‘Saccharification of Pine needle biomass using Commercial cellulase’

A PROJECT
Submitted in partial fulfilment of the requirement for the award of the degree of
BACHELOR OF TECHNOLOGY
IN
BIOTECHNOLOGY
Under the supervision of
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By
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MAY- 2019

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CERTIFICATE

This is to certify that the work entitled **“Saccharification of pine needles biomass using commercial cellulase”** pursued by Arunima (151804) in partial fulfilment for the award of degree Bachelor of Technology in Biotechnology from Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This part of work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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Acknowledgment

The research opportunity we had was a great chance for learning and professional development. We are grateful for having a chance to meet so many wonderful people and professionals who led us through this project. We have had so many rich experiences and opportunities that we believe will forever shape and influence our professional life while fostering personal growth and development.

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- Special thanks to our parents and friends for their motivation, sharing their experiences, time and commitment especially during the final stages of this project and being constant support throughout.

Sincerely,

Arunima (151804)

Date:

DECLARATION

We hereby declare that the project work entitled '**Saccharification of pine needles biomass using commercial cellulase'**, submitted to the Jaypee University of Information Technology, Waknaghat, Solan is a record of original work done by us under the guidance of Dr. Ashok Kumar, Assistant Professor, Department of Biotechnology and Bioinformatics. This project work is submitted in the partial fulfilment of the requirements of the reward of the degree of Bachelor of Technology in Biotechnology. The results embodied in this thesis have not been submitted to any other university or institute for the award of any degree or diploma.

Arunima (151804)

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Dr. Ashok Kumar
Assistant Professor
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LIST OF ABBREVIATIONS

Symbol	Abbreviation
°C	Degree Celsius
%	Percentage
pH	Power of Hydrogen
g	Gram
v/v	Volume/Volume
ml	Millilitre
h	Hour
min	Minute
dH_20	Distilled Water
RPM	Rotation per minute
NaOH	Sodium Hydroxide
HCl	Hydrochloric acid
OD	Optical density
PNB	Pine needle biomass

Abstract

Pine needles impose a problem in hilly regions. Dried pine leaves can cause forest fires or soil erosion. These pine needles are a good source of lignocelluloses which is an important precursor for biofuel production. Biofuels can replace fossil fuels which have become necessary with the increasing pollution and population. Biofuels have low carbon emissions, they are renewable and fuel distribution won't be concentrated to a few particular regions. The problem with biofuels is that we lack the technology to make the production easier although many countries have started production using corn, sugarcane, etc but these are edible materials and we cannot afford to utilize them for large scale production thus the requirement arises to produce biofuels using woody biomass which has high lignocelluloses content. The substrate was collected, washed and pre-treated with sulphuric acid at 0.2%, 0.4%, 0.6%, 0.8%, and 1% (v/v) after which incubation, centrifugation, oven dry was done to obtain dried biomass. The incubation time, pH, temperature, and agitation rate was optimized for saccharification of PNB using cellulase. Reducing sugar obtained after saccharification was calculated by plotting a standard curve of glucose. The incubation time 36 h was optimized for cellulase treated PNB at 45°C and cellulase work in the acidic medium thus the maximum amount of reducing sugar was obtained at pH 5.0. There is a lot to explore in the production of bio-fuel and to develop technology and infrastructure.

Keywords: Biomass, Pine needles, Bio-fuel, Cellulase, Lignocellulose

Chapter 1

Introduction

INTRODUCTION

Cellulose is the most abundant and renewable polymer present on our earth. It is a major component of the plant cell wall (Tony et al., 2008). Bioethanol is formed when cellulosic biomass is used to synthesize ethanol and in the coming future, bioethanol could be produced largely from cellulosic biomass only. Lignocellulose is the dried part of plants and the most abundant raw material for biofuel production. It comprises of cellulose, hemicelluloses lignin and is in demand for paper industry and biofuel production (Chris etal., 2009). There are lots of benefits of fossil fuels but the growing population highlights the one disadvantage of fossil fuels, which is non-renewability. Biofuels can be derived from edible and nonedible parts of plants so there is no problem related to availability but it has an environmental impact similar to fossil fuels, which can be avoided if preventive measures are taken. Fossil fuels are available in defined geographical regions which makes other regions dependent on them for energy but in case of biofuels this will not be an issue as several sources of feedstock can be used for their synthesis.

1.1 Various sources of biofuels

There are many sources available for biofuel production and one of them is algal oil which has high production and growth rate but it is still not used because there is no technology available to control the fast growth of algae. Another source is corn which is a good source for biofuel production and is even used in the United States as fuel feedstock but it creates conflict as it is an edible crop and therefore cannot be used in long term. In Brazil the popular choice for biofuel production is sugarcane and they are using it for biofuel production from a long period of time but as sugarcane can be grown in tropical regions only so this option is not available to every country. Another source is Camelina and Jatropha which can be grown in many regions and are also drought tolerant plants. Camelina is a tropical plant and Jatropha, on the other hand, is a temperate plant (Laxmipathi et al., 2010). Animal fat can also be a good source of biofuels as it has high saturation point so fat solidifies at a high temperature leading to high cloud point for biodiesel that is made from it. Contamination is an obvious challenge in this option so proper refining should be done before using it as fuel (Brown et al., 2012). In Europe and in some parts of the United States, methane is also used. It is produced by decomposing microorganism so production is easy but vehicles need to be adapted accordingly which can be challenging (Redman et al., 2008). Cellulosic biomass is considered to be most beneficial because of low contribution to greenhouse emissions, easy availability, low cost of raw materials and high energy gain (Pernick et al., 2007). There are several options to be chosen from and lots of possibilities to explore, we just need to decide the best one keeping in mind the country's economic growth, availability, and environment. Among available lignocelluloses feedstock, pine needle has proven to be very beneficial because it contains 75% polysaccharide that can be broken down into sugars and used for biofuel production. Pine needles are a problem for many areas especially hilly regions because apart from causing soil erosion and destruction to flora and fauna, the fallen and dried needles might cause forest fire.

1.2 Pine needles

Pine needles are an important part of hilly regions but pose a threat to agriculture and the environment. They fall during summers and almost the whole ground is covered by them which can be destructive because it can lead to forest fires even by small fire source and these fire can be the reason for the destruction of ecology, fertile soil loss and grazing grounds (Umesh, et al., 2017). Pine needles are coniferous trees with maximum height about 81.79m and that is of ponderosa pine (Fattig, et al., 2011). Typically pine trees age is 100-1000 years and pine to live longest is Great Basin bristlecone pine (Ryan et al., 1999). Pine needles have broad and scaly bark but some have narrow and flaky bark and branches appear in ring type. There are four leaf types present in pine trees seed leaves, juvenile leaves, scale leaves and needles. Pine trees have male and female cones on the same tree but in some cases only individual are expressed. Male cones have a smaller size, short life and they fall when their pollens are shed but the female has a long life like about 1.5-3 years and they mature after pollination only. The seeds arrangement is 2 seeds on each of fertile scale. The seeds are small in size and can be dispersed by wind but the larger ones can be dispersed by birds. For the dispersion of seeds, cones get opened and then dispersed by birds but in some cases, birds break the cones or in extreme cases, the cones are bound by resins which are melted by forest fire only (Michael et al., 2016). Some species of pine needles have large seeds and they are called pine nuts and they can be used for cooking and baking. Even the soft bark is edible and has high vitamin content. There is also a tea made from pine needles that are consumed in various regions (Zeng et al., 2011). The growth of pines is mostly in acidic or calcareous soil and they require good soil drainage. There are also some species of pine that require forest fire to regenerate and if not provided then decline in numbers. Many pine species need extreme conditions and are well adapted for growth in very high or low temperature. Pine has an important role in the regulation of food webs (Michał et al., 2016) and might be responsible for fungi activation for the decomposition of litter containing no nutrients (Filipiak, et al., 2016). They also play a role in exchanging plant matter between ecosystems (Michał et al., 2016).

Pine needles have various uses like they can be used in parks, wildlife sanctuaries for decorative purposes, some have large seeds that can be used as nuts for baking purposes (Solanki et al., 2017). There are lots of concerns associated with fossil fuels use like availability, uneven distribution, global warming, and many other factors contribute in this so as the fossils are depleting with time we need alternatives and biofuel is one of the major options available. Biomass has been used for a long time to produce heat and electricity but when used as a source for biofuel production there might be some energy loss.

1.3 Bio-fuels

The renewable energy production is seeing rapid increment and for transportation fuel the established options are ethanol derived from corn and already used cooking oil. The aim is to produce ethanol from cellulose which is still in initial phases and requires more effort and advance technology. The idea is to be not dependent on feedstock like grains and other raw materials because that would accomplish nothing and only give rise to competition between foods versus fuel. Bio-fuel production from algae is also a good option (Menetrez et al., 2014). Exploring algae bio-fuel industry has a lot of potentials and algae are already used for manufacturing food, in cosmetics, health products but not for bio-fuel. Microalgae can bring algae to a stage similar to that of cellulosic ethanol (Menetrez et al., 2012; Milledge et al., 2001). The synthesis from first-generation biofuels is comparatively easy as the feedstock is sugarcane, crops, corn, etc but they definitely create a dilemma of exhausting edible raw materials but on the other hand second-generation biofuels that constitute woody biomass, waste which are more suitable for synthesis but are hard to synthesize and require effort and advance technology. There is a difference between biologically derived ethanol and petroleum process derived ethanol. Ethanol is used as a solvent, disinfectant or additive to replacement for petroleum-based fuels (American Heritage et al., 2005). When exhaustibility is concerned, bio-ethanol definitely has an advantage over petroleum-based fuel as it is renewable, can be made from many raw materials, low carbon dioxide emission, harm to the environment is very less and it will not be geographically concentrated in case of availability. The trend that is going on is biofuel production from corn but it should not be our only option as many factors contribute in that like climatic changes such as rain, flood, drought which decide production of corn and if they are not in the order as expected then it will be a huge obstacle in production and there is an ethical issue attached if corn is used. Apart from corn there are other options like cellulosic biomass that require carbohydrate-rich feedstock like starch, cassava, grain, bagasse, beet, sorghum, potatoes, sunflower, molasses, wheat, etc.

