerol, 10 % water, 7 % ash and 1 % methanol) produced as a byproduct in the biodiesel industry is also being extensively researched (Bauer & Hulteberg, 2013; Uprety et al., 2017). Wastewater from dairy, breweries and municipality waste have also been investigated as an alternative source for lipid production

(Schneider et al., 2013). The presence of acids such as acetic acid, isobutyric acid, propionic acid, isovaleric, n-butyric acid and n-valeric acid in volatile fatty acid obtained from industrial waste makes it important contender for single cell oil production.

Chapter 3 : Materials & Methods

3.1 Primary paper mill sludge (PPMS)

The PPMS was provided by the local paper mill Resolute forest products, in Thundery bay, Canada. The mill follows Kraft pulping process and the sludge was collected from the primary clarifier of wastewater effluent treatment plant and stored in sealed buckets. The original sludge had high moisture content and was dried overnight at 50 ^{o}C. The composition analysis of the dried sample consisting (ash, carbohydrates and lignin content) was done using the standard National Renewable Energy Laboratory (NREL) protocol recommended by the Department of Energy (US) (Gurram et al., 2015).

3.2 Enzymes

Commercial enzymes Accellerase 1500(cellulase) and Accellerase BG(β-glucosidase) were used in this research. Both enzymes were provided by DuPont Industrial Biosciences, San Jose, CA, USA for research purposes. Both the enzyme was received in sealed containers and were stored at 4^{o}C until experimental work. The filter paper activity of the cellulase enzyme was measured using the method recommended by NREL (Ghose, 1987). The β- glucosidase activity of the enzyme was measured following a method listed in a book chapter by Zhang et al, (2009).

3.3 Microorganism

The oleaginous microorganism *Cryptococcus curvatus* ATCC 20509 were obtained from American Type Culture Collection (ATCC). The culture was grown on YPG media (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) for 24 hours in an incubator shaker at 30 ^{o}C and 200 rpm. The seed culture was stored in -80 ^{o}C in presence of pure glycerol (50 % v/v).

3.4 Analytical methods

3.4.1. Quantification of sugar using DNS methods

Dinitrosalicylic acid (DNSA) is used for the quantification of reducing sugar present in the sample. In this method, the reducing sugars reduces the DNSA to produce a reddish orange colored complex which is measured using spectrophotometer at 540 nm. In this study 3 ml of DNSA was

added to the 1.5 ml enzyme-substrate mixture and incubated in boiling water for 5 mins and then transferred to ice-water bath for cool down. The color formation was determined by measuring the absorbance at 540 nm. The sugars were estimated by comparing the absorbance with glucose standard prepared using same method (Miller, 1959).

3.4.2. Quantification of sugars using HPLC

The hydrolysate was analyzed for soluble sugars using an HPLC (Agilent Technologies 1260 Infinity) with Bio-Rad Aminex HPX-87H ion exchange column (300 mm x 7.8 mm) and a Refractive Index Detector (RID). The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.5 ml/min at 50 ^oC. The instruments were calibrated with the standards of varying sugar concentration and the response factor (RF) obtained for the standards was used to calculate the concentration of the sugars formed.

3.4.3. Determination of biomass, lipid and characterization of lipid

Estimation of biomass content in the fermentation broth was done by centrifuging 5 ml broth sample at 4400 rpm for 10 mins. After discarding the supernatant, the wet cells were washed with distilled water and dried over at 80 ^oC in a pre-weighed aluminum weighing boat. The quantification of lipid accumulated was done using modified version of Bligh-Dyer method, published by Uprety et al., (2017). 25 mg of dried biomass was weighed in 2 ml Eppendorf tube and 0.33 ml of 4 M HCL was added to it. The suspension was kept in a water bath at 80 ^oC for 1 hour and later centrifuged at 6000 rpm for 10 mins. The supernatant was disposed of and cells were resuspended in 0.2 ml of methanol and 0.1 ml of chloroform. After vortexing it for 2 mins, 0.1 ml of chloroform was added and rigorously shaken for another 2 mins. To make the final ratio of 2:2:1.8 (methanol:chlroform:water), 0.18 ml of distilled water was added to the mixture. The suspension was then centrifuged at 6000 rpm for 10 mins and chloroform layer was transferred to another pre-weighed Eppendorf tube. The extraction was again repeated by adding 0.2 ml of 10 %

(v/v) methanol in chloroform solution to remaining methanol-water mixture. The chloroform layer collected from both steps were combined and dried at 105 ºC for 4 hours and the final weight of the tube was taken. The biomass and lipid extracted was calculated using the equation given below:

$$\text{Biomass concentration}\left({}^{\text{g}}/_{\text{l}}\right) = \frac{\text{Weight of dried biomass(g)}}{\text{Volume of fermentation broth(l)}} \quad (1)$$

$$Lipid\ concentration\left({}^{g}/_{l}\right) = \frac{Weight\ of\ lipid\ (g)}{volume\ of\ fermentation\ broth(l)} \quad (2)$$

$$Lipid\ content(\%wt) = \frac{Weight\ of\ lipid(g)}{Weight\ of\ dried\ cell\ biomass(g)} \times 100\ \% \quad (3)$$

The extracted lipids were further analyzed for fatty acid profile using a method reported by Uprety et al., (2017). 1mg of extracted lipid was dissolved in 0.2 ml of toluene and 0.2 ml of methyl nonadecanoate (5 mg/ml) was added to the solution as an internal standard. Subsequently, 1.5 ml of methanol and 0.3 ml of 8 %(w/v) HCL solution in methanol was added to the mixture and was kept in a water bath at 100 ºC for an hour. After cooling down the samples the extraction was completed by adding 1 ml of each, hexane and water. The hexane layer was later used for the fatty acid characterization using Gas Chromatography-Flame ionization detector.

3.5 Preparation of standard solution for calibration curve of glucose and xylose

Glucose and xylose standards were prepared for the quantification of sugars in the enzymatic hydrolysate and for enzyme activity measurements. Two different glucose standards were required for the HPLC and DNS method. The concentration range of standard for glucose and xylose were 1-8 g/l and 0.5-2 g/l respectively. The samples were filtered using 0.2 µm nylon syringe filter and

the peak area on the HPLC obtained was plotted against the known sugar concentrations. The glucose standard prepared by DNS method was only used for determination of cellulase enzyme activity.

