Production of biofuels from lignocellulosic biomass in pulp and paper mill effluents for low carbon society

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Abstract

Carbon Dioxide (CO_2) is the most prominent Green House Gas (GHGs) in the Earth's atmosphere is responsible for climate change and other environmental problems. However, CO_2 may be converted into organic compounds and lignocellulosic biomass. The pulp and paper mill is a major industrial sector utilizing huge amount of natural product (woody and non-woody plants), inorganic and organic materials along with large volume of water in different stages of the paper manufacturing. In kraft pulping, sodium hydroxide and sulphide are used for pulping, forms dark brown colour due to lignosulphonics, resin acid and phenols. The lignocellulosic components of pulp and paper mill effluent may be extracted, degraded by microorganisms, and converted to ethanol and biodiesel. However, lignin content hinders degradation and bioconversion processes for production of biofuels. Various physiochemical methods like use of acids to precipitate lignin are in use, however, they are inefficient, costly and produce large amount of sludge. Lignin-degrading microorganism may be applied after optimization of processes for removal of toxic compounds and production of biofuels. It is important to isolate and characterize appropriate microorganisms for degradation of chemical compounds hinders the saccharification processes and further fermentation for biofuel production. The desired microorganisms should have a high growth rate, high lignocellulose degradation ability, high tolerance during fermentation processes, low operation cost and rich in valuable components in their biomass.

1. Introduction

Carbon dioxide (CO_2) is the most prominent Green House Gas (GHGs) in the Earth's atmosphere together with methane, nitrous oxide, chlorofluorocarbons and ozone. Total atmospheric emission of carbon dioxide from fossil fuel projected to increase from 6.79GtCyr^{-1} in 2000 to 8.35 GtCyr^{-1} in 2010 and 9.97GtCyr^{-1} in 2020. The total worldwide energy consumption in 2008 was 474 exajouls (474×10^{18} J) with 80 to 90 % derived from the combustion of fossil fuels emit carbon dioxide. Increasing amounts of GHGs in the atmosphere and global warming could also lead to more health concerns, vector-borne diseases, frequent and powerful extreme climatic events, probability of floods, a rise in sea level, decrease in Glaciers volume, Arctic ice thickness, ice mass and availability of water, retreat of sandy shorelines, extinction of flora and fauna and displacement of human. Therefore, it is important to reduce carbon dioxide in the environment through designing low carbon society (LCS). Sociopolitical and technology based remedial methods are available which can be enforced properly to contain carbon dioxide in the environment. Adaptation and sequestration of carbon would be significant possibilities for low carbon

society. Microbial communities, autotrophic and chemolithotrophic, and higher plants have the potency to sequester carbon dioxide by photosynthesis processes and forms organic compounds and biomass, which may be converted in to ethanol, biodiesels, biohydrogen and microbial fuel cells.

Forests cover around 3.9 billion ha of the earth's land area worldwide. Fifty percent of all biomass with an estimated annual production of 50 billion tons is lignocellulosic (Chen *et al.,* 2009). Half of the residues used as material, energy, green manure, feed for low producing ruminants, and production of pulp and paper and other related commercial items, however, half of the residue remain unused. Unused biomass is major source of "waste"- pose an environmental pollution problem which may be converted in to value added products by biotechnological methods. Human activity generates millions of tons of wastes every year; the most abundant is of biological character, namely biomass. Biomass refers to all renewable organic matter, including trees, herbs and associated residues, as well as animal, agricultural, industrial, and municipal solid wastes. All of these materials have great potential that enables them not only to meet energy needs or nutritional demands from farming but also to produce a wide variety of chemical feed stocks such as organic acids, solvents, or biopolymers (Singhal and Thakur, 2009b). Although some of these applications allow direct management of the wastes, physical, chemical, and biological treatments are necessary to derive full benefits from the use of these natural resources.

Lignocelluloses are the building blocks of all plants and are ubiquitous to most regions of our planet. The basic chemistry of lignocelluloses is cellulose, hemicellulose, and lignin (Pandey *et al.,* 2000). Cellulosic feed stock consists of lignocelluloses which are mainly comprised of cellulose, a polymer of six-carbon sugar, glucose; hemicellulose, a branched polymer comprised of xylose and other five-carbon sugars, and lignin consisting of phenyl propane units (Rana *et al.,* 1996). Lignocellulose such as sugar cane bagasse and wood has a complex structure, and is primarily composed of lignin (25%), hemicellulose (25%) and cellulose (40–50%) used for pulp and paper production (Pandey *et al.,* 2000; Neureiter *et al.,* 2002). Cellulose is made up of linear chains of β-1,4-linked D-glucose residues, however, hemicellulose is made up of branched heteroglycans with a backbone of β-1,4-linked D-xylopyranosyl residues with branches of α-1,3-linked L-arabinofuranosyl and α-1,2-linked 4-O-methyl-glucoronic acid residues. Lignins are heterogeneous, three dimensional polymers composed of oxyphenyl propanoid units connected by C-C and C-O-C linkages (Gnanamani *et al.,* 2006). It is formed by random coupling of coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. Productions of ethanol and biodiesel using lignocelluloses are well established, however, utilization of lignocelluloses from effluent of industries could make biofuel production more competitive with fossil fuel and also in mitigation of green house gases (Lynd *et al.,* 1991). One important requirement for biofuel production is an efficient microorganism which can able to degrade lignocellulose and ferment a variety of sugars (pentoses and hexoses) as well as to tolerate stress conditions (Wyman, 2003).