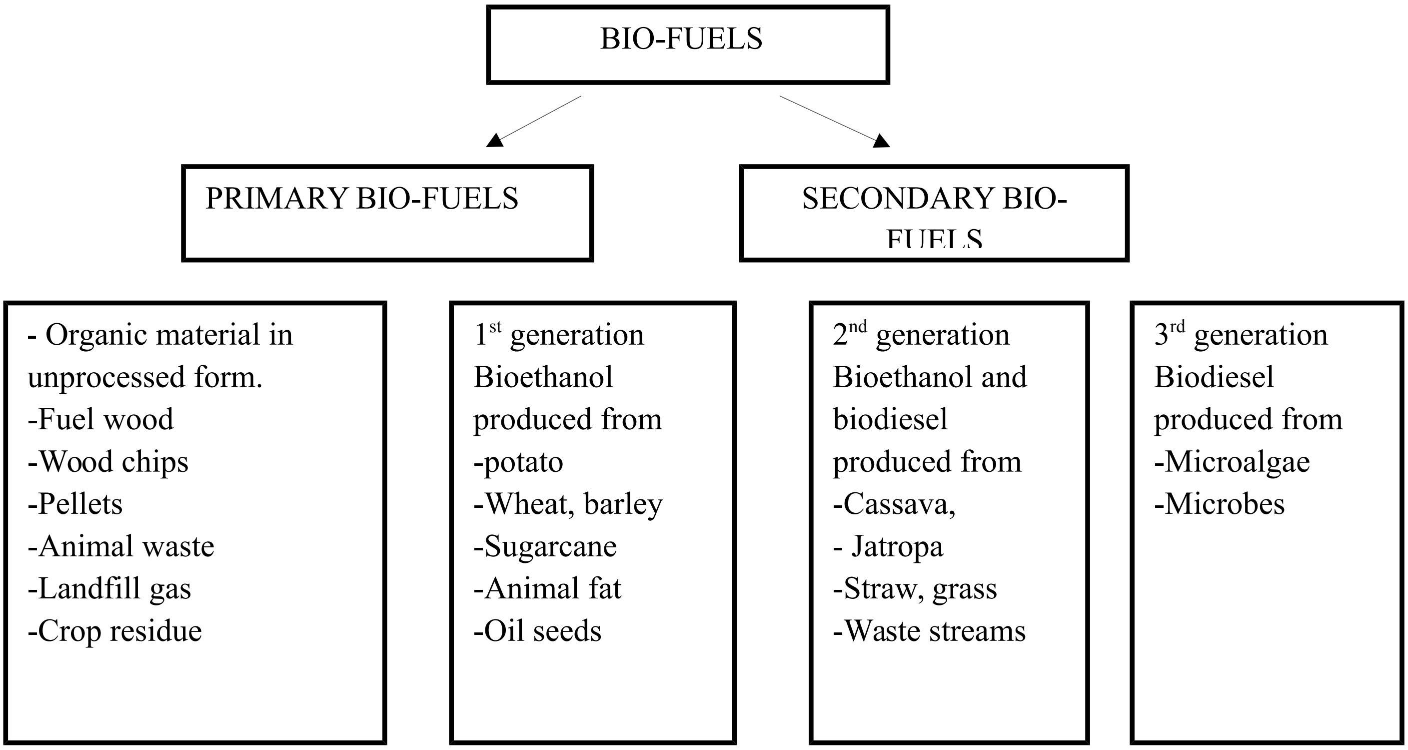


Figure1: Bio-fuel types and generations.

There are few factors that need to be accounted when producing ethanol like availability, cost, yield, the efficiency of the procedure, price of sales and in addition the transportation cost which is important and needs to be added when the operation is in the planning phase and the location is decided. Another energy source is oil from spent bleaching earth (SBE) and palm kernel (Knothe et al., 2005) but there are reasons why it cannot be used directly in the internal combustion engine and that is because of low volatility and high viscosity (Knothe et al.,2005; Atabani et al.,2012). Biodiesel is said to be the best substitute for petroleum fuel because of low toxicity, biodegradability, good lubricity, and it is compatible with the environment. The spent bleaching earth is the disposed waste in landfills and no attempt is made to recover the oil thus it is a huge threat to the environment as it can cause fire and pose as a pollution hazard. Spent bleaching earth contains oil that can be recovered and used as an energy source as it also decreases the cost that is attached with refining processes (Nursulihatimarsyila et al., 2010). Fresh palm kernel oil or recovered oil from spent bleaching earth can be used as bio-diesel. Biodiesel production from edible crops is not appreciated whereas production from non-edible crops requires lots of effort, technology, high cost, low yield but genetic engineering opens a completely new path for biofuel production. The aim is to increase the oil yield, quality and to improve tolerance towards biotic and abiotic stress. To increase the breeding process another new omics technology is introduced. It includes identifying and isolating genes used in lipid biosynthesis and their transfer to edible and not edible oil plants. To enhance oil yield and quality different genetic engineering strategies are introduced using two nonedible plants Jatropha and Camelina (Grover et al., 2013). There is an urgent need for another source of energy because of increasing population and pollution. Many countries are making huge efforts in this direction and one of them in Brazil. It has an efficient system based on sugarcane cultivation in the world (Garten Rothkopf et al., 2007). They have 40-year-old ethanol program and right now there is hardly any vehicle in Brazil that is running on gasoline. In the United States, corn is the main feedstock for the production of bio-fuel.

Chapter 2

Aim and Objectives

AIM

1. Collection and preparation of Pine needle biomass
2. Pre-treatment of cellulosic biomass using acid and alkali
3. Saccharification of pine needle biomass using cellulase

Objective

1. Biofuel production using pine needles
2. Pine needles can cause forest fires, soil erosion in hilly areas so it can be utilized.
3. Decreasing dependency on producing biofuels using edible materials
4. Bio-fuels causes less pollution than other fuels

Chapter 3

Review of literature

3.1. Biofuels and biomass

Biofuels are renewable sources of fuels with a biological origin and can be a good alternative to petroleum fuels. India has the potential to produce 2,171 million litres bioethanol while Brazil has a high bioethanol production rate with potential up 6,641 million liters [United States Department of Agriculture] (Michael et al., 2011). To boost production Indian government launched the blending of ethanol with petrol programme. The lack of resources is an increasing issue and biomass can be a solution for that. Biomass is a renewable resource because of its photosynthetic nature and in India; it fulfills about 75% of energy needs. Biomass refers to all biologically produced matter and all earth's living matter whose energy is derived from plant sources, such as agricultural, forestry, agro-industrial, human or animal wastes, which can be converted into convenient phases of fuel. Cellulase name is used for complex enzymes. It helps in catalysis and cellulose decomposition and it breaks down monosaccharides, polysaccharides, and oligosaccharides. Cellulose breakdown is economically very beneficial because it makes major constituent for plants (Klemm et al., 2005). Pine needles are waste of forest and they have no use as fodder. They cannot decay other biomass and are a major cause of wildfire but however, a rich source of biomass fuel. The groundwater gets depleted because of dry pine foliage and this foliage blocks sunshine preventing grass to grow on which cattle's feed. However pine needles biomass can be changed to renewable fuels using appropriate physical, thermo-chemical or biochemical conversion processes protecting the natural resources, flora, and fauna of the region and provide a rural livelihood.

Lignocellulosic biomass has the potential to end the conflict on food versus fuel. The waste can be used as biofuel substrates like in case of Rice straw which is a by-product of produced rice and is a great source of lignocelluloses (Kaparaju et al., 2009) and this rice straw was burnt before leading to air pollution and greenhouse gas emissions. Rice straws are becoming a popular choice for biofuel product because it is rich in carbohydrate polymers like cellulose and hemicelluloses but these polymers are bound by lignin that needs to be removed before saccharification is done (Ballerini et al., 1994). Cellulosic biomass saccharification is done using 3 enzymes endonucleases, exonucleases, beta-glucosidases that degrade cellulose to sugars. Commercial cellulase combines more than one source like *Trichoderma resei* and *Aspergillus niger* (Shishir et al., 2016)

3.2 Related research paper

(Danielle et al., 2015) used loblolly pine that is considered as potential feedstock in United States but when this pine was pre-treated, inhibitory products were formed that had to removed before hydrolysis and fermentation. To remove inhibitory products rinsing with dilute acid works. Citrate buffer strength was checked for rinsed and unrinsed biomass. Saccharification is still possible in unwashed biomass if buffer strength kept 50mM and result obtained was 65% indicating that inhibiting product is not the only factor responsible. **(Elsayed et al., 2013)** used rice straw to produce bioethanol using microorganism *Trichoderma reesei*. He dried the straws and both acidic and alkali pre-treatment along with ultrasound pre-treatment was done before enzyme addition. After all, this detoxification was done for charcoal removal and then fermentation was performed using