3.6 Experimental methods

3.6.1. Determination of enzyme activity

3.6.1.1 Filter paper activity

A standard procedure recommended by International Union of Pure and Applied Chemistry (IUPAC) for measuring the activity of all kinds of cellulase enzymes was used. (Adney & Baker, 1996). It is designed in such a way that an enzyme dilution releasing 2 mg of glucose, designated as an intercept for 4 % conversion from 50 mg of filter paper in 60 mins, is measured from the experiment. In this work, enzyme dilution of 0.01,0.025,0.05,0.075 and 0.1 were made by diluting the original enzyme in distilled water. 0.5 ml of each enzyme dilution was added to a centrifuge tube containing 50 mg of filter paper immersed in 1ml Citrate buffer(50mM) and was incubated in a water bath at 50 ^{o}C for 60 minutes. Along with these sample tubes, enzyme and substrate blanks were also incubated. After an hour of incubation 3 ml of DNS reagent was added to all the tubes and the tubes were transferred to boiling water bath for 5 mins to stop the enzymatic reaction. The tubes were subsequently transferred to ice cold water bath and samples were further diluted and analyzed for optical density using spectrophotometry at 540 nm. Using the absorbance value of diluted samples, the glucose concentration is calculated from the glucose standard graph. Using a semi log graph of enzyme dilution vs glucose concentration, the enzyme dilution releasing 2 mg of glucose is intercepted and used for the calculation of filter paper activity as follows:

$$Filter\ paper\ acitivity\ = \frac{0.37}{[enzyme] releasing\ 2.0\ mg\ glucose} units/ml \qquad (4)$$

3.6.1.2 β-glucosidase activity

The measurement of β-glucosidase activity was done following a method documented by Zhang et al, (2009). The method is similar to filter paper activity. However, instead of Whatman filter paper 15 mM cellobiose in citrate buffer (50 mM, pH 4.8) was used as a substrate. Enzyme dilution in the range 200 to 1000 were made by diluting the original enzyme in citrate buffer. Along with substrate and enzyme blank, centrifuge tubes containing 1ml of diluted enzymes and 1 ml of cellobiose solution was incubated at 50 ^{o}C for 30 mins. For termination of the enzymatic reaction, all tubes were transferred to boiling water bath for 5 mins and subsequently into ice bath for cool down. DNS method could not be used for the quantification of sugars, as it fails to distinguish between reducing sugars, glucose and cellobiose, and therefore HPLC was used for the same. From the semi log graph of enzyme dilution vs glucose concentration, enzyme dilution releasing 1mg of glucose was interpreted and the activity of enzyme was calculated using equation given below:

$$\beta - glucosidase\ activity = \frac{0.0926}{[enzyme] releasing\ 1\ mg\ of\ glucose} IU/ml \quad (5)$$

3.6.2. Composition analysis of paper mill sludge

The paper mill sludge used in this study was analyzed for moisture, carbohydrate, lignin and ash content. The procedure used for the analysis is well defined by NREL and most commonly used in research work related to lignocellulosic biomass (Sluiter et al., 2008). For moisture content analysis, 1 gram of sludge was weighed in a pre-dried aluminum dish and was further dried at 105 ^{o}C for 4 hours (Sluiter et al., 2008a). The sample was transferred into a desiccator for cooling down and the weight of the dried sample was measured. The samples were again placed in an oven at 105 ^{o}C and dried to constant weight. The moisture content of the sample was then calculated using equation given below:

$$\%Moisture = 100 - \left\{\frac{\text{Weight(dry pan plus dry sample)} - \text{Weight(dry pan)}}{\text{Weight(sample as recieved)}} \times 100\right\} \quad (6)$$

As a pre-requirement, the sample used for the carbohydrate and lignin analysis were prepared following Hames et al., 2008 method. The carbohydrate and lignin content of the sample is analyzed following double acid (concentrated and dilute) hydrolysis technique. 300 mg of sludge was weighed and transferred into a beaker. 3 ml of 72 % sulphuric acid was added to the beaker and mixed well using a glass stir rod. The beaker was kept at 30 ^{o}C water bath for 60 minutes. Stirring the sample is essential for uniform hydrolysis and it was done every 15 minutes without removing the samples from the water bath. 84 ml of distilled water was added in order to reduce the acid concentration to 4 %. The sample taken in an Erlenmeyer flask was autoclaved at 121 ^{o}C for 1 hour. The sample was later filtered using vacuum filtration and the residues filtered were transferred into a pre-dried and weighed crucible for drying in oven. The weight of the sample was noted after it attained constant weight. The crucible was then transferred to muffle furnace at 575 ^{o}C for 24 hours for analysis the ash content of the sludge.

For carbohydrate analysis, 20 ml aliquot was taken from the filtrate and was first neutralized using 3 M NaOH. The sample was quantified for sugars using a HPLC. The filtrate was also analyzed for acid soluble lignin (ASL) using UV-spectrophotometer. The equations used for the calculations of each component are as follows:

$$Cellulose(\%) = \frac{Glucose\ produced(mg)}{Oven\ dry\ weight(sludge)(mg)} \times 0.9 \quad (7)$$

$$Hemicellulose(\%) = \frac{Xylose\ produced(mg)}{Oven\ dry\ weight(sludge)(mg)} \times 0.88 \quad (8)$$

$$Acid\ insoluble\ lignin(\%) \quad (9)$$

$$= \frac{[Weight(crucible\ plus\ solid\ residue) - Weight(crucible)] - [Weight(crucible\ plus\ ash) - Weight(crucible)]}{Oven\ dry\ weight\ (sludge)}$$

$$Acid\ soluble\ lignin(\%) = \frac{UV(abs) \times Volume(filtrate) \times Dilution}{\varepsilon \times Oven\ dry\ weight(sludge) \times Pathlength} \quad (10)$$

Where:
UV(abs)= Absorbance measured at λ_{max}
ε = Absorptivity of biomass
Pathlength= Pathlength of UV-Vis cell in cm

3.6.3. Optimization of enzymatic hydrolysis

The optimization of enzymatic hydrolysis was done based on four parameters namely pH, temperature, solid loading and enzyme loading. The total working volume of the enzymatic reaction was 50 ml citrate buffer and all experiments were done in duplicate in 250 ml Erlenmeyer flasks. For pH optimization, four levels in the range of 4.0 to 6.0 pH in 5 mM citrate buffer was made. The temperature, solid loading and enzyme loading were 50 ^{o}C, 5 % and 20 FPU/g oven dry (OD) sludge respectively, was chosen based on the literature. For optimizing temperature, the enzymatic reaction was carried out in 4 levels of temperature 30,40,50 and 60 ^{o}C. Optimized pH (4.5) conditions were used with 5 % solid loading and 20 FPU/g OD sludge. Using the optimized pH (4.5) and temperature (40 ^{o}C), the effect of solid loading was studied for 2.5,5.0,7.5 & 10 % solid concentration(w/v). Finally, enzyme loading was optimized with varying enzyme input of 10,20,30&40 FPU/g OD sludge. All enzymatic reaction was done for 96 hours and samples were withdrawn every 24 hours for sugar analysis using HPLC. The conversion of cellulose to glucose was calculated using the equation 11.

Table 3.1: Optimization parameters for enzymatic hydrolysis.