Saccharomyces cerevisiae. **(Zeng et al., 2012)** evaluated the chemical composition, antioxidant and antimicrobial activities of oil obtained from pine needles. Pine needle oil showed remarkable antioxidant activity and showed antimicrobial activity against food-borne microorganism with minimum inhibition concentrations revealing that pine needles oil has the potential to be a natural antioxidant and antimicrobial agent in food processing. (**Wayman et al., 2014)** worked on bioethanol production from lignocellulosic biomass where cellulose required 320˚C to transform rigid crystalline structure to amorphous one. Pre-treatment and first stage hydrolysis was done before fermentation and after that second stage hydrolysis and then purification. (**Elizabeth et al., 2016)** used 4 lignocellulosic biofuel manufacturing plant. Lignin is the primary molecule that contributes to recalcitrance because of complex structure. The new solvent is used for treating biomass gamma valerolactone. It is derived from biomass itself and can pre-treat any type of biomass. The advantages are neutral carbon balance and can manufacture co-product from biomass. (**Lu et al., 2012)** performed enzymatic hydrolysis at 36˚C and 50˚C for 72 hours in a 100ml flask and each flask contained 20ml to 50ml of 0.05 sodium citrate buffer and had solid to liquid ratio of 1:50, enzyme loading was 10-30 filter paper unit and a sample collected at 1,5,9,12,24,36,48 and 72 hours. Different parameters were observed and treatment was done to increase the digestibility of reed and yield obtained was 99.5%. (**Sulaiman et al., 2016)** performed enzymatic saccharification of date palm trees; he mixed 2% cellulosic waste with the appropriate amount of enzyme in a 100ml flask containing 20ml acetate buffer (pH 5) and sodium azide (0.3g/L). Hydrolysis carried out for 24hour at 50˚C using shaking incubator at 100 rpm. The reaction mix was centrifuged at 4000 rpm for 30min. Amount of reducing sugar was calculated using the DNS method. (**Juan et al., 2016)** wrote about the benefits of biofuels and how first and second generation biofuels can be used to decrease greenhouse emissions, our reliance on diesel, petrol and can increase energy diversity.

3.3 Cellulose

It is an organic compound with the formula ($C_6H_{10}O_5$) n (Crawford et al., 1981). It is a structural component of cell walls of green plants and of bacteria secrete for bio films secretion (Romeo et al., 2008). For industrial use, it is mainly obtained from wood pulp cotton and is used to produce paperboard and paper. Some animals can digest cellulose like ruminants and termites with the help of symbiotic bacteria. Humans can digest cellulose to only some limit.

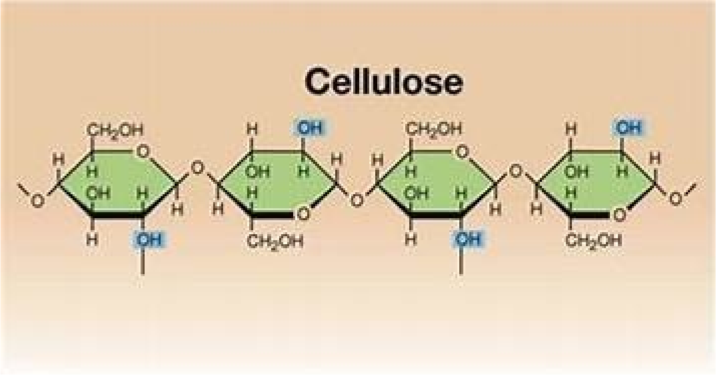


Figure2: structure of cellulose

Cellulose was discovered in 1838 by Anselme Payen who isolated it from plant matter (Crawford et al., 1981). The cellulose was used to produce the thermoplastic polymer, celluloid. Herman Staudinger determined polymer structure of cellulose in 1920. Kobayashi and Shoda were the ones who chemically synthesized the cellulose (Shiro et al., 1992). Cellulose is tasteless, odourless, hydrophilic and insoluble in water (Bishop et al., 2007). It is derived from D glucose units and is a straight chain polymer with no tangles. There is high tensile strength and lignin glues the cellulose fibres. Cellulose needs 320˚C and 25MPa to work as an amorphous in water. It has different hydrogen bonds location that decides the crystalline structure (Deguchi et al., 2006). Natural cellulose is cellulose I and it is converted to cellulose II irreversibly meaning cellulose I is meta-stable and cellulose II is stable. Many cellulose properties depend on the length of chain, degree of polymerization and the number of glucose units that constitute one polymer (Klemm et al., 2006). Plant-derived cellulose has a mixture of lignin, hemicelluloses (Klemm et al., 2006), pectin while bacterial cellulose is almost pure with high water content, high tensile strength and amorphous region of cellulose can be broken by treating with strong acid and producing nano-crystalline cellulose which has many important and desirable properties (Peng et al., 2011) and it is recently used as filler phase in bio-based polymer matrices for nano-composites production with superior thermal and mechanical properties. In the plant, cellulose is synthesized at the plasma membrane by rosette terminal complexes and in the animal it can be synthesized by tunicate animals (Kimura et al., 1999). Cellulolysis is cellulose breakdown into cellodextrins, it is a hydrolysis reaction. Cellulose temperature above 350˚C makes it undergo thermolysis, making it decompose into char, vapours, aerosols, etc (Czernik et al., 2004).

3.4 Cellulose application and sources

There are two sources of cellulose Natural and Synthetic. Natural fibres areas are fibres made from plants, vegetables, and other mineral sources.

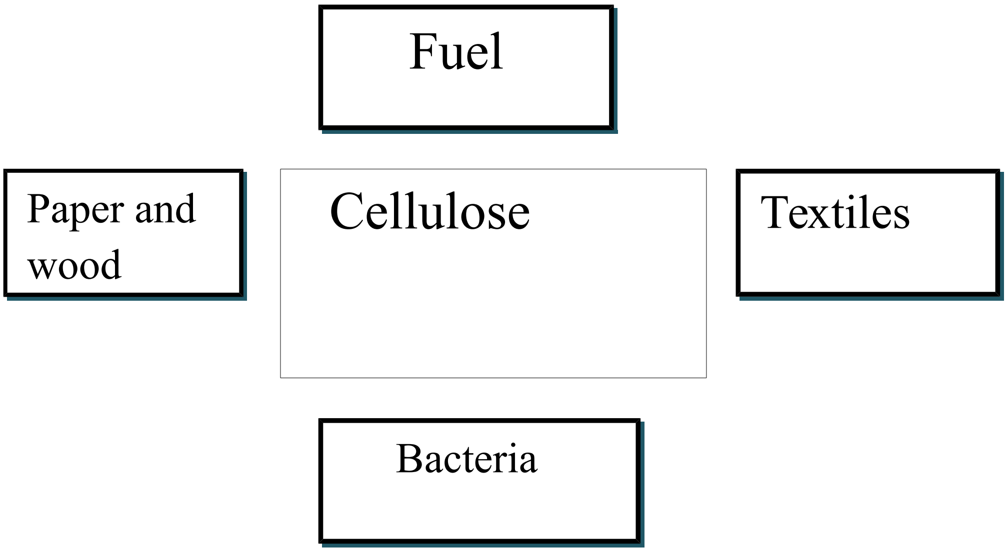


Figure 3: Celluose sources and applications

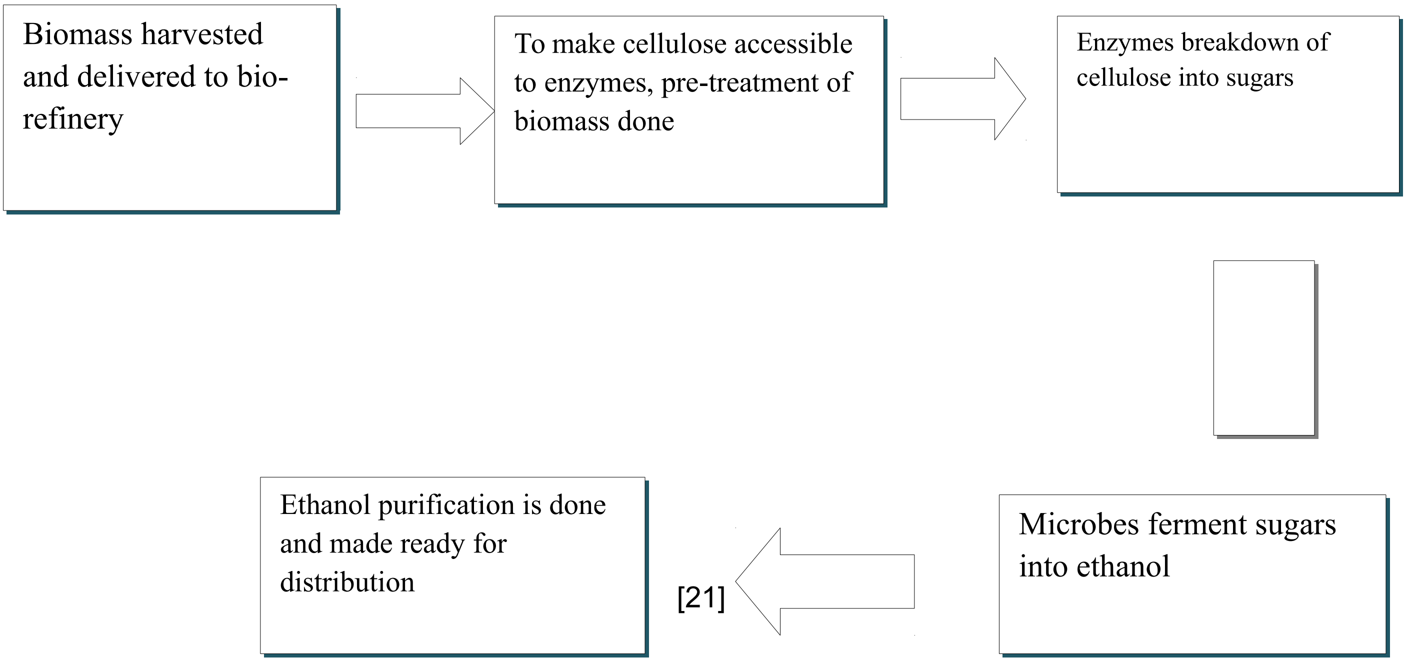
Table 1: Natural fibers sources and percentage