	Effect of pH	Effect of Temperature	Effect of Solid Loading	Effect of Enzyme loading
PH	4, 4.5, 5 & 6	4.5	4.5	4.5
Temperature (^oC)	50	30,40,50,60	40	40
Solid loading (%w/v)	5	5	2.5, 5,7.5&10	10
Enzyme loading (FPU/g OD sludge)	20	20	20	10, 20, 30 &40

$$Cellulose\ Conversion\ \% = \frac{Glcose\ released(g)x\ 0.9}{Cellulose\ content\ in\ the\ sludge(g)} x100 \quad (11)$$

3.6.4 Effect of β-Glucosidase on enzymatic hydrolysis

The effect of β-glucosidase on enzymatic hydrolysis was studied by changing the ratio of β-glucosidase enzyme to cellulase enzyme in the enzymatic reaction. Optimized reaction conditions (pH, temperature, cellulase enzyme loading & Solid loading) were used for this experiment. The ratio of β-glucosidase to cellulase enzyme studied were 1:0.5,1:1,1:1.5 & 1:2. All enzymatic reactions were done for 96 hours and samples were withdrawn every 24 hours for sugar analysis using HPLC.

3.7 Fermentation of hydrolysate to microbial oil

The inoculum for the fermentation was prepared in the YPG media (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose). The *Cryptococcus curvatus* culture was incubated for 24 hours in rotary shaker at 30 ^oC and 200 rpm until cell count reached $1x10^8$ cells/ml. All experiments were

carried out in 125 ml Erlenmeyer flasks with 50 ml of media for 168 hours. The sugar concentration and C:N ratio for the fermentation was optimized using pure glucose and ammonium chloride. The optimized conditions were used to ferment the sludge hydrolysate by preparing a minimal media. The minimal media was prepared following Uprety et al., (2017) method, containing (g/l): Glucose 30.0, KH_2PO_4 2.7, $Na_2HPO_4 \cdot 12H_2O$ 0.95, $MgCl_2 \cdot 6H_2O$ 0.2, yeast extract 0.1 and pH 5.5. The media was supplemented with 10 ml/L of trace element. The trace element consisted of CaCl2.2H20 4.0 g, FeSO4.7H2O 0.55 g, citric acid monohydrate 0.52 g, ZnSO4.7H2O 0.10 g, MnSO4.H2O 0.076 g, 18M H2SO4 100µL. Samples were taken every 24 hours for lipid and biomass analysis.

Chapter 4 : Results and Discussion

4.1 Composition of paper mill sludge

The composition analysis of PMS was done using the double acid hydrolysis method proposed by NREL (Sluiter et al., 2008). The carbohydrate, lignin, ash and moisture content were quantified and reported in table 4.1. Glucose and xylose were the major monomeric sugars that were detected in HPLC as shown in the figure 4.1. The cellulose and hemicellulose together accounted for 50.6 % (dry weight basis). The cellulose content is usually high in the primary paper mill sludge. The cellulose content in the sample of PMS used in this study is around the average content found in other published reports (Alkasrawi et al., 2016; Kang et al., 2010).

Table 4.1: Composition of primary paper mill sludge.

Components	Cellulose	Hemicellulose	Lignin		Ash	Moisture
			Acid soluble	Acid insoluble		
Percentage (%)	38.5	12.1	3.3	35.1	8.0	62.4

The moisture content of the sludge was quantified using oven drying technique and was observed to be 62.4 %. Generally, the ash content is detected in the primary paper mill sludge is high due to the filler material used in the pulping process. However, the 8 % ash content measured in this study is substantially low as compared to report published by Carvalho et al, (2011). The presence of ash has a significant influence on the cellulose enzyme hydrolysis. The low ash content was an advantage as no pretreatment was required for enzymatic hydrolysis. The lignin content observed in this study was relatively high, similar to numbers reported by Li et al, (2015) and Alkasrawi et al, (2016). Both publications did not report any hindrance caused by lignin during the enzymatic hydrolysis and similar observations were made in this study as well. The high lignin content could be attributed to more mechanical pulp fibre (Li et al., 2015). The composition of paper mill sludge varies and is highly dependent on the pulping process followed at different paper mill. The large

amounts of highly accessible carbohydrates present in the primary paper mill sludge in this study makes it an ideal contender for further hydrolysis and microbial lipid production.

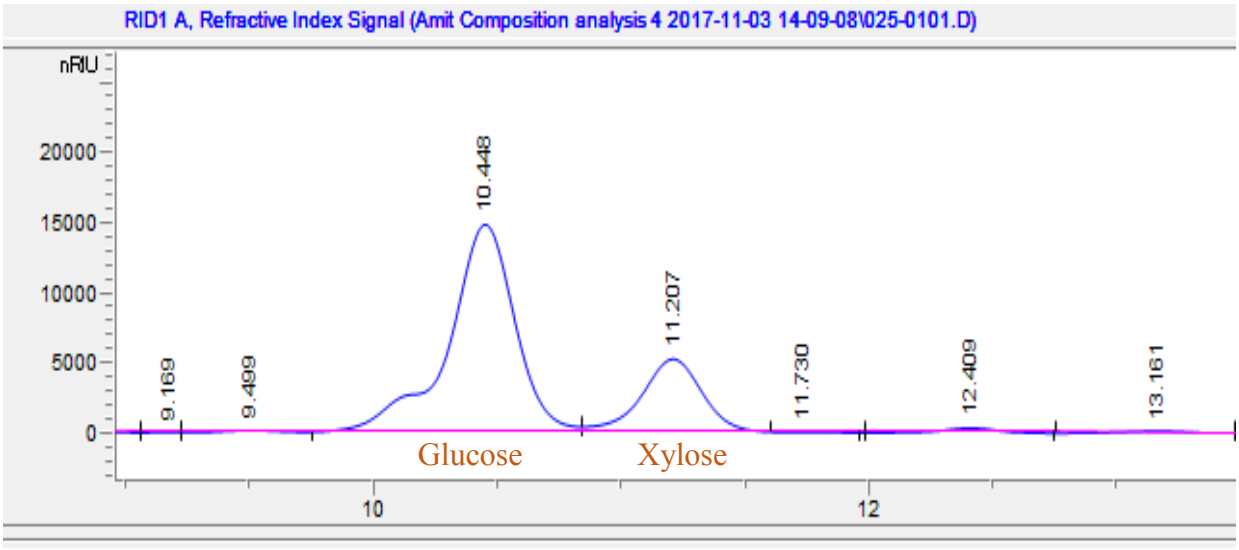

Figure 4.1: Chromatogram of sugar analysis after double acid hydrolysis

4.2. Determination of enzyme activity

The cellulase enzyme activity of the commercial enzyme used in this study was quantified using well described and recommended publication by Adney & Baker, (1996). The reducing sugar concentration for different enzyme dilutions were measured using DNS method and was plotted as shown in figure 4.2. The enzyme dilution releasing 2 mg of glucose was interpreted from the semi- logarithmic graph and used in the equation 4. The filter paper units (FPU) for the Accelerase 1500 used in this study was found to be 28 units/ml. The activity of enzyme is highly dependent on the purity of enzyme. Hence, the activity obtained for Accelerase 1500 is way low as compared to 77 FPU/ml reported by Alkasrawi et al, (2016). This confirm the need to measure enzyme activity before use, even if they are from the same source.