Category	Description	Examples	% of cellulose
Seed fibres	collected from seeds or seed case Fibers collected from seeds or seed case Fibre collected from fibre cases and seeds	Cotton, kapok	90
Leaf fibres	Fibre collected from leaves	Sisal, fique, agave	33
Bast fibres	Fibre collected from bast	Flax, jute, kenaf, Hemp,	33
Skin	Fibre collected from the skin	Ramie, rattan	33
Fruit fibres	Fibre collected from fruits	Coconut fibres	33-50

Synthetic fibres come from synthetic materials like petrochemicals and some are manufactured from natural cellulose. Cellulose fibres are generated from cellulose fibres and it comes from many sources.

3.5 Cellulose-based biofuels

Bioethanol is the most abundant bio-fuel. Bio-fuel has many applications and few of them include reducing carbon emission, nitrous oxides, and hydrocarbons. Biofuel can be used for increasing frequency emissions. It exhibits high vapour pressure and vaporization heat, thus increasing power outputs while ethanol use.



[21]

Figure 4: Cellulosic Ethanol production

The above figure shows the representation of cellulosic ethanol formation. The first step shows biomass delivery to bio refinery and then to make it accessible to enzymes and the enzymes break cellulose chains to sugars, later sugar is fermented into alcohol by microbes and purification is done.

3.6 Nano-cellulose uses

Nano-cellulose is used in the paper industry as nano-cellulose increases fibre bond strength having a strong effect on paper. It may be used as a barrier in greaseproof paper and as a wet added additive (Tapiale et al., 2010). Another use of nano-cellulose is in the food industry as it can be used as a replacement for low calorie. It can also be used for food processing of chips, fillings, wafers, etc. In pharmaceuticals nano-cellulose can be used as frozen gels in tampons, sanitary napkins, etc. Nano-cellulose tablet is also used for the intestinal purpose in dry form and nano-cellulose powdered form is part of many compositions (Kirsebornet al., 2011). It can also be used to make tobacco filter, battery separators, loudspeaker membranes and as super water absorbent.

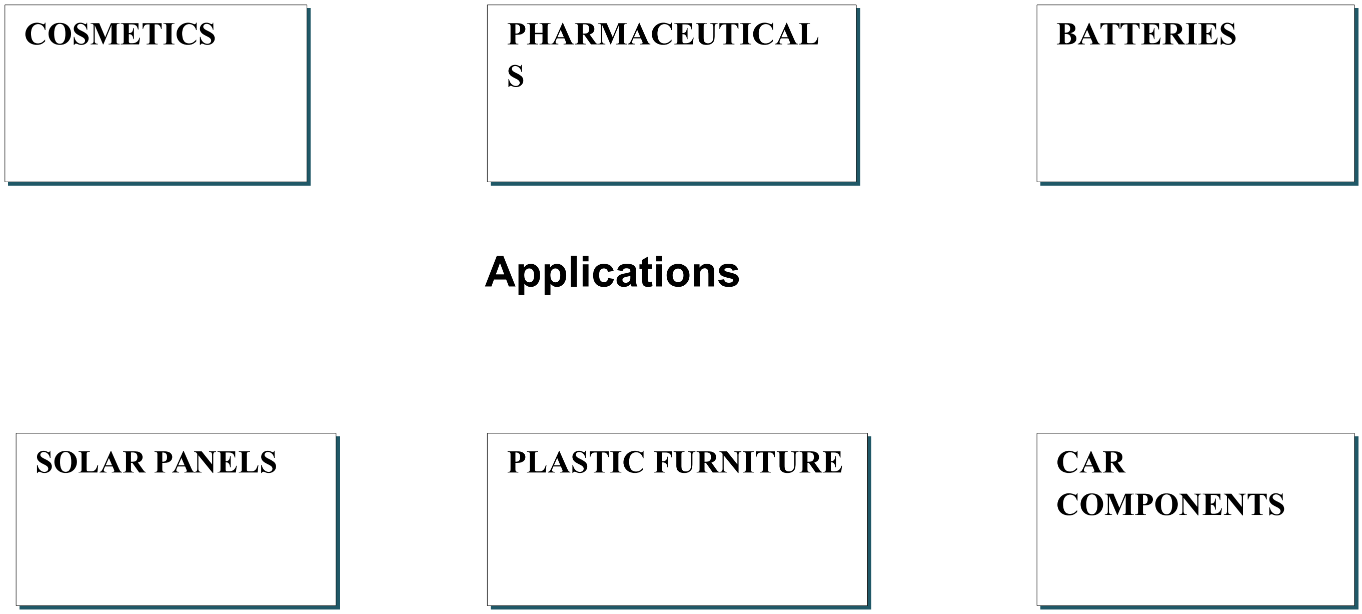


Figure 4: Nano-cellulose uses and applications.

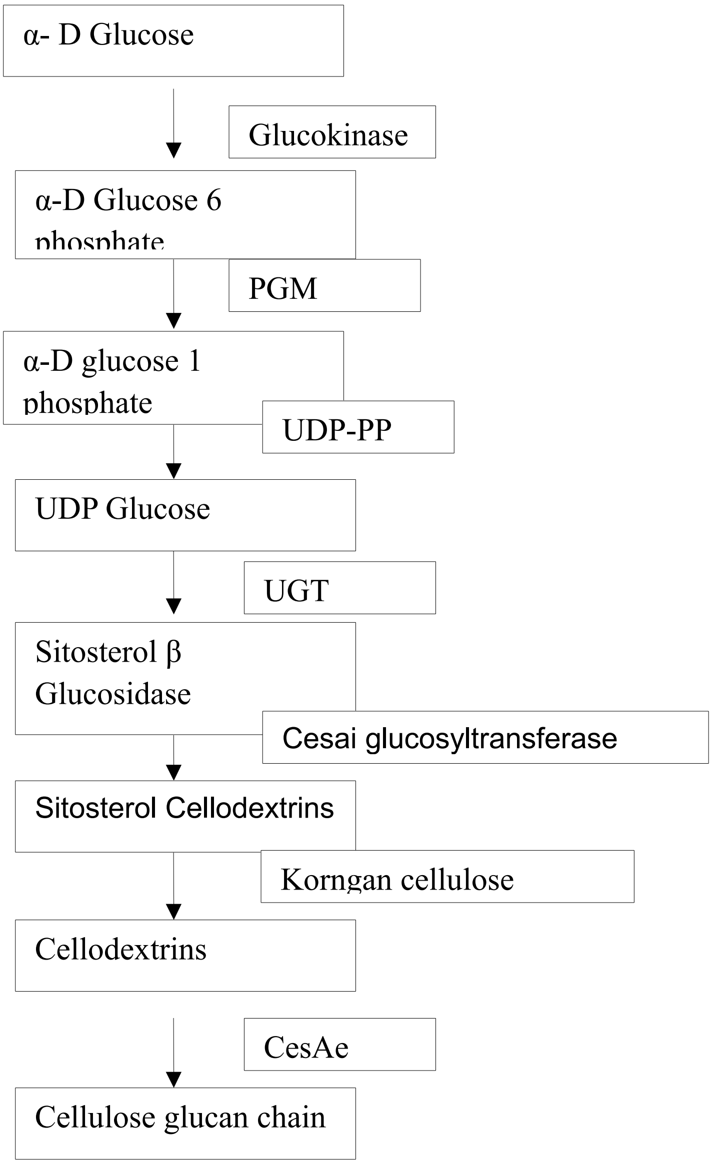


Figure 5: Biosynthesis of glucose

3.7 Cellulase

Cellulase enzyme has many uses in the biofuel industry. Cellulase has 3 components endoglucanase, exoglucanase, beta-glucosidase and these convert lignocelluloses into fermentable sugars. Cellulase enzyme is considered to be the third largest global enzyme in the market (Yoon et al., 2014). For the production of bio-fuel, cellulose needs to be hydrolyzed and cellulase enzyme breaks down cellulose (Srivastava et al., 2015). Nowadays cellulase production is done using fungi as it can produce complete cellulase system in comparison to bacteria who have less penetration ability than fungi although fungi might have some component loss like in case of *Aspergillus niger* which produces beta-glucosidase efficiently but not cellobiohydrolase whereas *Trichoderma* reset produces low beta-glucosidase.

3.8 Applications of cellulase

It is used in the textile industry as detergent, in helping with digesting capacity of animals, in the paper industry, in the pulp industry and there is a huge demand for this enzyme in the market. It also plays a role in the commercial processing of coffee.