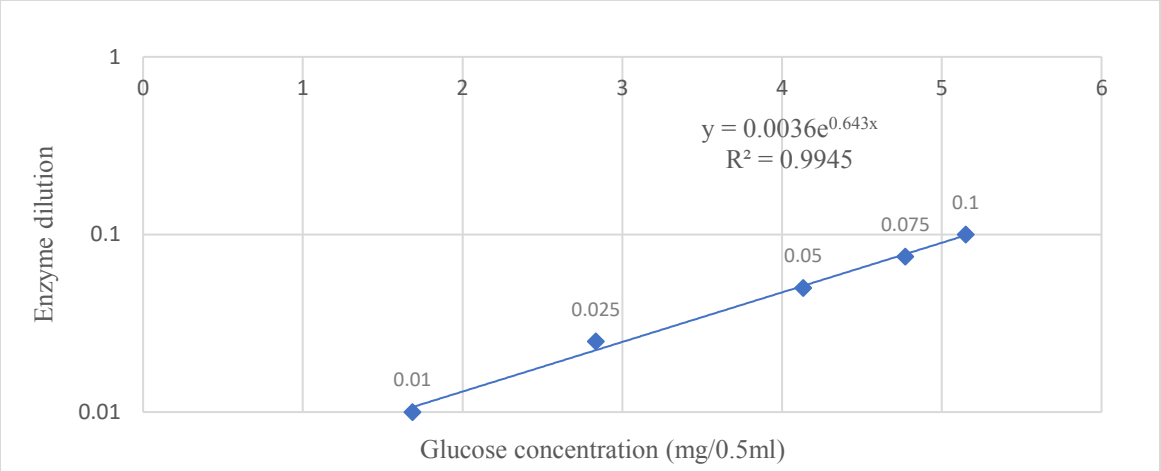

Figure 4.2: Relationship between enzyme dilution and glucose concentration detected using DNS method

The β-glucosidase activity of Accelerasse BG enzyme was determined using the method published by Zhang et al, (2009). The glucose concentration quantified using HPLC was plotted against enzyme concentration as shown in the figure 4.3. Similar to filter paper assay, the enzyme concentration releasing 0.5 mg/ml was interpreted and the β-glucosidase activity was calculated using equation 5. The activity of Accelerase BG enzyme was found to be 79 IU/ml.

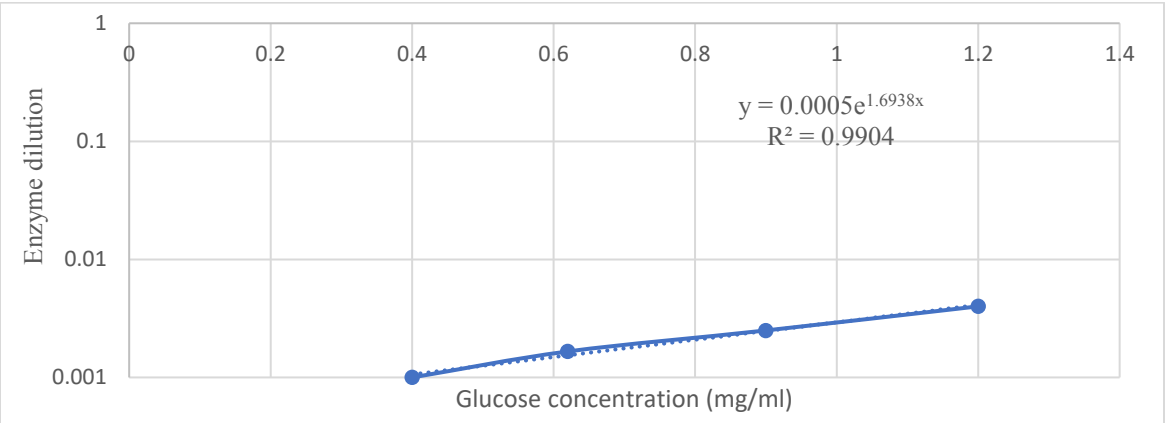

Figure 4.3: Relationship between enzyme dilution and glucose concentration detected using HPLC

4.3. Optimization of enzymatic hydrolysis

Experiments were carried out to study the effects of pH, temperature, solid loading and enzyme loading on the Accelerase 1500 enzyme. The experiments conditions listed in the table 3.1 were applied to learn its effects. These conditions were selected based on a thorough literature review conducted on enzymatic hydrolysis of paper mill sludge (Table 2.5).

4.3.1. Effect of pH on Accelerase 1500 enzyme

The effect of pH in the range 4 to 6 on the Accelerase 1500 enzyme was studied. Monohydrate citric acid was used as a buffer to control the pH. The reaction was carried out at 5 % solid loading, 20 FPU/g substrate at 50 ^{o}C. These conditions were selected based on the thorough literature review conducted (Table 2.5). Maximum glucose concentration of 7.43 g/l was observed at pH 4.5 after 9 hours of enzymatic hydrolysis (Fig 4.4). Slightly less, 7.1 g/l of glucose was produced at pH 5 suggesting the range 4.5 to 5 best suited for the enzyme, Accelerase 1500, used in this study. There was no significant increase observed in glucose level after 96 hours, suggesting the saturation of enzyme. Most of the studies conducted using cellulase enzyme have reported the similar range (4.5to 5). However, Alkasrawi et al, (2016) reported pH 5.5 as optimum condition for the enzymatic hydrolysis. Using water instead of buffer for the hydrolysis medium did not yield good results. The mixture of primary paper mill sludge with water had a pH of 3.7 and produced 4.16 g/l of glucose.

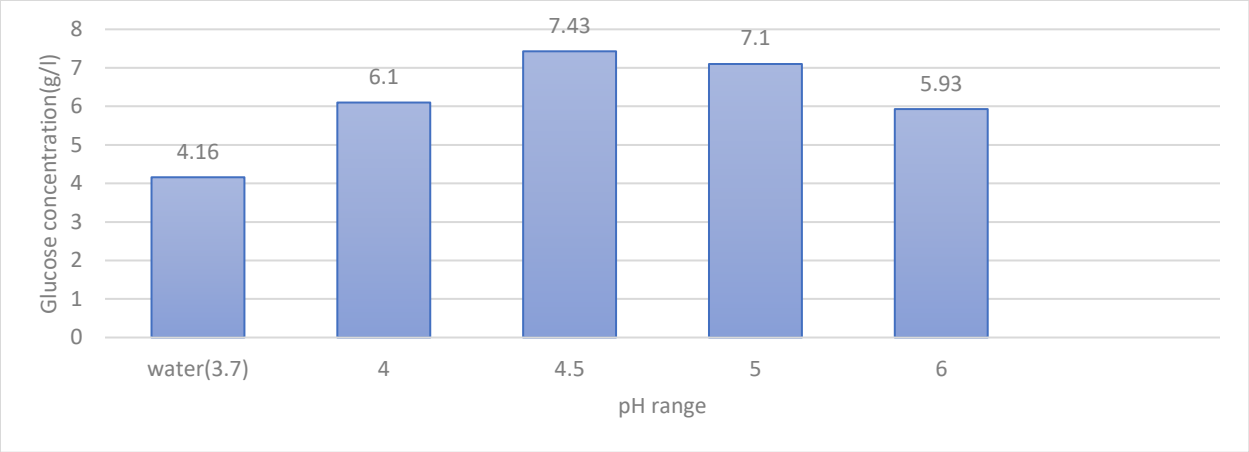

Figure 4.4: The concentration of glucose produced as function of pH at 50 ºC, 5 % solid load and 20 FPU/ g substrate of cellulase enzyme loading.

4.3.2. Effect of temperature on Accelerase 1500 enzyme

Most reactions involving cellulase enzyme have known to be conducted at temperature higher than room temperature. Higher temperatures not only improve the catalytic reaction but also alleviates the product inhibitions caused during the enzymatic reaction (Teugias & Valjamae,2013). Maintaining higher temperature requires lot of energy, making it necessary to optimize the temperature of the reaction. Hence determination of the optimum temperature is very important.

To determine the effect of temperature on Accelerase 1500, the trials were conducted at 30,40,50 & 60 ºC. Other experiment conditions used were optimized pH 4.5, 5 % solid loading and 20 FPU/ g substrate. As shown in the figure 4.5, increase in temperature improved the glucose concentration with 7.9 g/l of glucose producing at 40 ºC. However, further increase in temperature to 60 ºC produced 3.82 g/l of the glucose, that is almost 50 % reduction in glucose concentration. Deactivation of enzyme at high temperature could be the reason for this reduction. Similar observation was made by Zhu et al, (2011) in their optimization experiments.

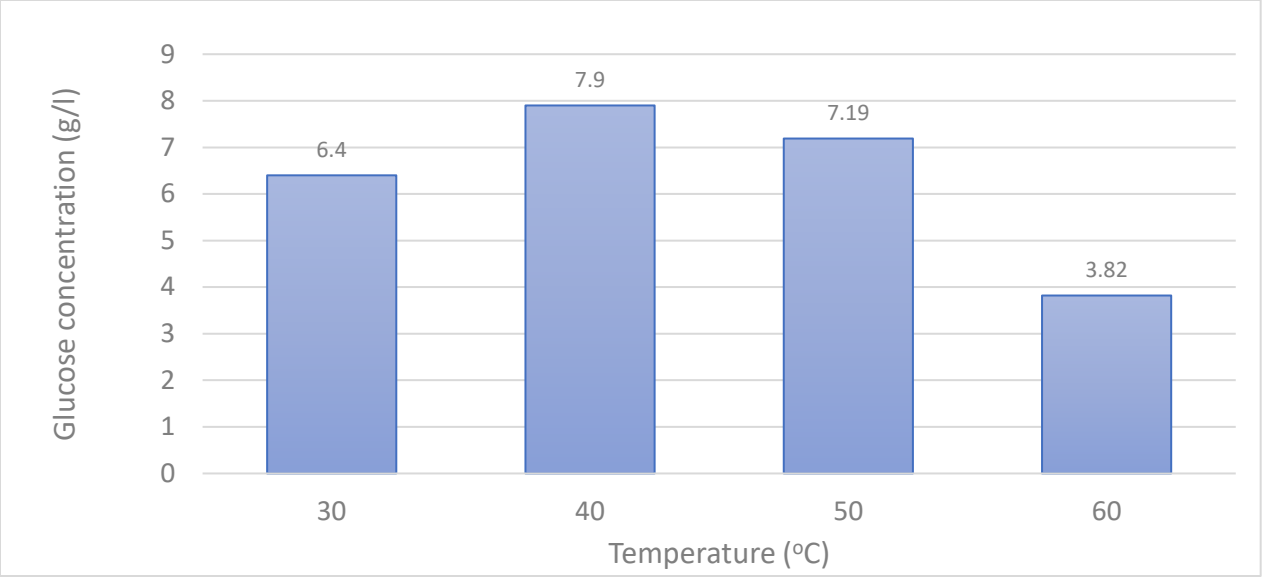

Figure 4.5: The concentration glucose produced as function of temperature at 4.5 pH, 5 % solid load and 20 FPU/ g substrate of cellulase enzyme loading.

4.3.3. Effect of solid loading on enzymatic hydrolysis

Percentage solid content present in the reaction for the enzymatic hydrolysis has a great influence on the amount of sugars produced. For the biochemical reactions to be economically feasible, reactions with higher solid content are desirable (Teugjas & Valjamae, 2013). Experimental trails were carried out for the solid loading of 2.5,5,7.5 and 10 % (w/v). Increasing the solid content amplified the glucose concentration to approximately 6 times with the enzyme producing 3.2 g/l of glucose at 2.5 %(w/v) solids to 17.6 g/l at 10 % (w/v) solids (Fig 4.6). The increase in glucose concentration can be attributed to higher cellulose availability in reaction. Gurram et al, (2015) in their work tried 20 % (w/v) solid loading and observed a dip in cellulose to glucose conversion. The reduction in conversion was associated with rheological challenges and product inhibition. Similarly, 12.5 % (w/v) solid loading was initiated in this study and subsequently stopped due to irregular mixing as shown in figure 4.7.