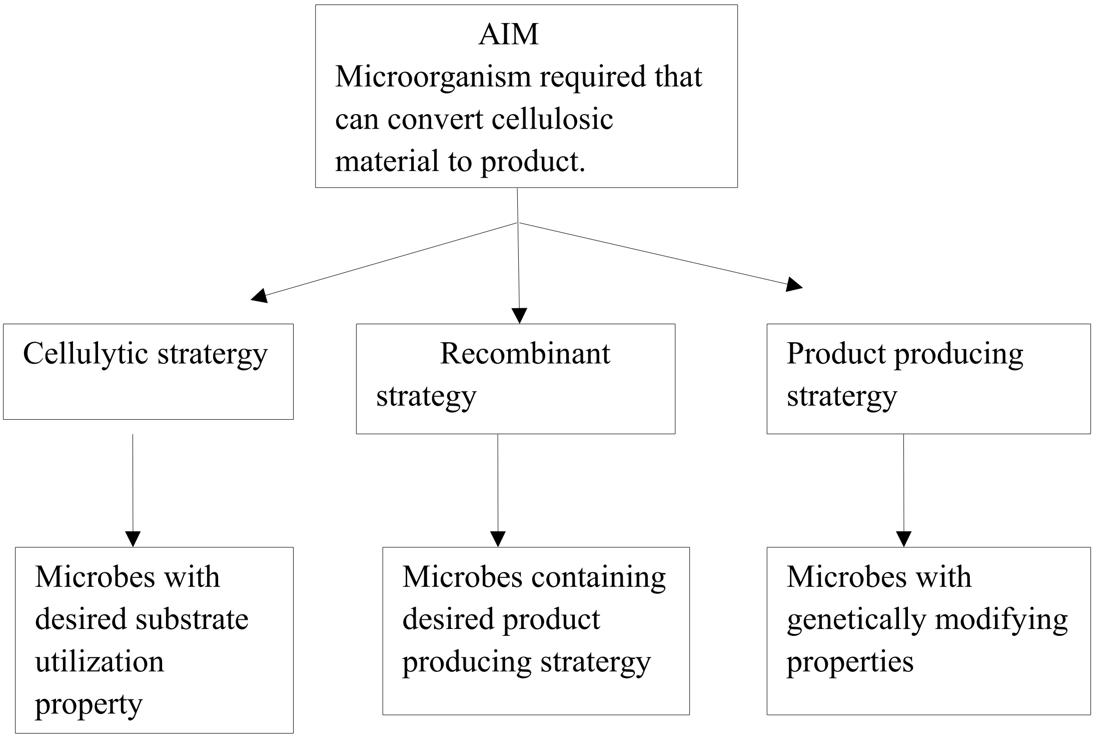


Fig 6: Bioprocessing stratergies to obtain microorganism of desired qualities

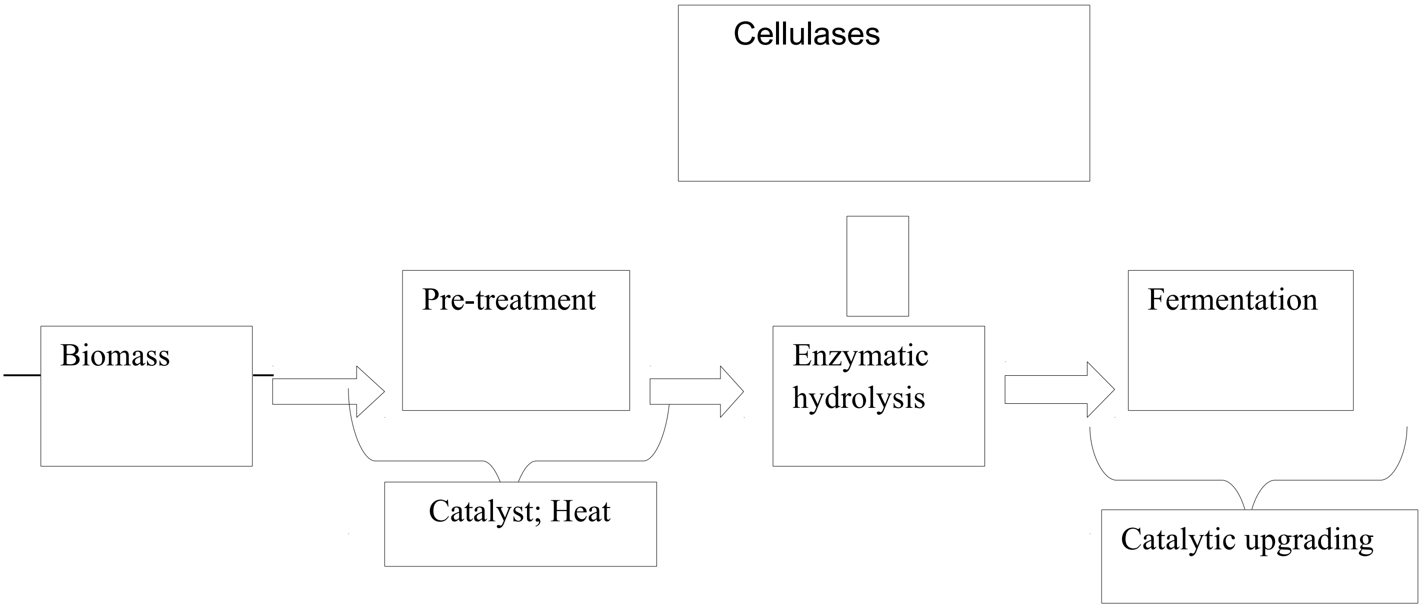


Figure 7: Cellulase uses in bio-fuel production

3.9 Future of biofuels

Bio-fuels have already eliminated 232 million metric tonnes of carbon [Renewable fuels association]. First generation biofuels are now added to 10% gasoline and in U.S fuel production from corn has increased. However fuel produced from edible sources loses impact in comparison with fuel produced from biomass and one factor that contributes the most is the low carbon footprint of cellulose. There are lots of projects being done keeping cellulose in mind and few of them are almost in the last stage like DuPont who opened world's largest cellulosic plant in Nevada and already has two commercial plants in Brazil, three in the U.S, two in Europe and one in China. Sweetwater energy of Rochester, New York has patented technology for production of low-cost sugars and lignin fibres from nonfood materials. Another company working on advanced biofuels is Red Rock bio-fuels and they convert biomass residues from forests and sawmills into jet fuel and diesel. There is research going on biofuel production using different waste like in Ethiopia waste of coffee is processed and used to synthesise fuel. These wastes are harmful to the environment as it is discharged into water bodies and can cause health problems. Sulphuric acid is used to hydrolyze waste and then sugar content is determined. It is believed that Africa will be the largest in bio-ethanol production by 2050 depending on advancement in the agriculture industry (Chimphango et al., 2011). The current research focuses on waste utilization, not on edible available options. Coffee by-products of wet processing constitute around 40% of the wet weight of the fresh fruit.Wet processing of coffee uses a lot of water to produce one ton of clean beans (Assefa et al., 2010). Coffee processing waste contains large amounts of organic substrates which are suitable for bioconversion into value-added bio-products (Kivaisi et al., 2010). Another research that's been going on for biofuel production is on Cassava. Biodiesel is produced from oil plants like *Jatropha, curcas* where the oil is blended with diesel to produce fuel (Davies et al., 2006). Crops grown for energy production purposes may be sugar cane (Hall et al., 2009), cassava, corn, and sweet potato (Ziska et al., 2009). However, the choice of feedstock is based on availability, and cost (Londo et al., 2010). The high productivity and yield of cassava (Ziska et al., 2009), along with its ability to grow on marginal soils (Dixon et al., 2002), requiring a minimum of labour (Karltu et al., 1998) and management costs (Jannson et al., 2009), have placed it among the candidates for bio-ethanol production. In terms of cassava, the above-ground biomass, including stem and leaf residues, is often not utilized for economic purposes (Ahamefule et al., 2005), apart from being a source of planting material (Pattaya et al., 2007) and the unintended use as fertilizer (Fermont et al,. 2008). Root residues, especially peels, which are poisonous due to high levels of cyanogenic glycosides (Guo et al., 2008; Pattiya et al., 2007), may be exploited for energy production taking into account their role in nutrient recycling (Fermont et al., 2008).

Another area that holds lots of potentials is Microbial bio-fuels especially Microalgae and cyanobacteria, these bacteria produce other by-products under stressed conditions. Microbes can convert acetic acid into methane gas, this methane or hydrogen gas can be converted to methanol and as hydrogen and methanol are water-insoluble so they can be recovered from the water.

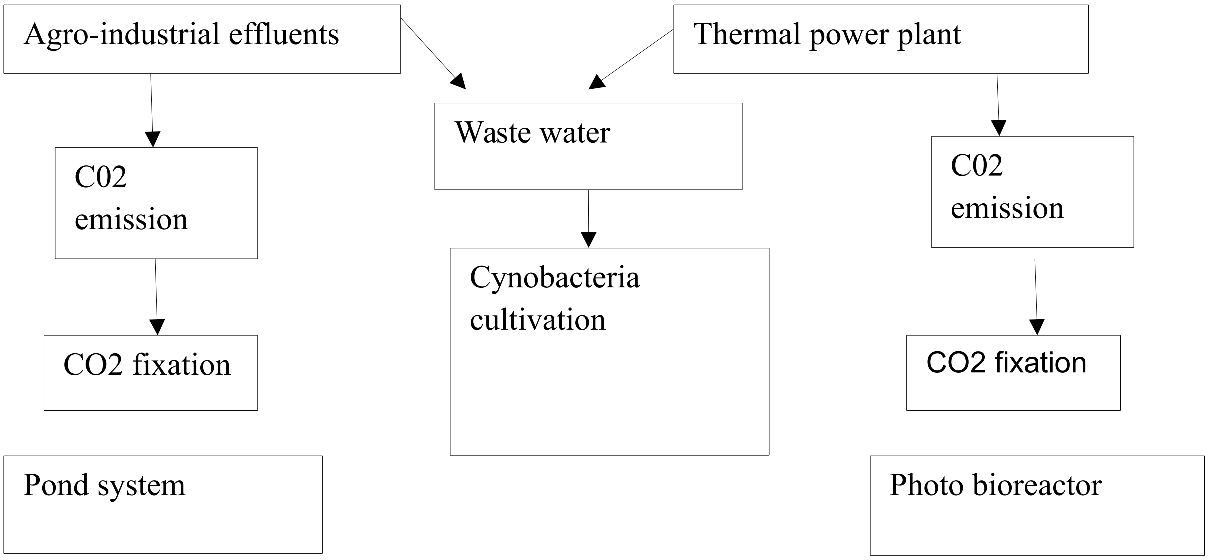


Figure 8: Sources of Cyanobacteria

3.10 Crops available for bioethanol production

Challenges that Biofuel production poses is increasing energy density on mass, work on present infrastructure, environment-friendly and not made by food supplies. Nowadays the biofuels produced are made from starch or vegetable oils and they have several issues like limited availability, minimum contribution to carbon dioxide emissions and fossil energy consumption. So in such conditions lignocellulose is a good alternative because it does not compete with any food source and has a wide availability but the conversion from woody biomass is definitely more complex as more hemicelluloses are present as well as the presence of crystallized cellulose structure (Taiichiro et al., 2010).

Table 2: Crop residue production and their availability for ethanol production in India

Crop	Total residue production	Non-fodder crop residue
Rice	114.28	20.57
Wheat	87.62	6.96
Cereals	33.29	0.0
Pulses	16.65	16.65
Other food grains	210.08	123.85
Sugarcane	147.10	132.36
Cotton+Jute+Mesta	32.63	32.63
TOTAL	641.65	333.0

Table 3: Estimated quantities (million-ton dry air weight) of major crop residue produced in the world.

Crop residue	USA	CHINA	INDIA	BRAZIL	OTHERS	TOTAL
Rice straw	12.81	221.76	157.69	16.01	340.73	749
Corn stover	233.92	101.40	10.92	31.98	185.78	564
Wheat straw	68.68	107.52	84.24	_	475.76	736
Sugarcane bagasses	11.20	38.34	100.54	177.21	237.71	565
TOTAL	326.61	469.02	353.39	225.20	1239.78	2614

All the above-mentioned countries have the mentioned crops grown in the majority and they have established or in the process of establishing infrastructure accordingly. According to Table 2, almost 50% of crops can be utilized or is available for bioethanol production

Table 4: Cellulose, hemicelluloses and lignin content of crop residues and other lignocelluloses

Agricultural residues	Cellulose (%)	Hemicellulose(%)	Lignin(%)
Hardwood stem	40-50	24-40	18-25
Softwood stem	45-50	25-35	25-35
Nut shell	25-30	25-30	30-40
Corn cobs	39-45	30-35	15-17
Grasses	25-40	35-50	10-30
Wheat straw	33-40	20-25	15-20
Rice straw	33-40	15-26	5-10
Leaves	15-20	80-85	0
Cotton seed hair	80-90	5-20	0
Coastal Bermuda grass	25-26	35-37	6-7
Switch grass	30-50	10-40	5-20

3.11 Plastid transformation technology

With the increase of interest in renewable resources, biofuel production is receiving special recognition. There are many enzymes that are used to convert cellulosic biomass to fermentable sugar and many of those enzymes can be expressed from the plastid genome in very high levels. Enzymes like cellulases, xylanases, glucosidases, pectate lyases, and cutinases can be expressed by the plastid genome. Many of them are thermostable as they were taken from thermophilic organisms adding a unique and important feature (Maliga P et al., 2004).

3.12 Genetically engineered crops for biofuel production

Genetic engineering of most feedstock is done or is in the process like rice, wheat, maize, switch grass, *etc.* Even though the foundation has been laid the challenge lies in the development of the efficient genotype and nonspecific system. The breeding plan might also have a role in the production of genetically engineered feedstock.Another point to be included in the production of the enzyme for hydrolysis in plants is that till now they are made in a bioreactor for commercial use but they can be produced in feedstock crops making the production little cheap in comparison to the present scenario. Increasing the biomass of plants is also possible by alteration of growth regulators and other factors that play a role in this is carbon allocation, carbon dioxide emission, water, nitrogen uptake, etc (Lee et al., 2008).

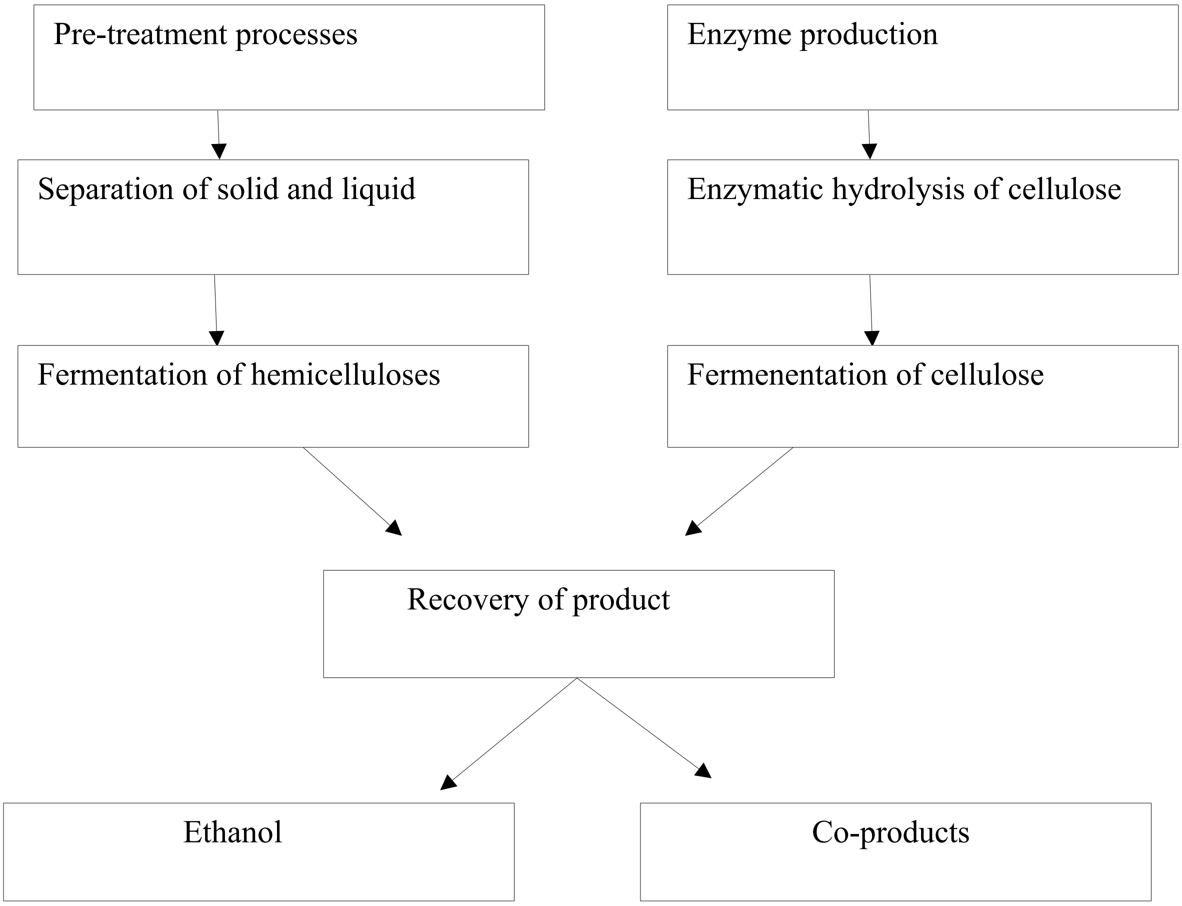


Figure 9: Ethanol production from cellulose

Chapter 4

Materials and Methodology

4.1 Chemicals used

For pre-treatment purpose sulphuric acid was used in different concentrations, ranging from 0.2% to 1%. The sulphuric acid concentration was 10 %. For assay purpose DNS was prepared, for which sodium hydroxide (2N) was used in amount 20ml, DNS 1g, and sodium potassium tartrate 30g. Sodium potassium tartrate was manufactured from Fisher Scientific and sodium hydroxide from EMSURE. Then for Citrate buffer preparation (0.1L), sodium citrate dihydrate was used in amount 2.409g and citric acid in 0.347g. Sodium citrate dihydrate and citric acid, both were obtained from Fisher Scientific. For phosphate buffer preparation (0.1L), Sodium phosphate dibasic heptahydrate was used in amount 2.021g and Sodium phosphate monobasic monohydrate in 0.339g. Then sodium acetate buffer was prepared using sodium acetate in amount 0.772g and Acetic acid in amount 0.035g. For Tris HCl (0.1L) 121.14 g of Tris was used and 100% concentrated HCl and NaOH was used to maintain pH.

4.2 Types of equipment used

Test tubes, Flasks, Beakers, Spatula, 96 well plates, Tarsons, Spectrophotometer, Hot air oven, Incubators, Water bath, Centrifuge, Weigh balance.