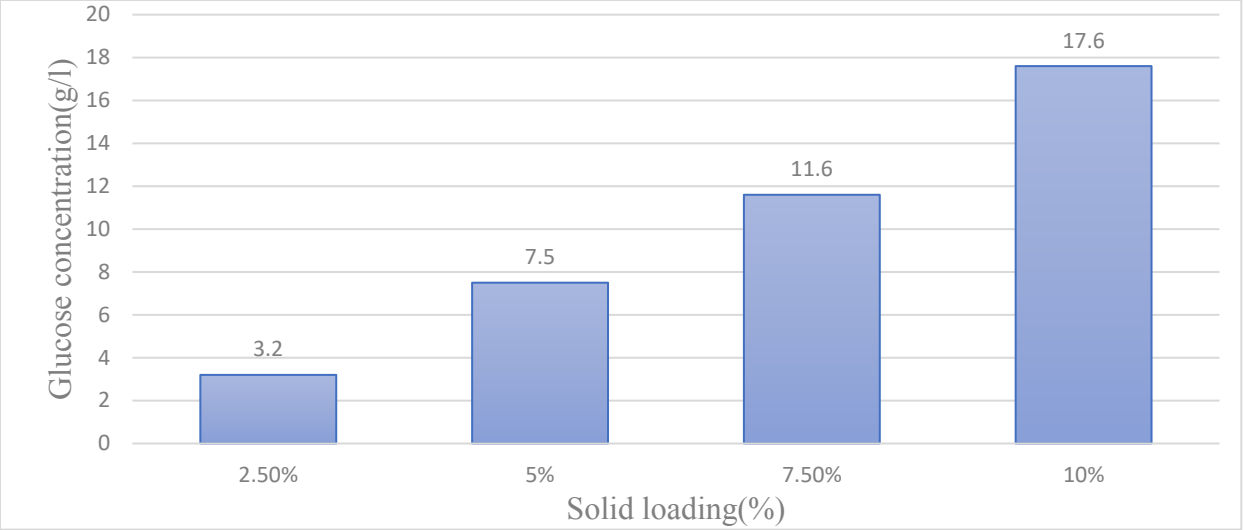

Figure 4.6: The concentrations of glucose as a function of solid loading at 4.5 pH, 40 ºC temperature and 20 FPU/g substrate of cellulase enzyme loading.

Sheading the sludge before the enzymatic hydrolysis have been found to ameliorate the accessibility of cellulose fibers for enzyme digestion. However, grinding the sludge in a blender, expands in volume thereby restricting the solid loading capacity. Enzymatic hydrolysis with 5 % shredded sludge was also investigated in this study. Contrary to Chen et al, (2014a) no significant improvement in glucose production was observed as compared to the non-grinded sludge. By not fractionating the sludge we were able to use 10 % solids, thereby producing twice the glucose concentration. Gurram et al, (2015) recommended specially designed reactors for reactions containing more than 20 % (w/v) solid content.

Figure 4.7: Irregular mixing pattern caused due to high solid loading.

4.3.4. Effect of enzyme loading on enzymatic hydrolysis

One of the major factors that influences the economic feasibility of the enzymatic hydrolysis is the amount of enzyme used. Optimization plays an important role in finding appropriate quantity enzyme for saccharification thereby avoiding the over dosage. Enzyme loading of 10,20,30 and 40 FPU/g substrate were examined under optimized conditions of pH, temperature and solid loading. The results are shown in fig. 4.8.

20 FPU/ g substrate was found to be the optimum enzyme loading condition, producing 16.2 g/l of glucose. Even though an adequate increase in glucose was observed with increasing enzyme loading from 10 to 20 FPU/g substrate, minuscule increase was observed with further addition of enzyme. Saturation of all substrate binding sites could be the major reason. Inhibition from glucose and cellobiose could also another reason for the stagnation. However, cellobiose content was not observed in chromatogram obtained from HPLC. Similar observations were made by Elliston et al., (2014) about Accelerase 1500 enzyme not producing enough cellobiose. Moreover, it is also report that cellobiose with low concentration of 0.6 g/l have been found to have inhibitory effect

on cellulase, especially cellobiohydrolase (Zhang et al., 2010). The optimum condition for cellulase enzyme loading found in this study was well within the range, 5-35 FPU/g substrate (Kumar et al., (2017).

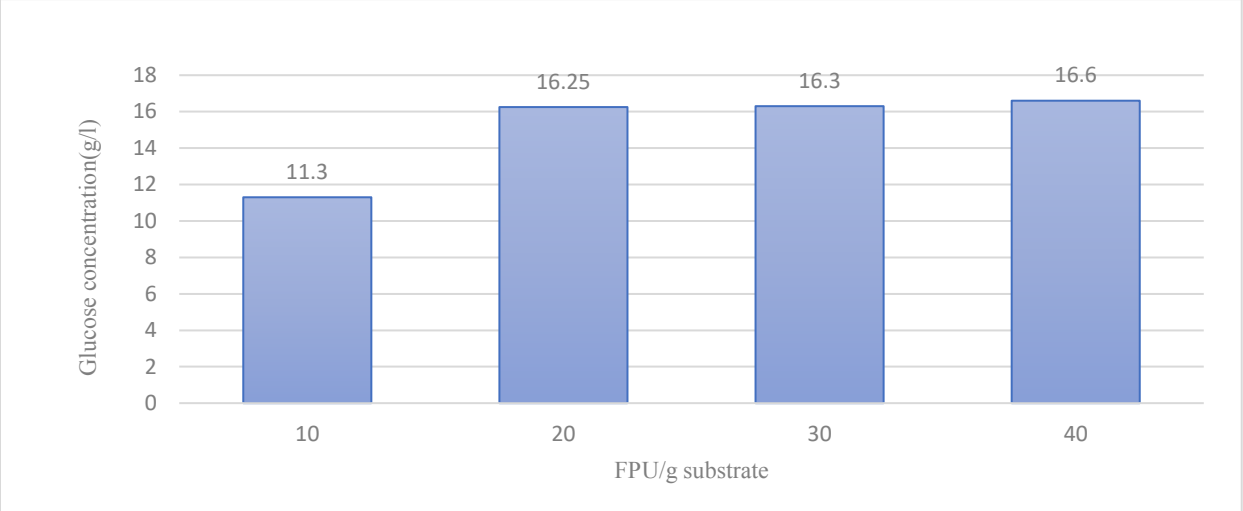

Figure 4.8: The concentrations of glucose as a function of enzyme loading at 4.5 pH, 40°C temperature and 10% solid loading.

4.4 Effect of adding β-glucosidase enzyme on cellulase enzymatic hydrolysis

In order to maximize the cellulose to glucose conversion and to enhance the glucose concentration in the sugar syrup, addition of β- glucosidase is necessary. Even though cellulase enzyme consist a portion of β- glucosidase enzyme, it's not enough for an efficient enzymatic reaction. Trials were conducted to study five different ratio of cellulase to β- glucosidase. The optimized pH, temperature, solid loading and enzyme loading used for this reaction were 4.5, 40 °C, 10 % and 20 FPU/ g substrate, respectively.

Supplementation with β-glucosidase yielded a substantial increase in the glucose concentration and conversion. A linear increase in glucose concentration and cellulose to glucose conversion was observed with additional supply of β-glucosidase. As compared to experiments with no addition of β-glucosidase, twofold increase in glucose concentration was observed for flask

containing cellulase and β-glucosidase enzyme in the ratio 1:2 (v/v). Moreover, 81 % of cellulose to glucose was also observed for the same. The low conversion rate (35.1 %) during only cellulase enzyme hydrolysis could be because of low β- glucosidase activity and product inhibition of cellulase enzyme. Another critical observation made from Fig 4.9 was that by mere addition of 0.5 % β-glucosidase enzyme the reaction time dropped from 96 hours to 24 hours for attaining glucose concentration equal to the experiment without any β-glucosidase supplementation.

There was no change observed in the xylose concentration by addition of β-glucosidase. The results obtained in this study were not as high as Elliston et al, (2014) or Zhu et al, (2011). Both those groups achieved almost 99 % glucose conversion in their study. However, initial surge in glucose concentration was observed in both studies. The results obtained in this study are in accordance with Zhang et al, (2010), who also observed a rise up to 80 % glucose yield by addition of Novozyme 188(β-glucosidase) in their enzymatic hydrolysis of corncob. Immobilization of β-glucosidase enzyme not only improved the glucose production but also for provides thermal stability to the enzyme (Borges et al., 2014). However, immobilization was not tried in this study because using immobilized enzyme against a solid substrate would have reduced the efficacy of the enzyme.

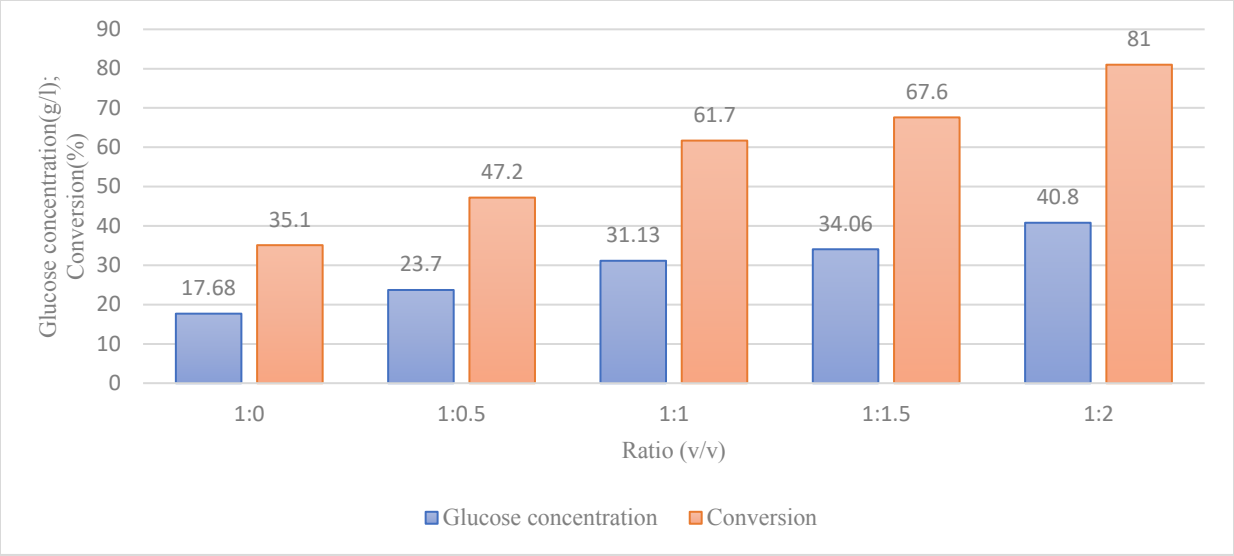

Figure 4.9: The concentration of glucose and conversion factor as a function of cellulase to β- glucosidase enzyme ration at 4.5 pH, 40°C temperature, 10% solid loading and 20FPU/g substrate cellulase loading.

4.5. Production of microbial lipids from paper mill sludge hydrolysate

Most of the research work that has carried out on the PMS has been for the production ethanol. The abundant availability of low cost ethanol makes it necessary to look for an alternate application. Thus, microbial production of lipids using *Cryptococcus curvatus* was thus carried out on PPMS hydrolysate. The amount of sugar present and carbon to nitrogen ratio has a great influence on the growth of an oleaginous strain. Both these parameters were optimized employing a minimal media and the optimized conditions were then used for the fermentation of primary paper mill sludge hydrolysate.

4.5.1 Optimizing glucose concentration for fermentation

The glucose concentration range studied in this experiment was 10-40 g/l. The minimal media composition was adapted from Uprety et al., (2017) and the C:N was adjusted to 100 using ammonium chloride for the fermentation reaction that was carried out for 168 hours. The C:N selected for these trials were based on the literature review conducted (Table 2.6).

The maximum biomass and lipid concentration obtained in these experiments were 10.14 g/l and 4.6 g/l at a glucose concentration of 30 g/l. Increase the glucose concentration to 40g/l led to substrate inhibition of the microorganism, thereby producing 6.32 g/l of biomass and 1.58 g/l of lipids. Chang et al, (2013) reported 40 g/l as their optimum glucose concentration, further increase up to 100 g/l saw a similar decrease in biomass and lipids. Chang et al, (2013) also proposed fed batch as a preventive technique for substrate inhibition.

Table 4.2: The quantity of biomass(g/l), lipid content (%) and lipid concentration(g/l) produced by *Cryptococcus curvatus* after 6 days of fermentation for varying glucose concentration

Glucose conc. (g/l)	C:N	Biomass (g/l)	Lipid Content (%)	Lipid Conc. (g/l)
10	100	5.56	42	2.3
20	100	7.64	44	3.3
30	100	10.14	46	4.66
40	100	6.32	25	1.58

4.5.2 Optimizing C: N for fermentation

The presence of nitrogen has considerable effect on the ability of the microorganism to produce lipids. The carbon to nitrogen in the ratio 50-300 were studied in this experiment. Minimal media without any addition of nitrogen was also included in this study.

There was no major impact observed on the microbial biomass with increasing the C:N ratio. C:N ratio of 50 produced maximum biomass of 11.43 g/l following by 10.3, 10.4 and 10.2 g/l for the C:N of 100, 200 and 300 respectively. However, a notable impact on the

cellular lipid content was observed in this study. High lipid content (above 60 %) was observed for reaction with C: N ratio above 200. A maximum of 64 % lipid content was obtained for fermentation flask with C:N ratio of 200. Further increase in this ratio or no addition of nitrogen, made it difficult for the separation of biomass from the fermentation broth. Hence C:N ratio of 200 was considered as the optimum condition. Another observation made from table 4.3, is the requirement of miniscule quantity of nitrogen by *Cryptococcus curvatus* for producing lipids as high as 64 percent. Less requirement of nitrogen is a positive sign from an economic point of view.