4.4 Methodology

4.41 Preparation of substrate

Pine needles were collected from outside campus. It was washed properly with distilled water, air dried and then grinded properly. 0.25g Pine needle was added in 5 tubes, 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1ml sulphuric acid was added and water added to make up the volume 10ml. The tubes were kept in incubation for 24hours and after that centrifuged at 8000 rpm for 10min at room temperature. Supernatant separated, sample put on aluminium foil and oven dried for 24 hours.

4.42 Preparation of glucose standard curve

Glucose standard curve was prepared with concentrations 0.2%, 0.4%, 0.6%, 0.8%, 1%. Glucose stock was prepared with 1g glucose in 100ml water. Water was added and incubated for 10min at boiling point and after cooling down 2ml DNS was added in each tube and O.D calculated at 540nm.

4.43 Optimization of incubation time

For optimization of Incubation time to measure the effect on saccharification of pine needles, 7 incubation periods were taken from 12 hours till 60 hours. Incubation of pine needle biomass was done by adding cellulase and sulphuric acid. After 24 hours incubation, pine needles were centrifuged and later oven dried to obtain dried biomass. Two samples types taken, treated and pre-treated. Treated samples were incubated with cellulase enzyme (1mg/10ml) and pre-treated were incubated with acid (sulphuric acid). Different incubation time 12hours, 24hours, 36hours, 48hours, 60hours were taken and samples were incubated according to them, then they were incubated in a water bath at 100˚C and after cooling 3ml DNS was added to each, later centrifuged at 8000 rpm for10 min then the supernatants were taken and absorbance measured at 540nm separately. Then we calculated reducing sugars using a standard glucose curve and checked that at what incubation time highest amount of reducing sugars were obtained and for which sample type.

4.44 Optimization of pH

3 buffers were prepared sodium acetate buffer (3-5 pH), phosphate buffer (6-7 pH) and Tris- HCl buffer (8-9pH). All these buffers, each 3ml along with pine needles dried biomass were boiled for 10min at 100˚C, then 3ml DNS added in each and 1ml sodium potassium tartrate added in each of them. Centrifugation was done at 8000 rpm for 10min, supernatants were taken and absorbance measured at O.D 540nm and then we calculated reducing sugars to know that at what pH maximum reducing sugar obtained.

4.45 Optimization of temperature

8 different temperatures were taken 25˚C, 30˚C, 35˚C, 40˚C, 45˚C, 50˚C, 55˚C, 80˚C. Samples were incubated at the mentioned temperatures then assay was done and O.D measured at 540nm. Reducing sugars were calculated using glucose standard curve and we identify that at what temperature we get the maximum amount of reducing sugars.

4.46 Optimization of agitation rate

Agitation rate on pine needle biomass was optimized, temperature was kept 130˚C till 300˚C. Samples were exposed to mentioned agitation rates and later incubated at 100˚C in a water bath. Centrifugation was done and absorbance measured at 540nm. Amount of reducing sugar was calculated with the help of the glucose standard curve.

Chapter 5

RESULTS

1. Preparation of substrate

Acid pre-treatment motive is to increase enzyme accessibility. It can be done using concentrated as well as diluted acid but diluted acid pre-treatment is preferred in order to ensure that no biomass is damage and it could also be a reason for the synthesis of microbial inhibitors. The sugar yield is not very high in the case where high acid concentration is coupled with high temperature. Dilute sulphuric acid is a cheap and effective method for the pre-treatment process. After pre-treatment, washing is done multiple times to obtain a neutral pH.

2. Glucose standard curve

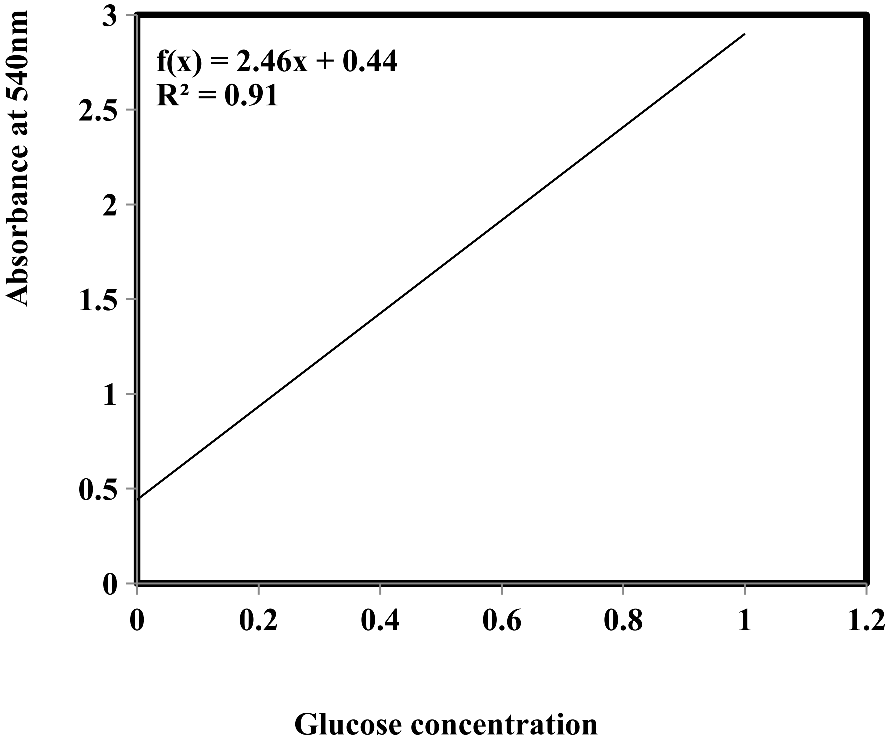


Figure 10 : glucose standard plot

Regression value square is 0.909. 10ml glucose stock was prepared with concentration being 10g/l. Absorbance was measured at 630nm. Glucose standard curve was required to calculate the amount of reducing sugars present when substrate exposed to different parameters.

3. Optimization of Incubation time

Incubation was done for different time periods using shaker incubator (Eppendorf). The treated biomass contained cellulase enzyme and pre-treated contained sulphuric acid. The incubator used is a bacteriological incubator. 5 different time periods were set for incubation and substrate containing cellulase enzyme and another one containing sulphuric acid was exposed to them. The highest amount of reducing sugar was obtained at 36 hours. The sulphuric acid concentration was 0.2% because high concentration might damage the biomass or burn it making it unsuitable for use.

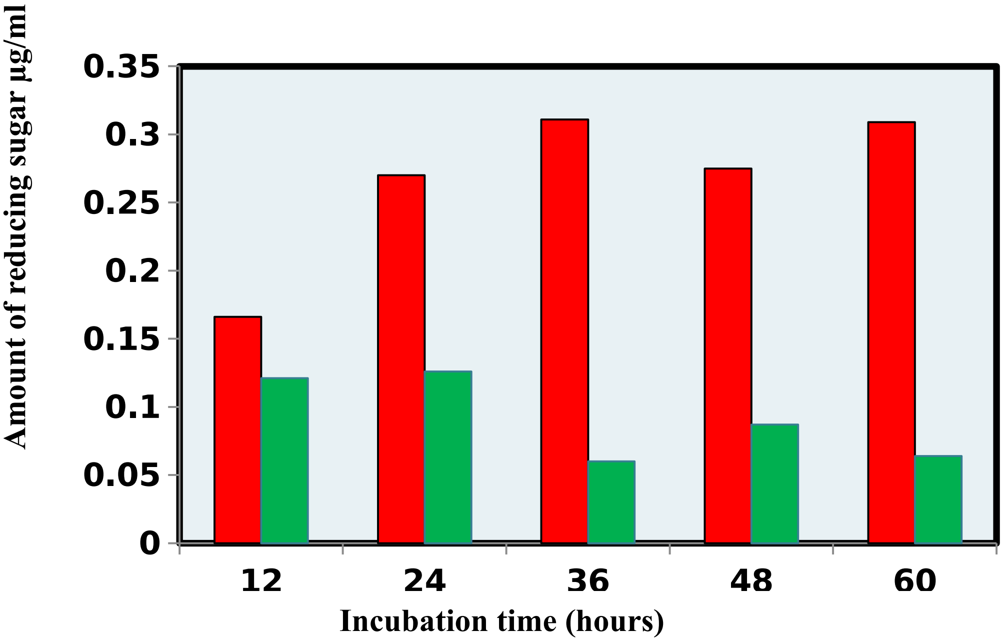


Figure 11: Incubation effect on PNB saccharification

Incubation time kept was 48 hours , highest sugar yield obtained was 286mg/g at 5min (Bijender et al., 2015). They used a high amount of substrate and incubation time difference was kept small. The best result obtained at incubation for 6min (Jonsson et al., 2014).

4. Optimization of pH

3 buffers were taken [illegible] Tris HCl. Treated and pre-treated biomass [illegible] and observed. Maximum reducing sugar was obtained at pH5 for treated and pH7 pre-treated. pH parameter

Table 9: Amount of reducing sugar obtained in treated and pre-treated biomass

helps in identifying the buffer and the pH at which amount of reducing sugar is obtained at most. The highest amount of reducing sugar was obtained at pH 3 (Jonsson et al., 2014). Substrate amount was 0.25g for pre-treated and treated for each buffer at different pH and then buffers were added 3ml each at different pH and later DNS added for absorbance measurement at 540nm.