Table 4.3: The quantity of biomass(g/l), lipid content (%) and lipid concentration(g/l) produced by *Cryptococcus curvatus* after 6 days of fermentation with varying C:N ratio

Glucose conc. (g/l)	C: N	Biomass (g/l)	Lipid content (%)	Lipid conc. (g/l)	Lipid yield
30	50	11.43	45	5.1	0.17
30	100	10.3	57	5.8	0.19
30	200	10.4	64	6.6	0.22
30	300	10.2	63	6.4	0.21

4.5.3 Time profile of lipid production by *Cryptococcus curvatus* on PPMS hydrolysate

The PPMS hydrolysate obtained after the enzyme hydrolysis was first diluted to optimum glucose concentration and then filtered using 0.2 µm micro filter before it was investigated as substrate for microbial lipid production. Ammonium chloride was added to adjust the C:N ratio to 200 along with minimal media salts and trace element. After seven days of fermentation the biomass, lipid content, lipid concentration was 15.8 g/l, 38.7 % and 6.1 g/l, respectively. However, maximum

lipid content and lipid concentration of 45.2 % and 6.4 g/l respectively (Fig 4.10), was noticed on the sixth day of fermentation. The lipid yield calculated for the sixth day in this study was 213 mg/g indicated that the *Cryptococcus curvatus* favored lipid synthesis rather than cell growth. Similar finding were reported by Zhou et al, (2017). The quantity of biomass obtained, and the cellular lipids produced in this study were high as compared to research conducted on low cost substrate such as wheat straw hydrolysate (Yu et al., 2011), industrial fats (Papanikolaou et al., 2001) and elephant grass (Chen et al., 2016). Huang et al, (2012) in their study using corncob hydrolysate yielded higher biomass concentration, but lipid content reported in this work are higher. The factors that contributed to positive results could be the low levels of nitrogen and the inhibitor free substrate. Slow consumption of glucose for five days was observed. However, no consumption of xylose took place until all glucose was consumed as reported by Yu et al, (2014b) was also noticed in this work. PPMS hydrolysate without any addition of salts or nitrogen was also experimented in this work. Surprisingly, this robust strain is capable of accumulating lipid upto 35.7 % without adding nitrogen or minimal salts which are required for almost all strains. Therefore, we can conclude that PPMS hydrolysate contains enough nutrient element for proliferation of *Cryptococcus curvatus* cells. This makes the general process economically feasible.

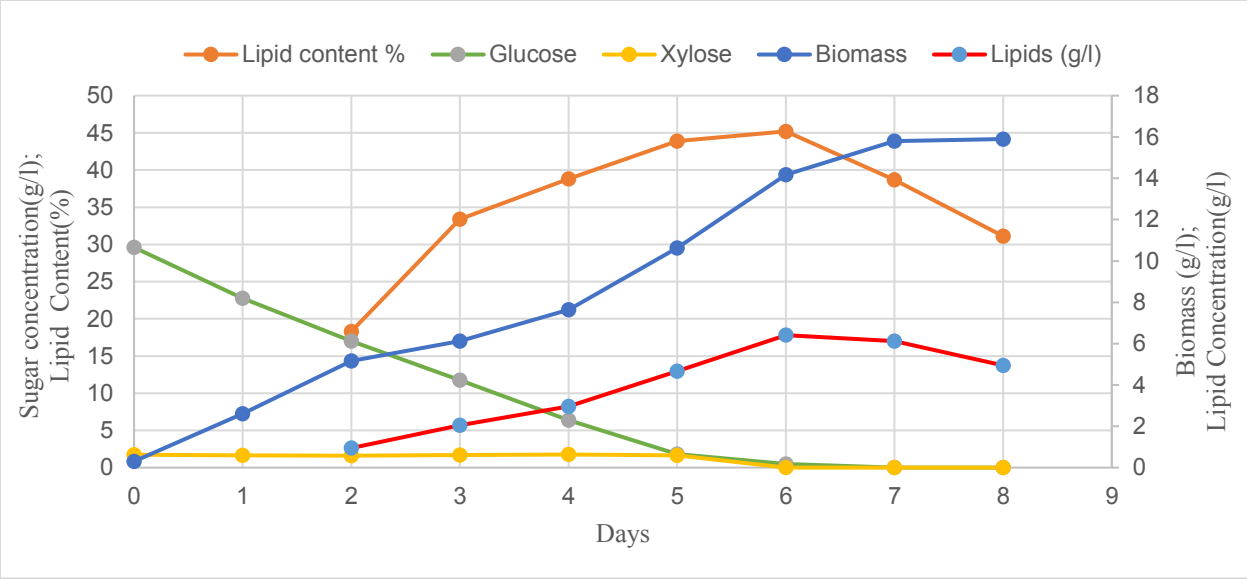

Figure 4.10: Time profile of lipid content (%), biomass(g/l), lipid concentration(g/l) by *Cryptococcus curvatus* on PPMS hydrolysate.

4.5.4 Fatty acid profile analysis

Using gas chromatography, the harvested samples were analyzed for fatty acid profile. As shown in table 4.4, the lipids synthesized by *Cryptococcus curvatus* in this study mainly consisted of oleic acid and palmitic acid. Although, noticeable reduction in myristic acid and linoleic acid can also be observed. The fatty acid profile for lipids produced from PPMS hydrolysate had similar composition as compared to vegetable oils (Sitepu et al., 2014). This similarity makes this microbial lipid a promising feedstock for biodiesel and oleochemical industries.

Table 4.4: Fatty acid profile comparison of lipids produced from pure glucose and PPMS hydrolysate

Sample	Fatty acid content (%, w/w)				
	Myristic acid (C14)	Palmitic acid (C16)	Stearic acid (C18)	Oleic acid (C18:1)	Linoleic acid (C18:2)
Pure glucose	8.9	15.3	10.0	38.9	12.6
Sludge hydrolysate	1.1	24.6	13.7	35.2	4.4

Conclusion

A lab-scale method for enzymatic hydrolysis of primary paper mill sludge and the fermentation was established in this study. A thorough composition analysis of PPMS indicated the presence of over 50 % carbohydrates, 38 % of lignin and low quantity of ash present in the sludge. The optimized conditions of pH, temperature, solid loading and enzyme loading were 4.5, 40 ^{o}C, 10 % and 20 FPU/ g substrate respectively. Supplementation of β- glucosidase to cellulase enzyme reaction yielded a positive outcome. A range of cellulase to β- glucosidase enzyme ratio was tested and 1:2 (v/v) was determined as optimum. The sugar syrup obtained after enzymatic hydrolysis contained both hexose(glucose) and pentose(xylose) sugar. The addition of β- glucosidase in the optimum ratio resulted in 2-fold rise in the glucose concentration to 40.8 g/l. Preliminary to fermentation of the sludge hydrolysate the glucose concentration and the C:N ratio of the fermentation reaction of optimized. The optimized glucose concentration and C:N ratio of 30 g/l and 200 respectively were utilized to produce microbial lipids. The *Cryptococcus curvatus* was able to produce as high as 64 % while grown on pure glucose. However, while grown on sludge hydrolysate the biomass, lipid content, lipid concentration was 15.8 g/l, 38.7 % and 6.1 g/l, respectively. The fatty acid profile of microbial oil studied using GC was found to be rich in oleic acid and palmitic acid. The microbial oil produced showed resemblance to vegetable oil based on the fatty acid profile. Further application of the microbial oil needs to be investigated.

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