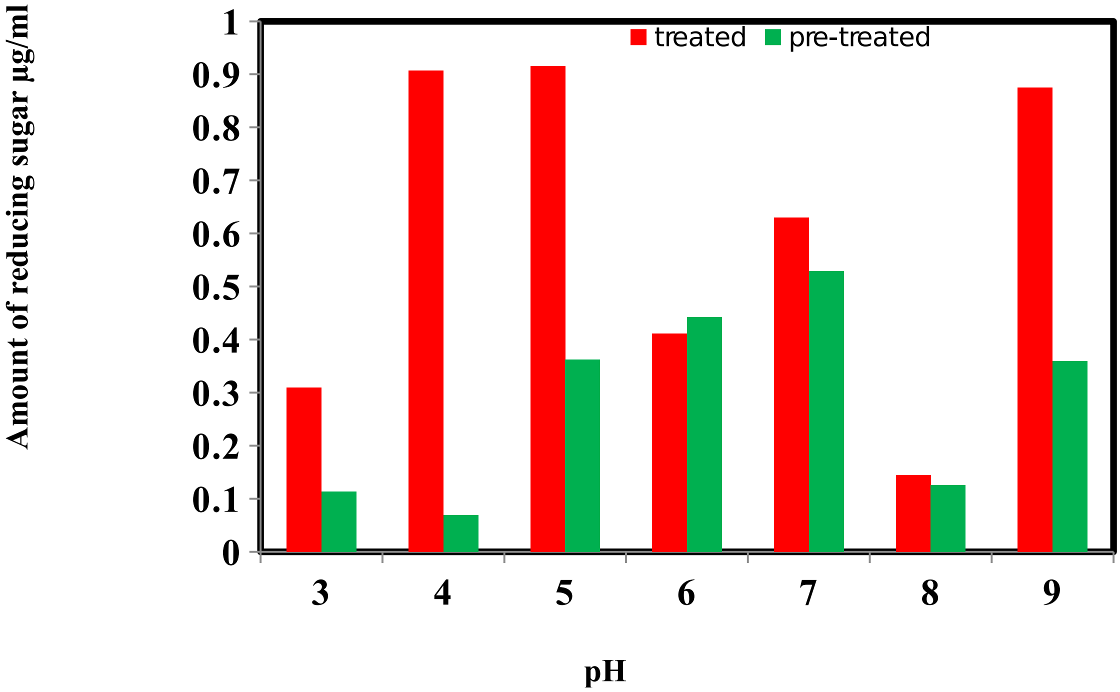


Figure 12: Ph effect on PNB saccharification

5. Optimization of temperature

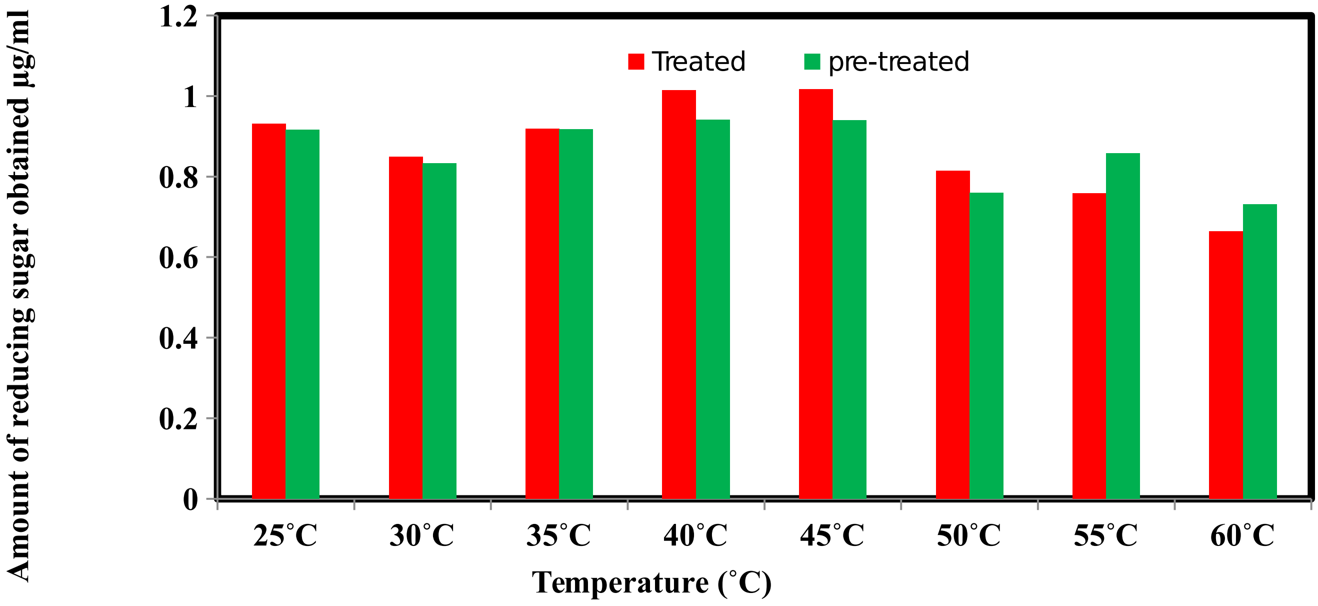


Figure 13: Temperature effect on PNB saccharification

Treated and pre-treated biomass was exposed to a temperature between 25˚C till 60˚C. The highest amount of reducing sugar was obtained at 45˚C. If exposed above 60˚C then there is a chance that enzyme might get deactivated as it needs proper handling and temperature. 0.25g substrate added for each temperature and then citrate buffer at pH 4 used in amount 3ml, later 3ml DNS added and after centrifugation absorbance was measured at 540nm.

Highest temperature taken was 85˚C and yield obtained was 286mg/g (Bajaj et al., 2015)

The highest temperature taken was 180˚C and yield obtained was 0.33mg/g (Jonsson et al., 2014).

6. Optimization of agitation rate

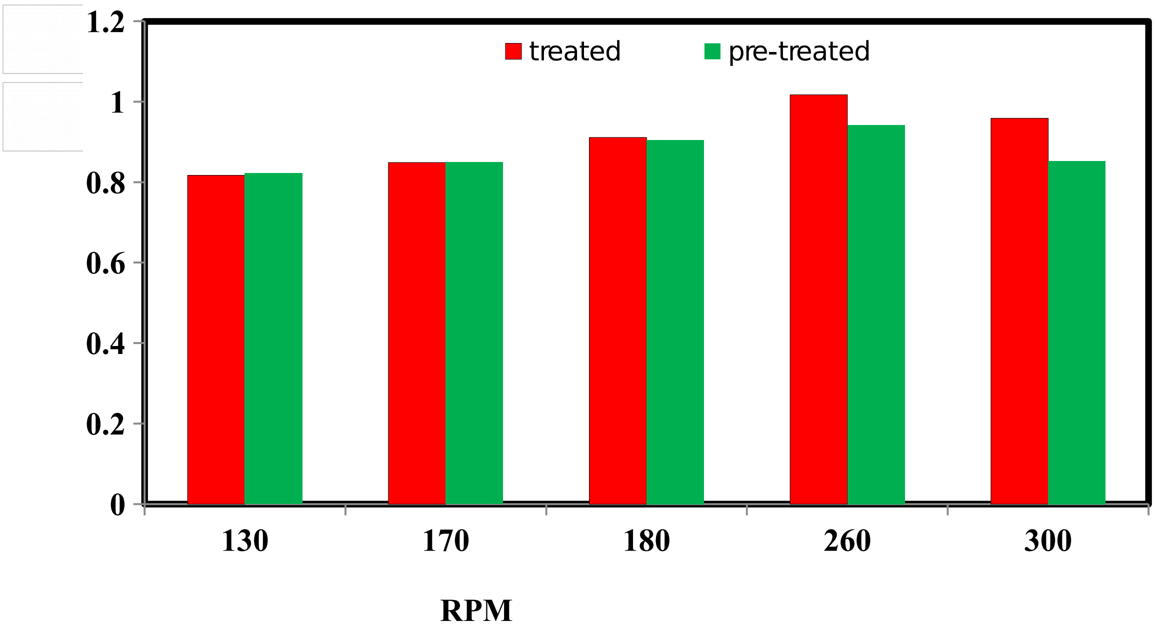


Figure 14: Effect of RPM on saccharification.

Amount of substrate taken was 0.25g, 3ml citrate buffer at pH 4 was added and the composition was put at different rpm ranging from 130 till 300 to measure rotation frequency and annoting the number of rotations done in one minute around a fixed axis. And then centrifugation was done at 8000 rpm for 10min to make sure that substrate settles down so absorbance can be measured at 540nm and later reducing sugar is calculated using glucose standard curve. The maximum yield obtained at 170 rpm (Jonsson et al., 2011)

CONCLUSION

It may be concluded that the best parameter for pine needle biomass saccharification is 36 hours and definitely at moderate temperate with low sulphuric acid. The pH optimum for this is 5 and temperature is 45˚C. The optimal concentration for sulphuric acid is 0.2 %. Pine needles are waste that can be a cause for many problems and with the increasing requirement for fuels and the struggle to minimize pollution caused by them, biofuels are definitely an option. Even though their processing requires technology and time but the end result is worth all the hassle. With the increasing population, production of biofuels from edible raw materials will not be an answer so more research needs to be done on biofuel production from non-edible materials and advancement in technology is required to minimize effort in their production. There are many countries like Brazil and the United States that are taking huge steps in biofuel production making efforts to be well established in this and trying to build infrastructure and vehicles keeping bio-fuels in mind. Bio-fuel holds a promising future and with the speed, the non-renewable energy sources are declining having an alternate energy source as an option is going to pay off.

Chapter 6

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