Conversion of lignocellulose into fermentable sugars is possible through thermal, chemical, or enzymatic hydrolysis (Laser *et al.,* 2002). Pretreatment technology is important steps for improving the conversion of cellulose to glucose in following enzymatic hydrolysis (Eggeman and Elander, 2005). Prior to ethanol fermentation by a microorganism, the feed stock needs to be processed by saccharification technology in order to release fermentable sugars. Industrial bioconversion of renewable resources is mediated primarily by enzymes. There are three classes of enzymes acting synergistically in cellulose hydrolysis: endoglucanases, exoglucanases and β-glucosidases, inhibited by production of end products during saccharification and fermentation. Lignocellulose as a feed stock presents two major challenges for ethanol production due to recalcitrant to biodegradation and presence of pentose and hexose sugars (Laser *et al.,* 2002; Chen *et al.,* 2009). Industrial biocatalysts, such as the common yeast, *Saccharomyces cerevisiae*, rarely possess native pathways able to efficiently ferment both hexoses (such as glucose) and pentoses (such as xylose). Metabolic engineering through genetic modification may be an effective means of manipulating the capabilities of the microorganisms (Chen *et al.,* 2009).

The cellulosic and hemicelluloses sugars obtained through acid and enzymatic hydrolysis can efficiently be used for ethanol fermentation either by separate fermentation of individual hydrolysate or fermentation of mixed hydrolysate using co-culture (Herna´ndez-Salas *et al.,* 2009). However, in co-culture cultivation, optimum growth conditions of the yeasts would be different and might result in lower efficiency and lower product yield. Therefore, proper optimization of fermentation processes is significant for ethanol preparation. *S. cerevisiae* efficiently converts both glucose and mannose into ethanol, but is unable to convert xylose into ethanol (Thakur, 2009). Other fungal yeast-like species, e.g. *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus*, have been found to be highly efficient xylose-fermenting strains that can be used in ethanol production (Agbogbo *et al.,* 2006). However, these yeasts have a relatively low ethanol yield and inhibitor tolerance. For enhanced production of sugar and ethanol it is essential to optimize the composition of culture media and process conditions. There are several factors that affect enzymatic hydrolysis of cellulose including substrates, cellulase activity and reaction conditions (temperature, pH, etc.). To improve yield and rate of the enzymatic hydrolysis, research has focused on the optimization of the hydrolysis process and enhancement of cellulase activity. Taguchi methods have been widely used to

optimize the reaction variable by devising minimum number of experiments (Prakasham *et al.,* 2005). This approach also facilitates to identify the impact of individual factor and find out the link between variables and operational conditions. Analysis of the experimental data using the ANOVA (analysis of variance) and factors effect, gives the output that is statistically significant (Kackar, 1985; Phadke and Dehnad, 1988). Hence, for better efficiency of ethanol production, the approach of separate hydrolysis and fermentation was preferred (Olsson and Hahn-Hagerdal, 1993). In this paper attempts have been made to review prospects of lignocellulosic waste resources present in pulp and paper mill effluent (Thakur, 2004) for production of bioethanol and biodiesel. The efficient saccharification and fermentation processes are also discussed by optimization of process parameters for enhanced production of biofuels by microorganisms.

For our goal of low carbon societies (HICEC, 2010), we reviewed minutely the fuel production possibility from lignocelluloses as one of renewable energy (Boyle, 2004; Pimentel, 2008).

2. Pulp and paper mill effluents

2.1 Pulp/paper mill effluent

The pulp and paper sector represents one of the energy intensive and highly polluting sectors within the economy of a country and is therefore of particular interest in the context of both local and global environmental discussions (Pokhrel and Viraraghavan, 2004; Singhal and Thakur, 2009b). Increases in productivity through the adoption of more efficient and cleaner technologies in the manufacturing and bioconversion sector will be most effective in emerging economic, environmental and social development objectives in current scenario of climate change and designing a low carbon society. There are two main steps in the process of paper making that generate pollutants. Firstly, pulping that generates highly coloured effluent rich in lignin, its breakdown products and hemicellulose. Secondly, bleaching that generates effluent depending on the type of bleaching agent used, for example, use of chlorine leads to formation of chlorinated aromatic compounds (Figure 1). Both the

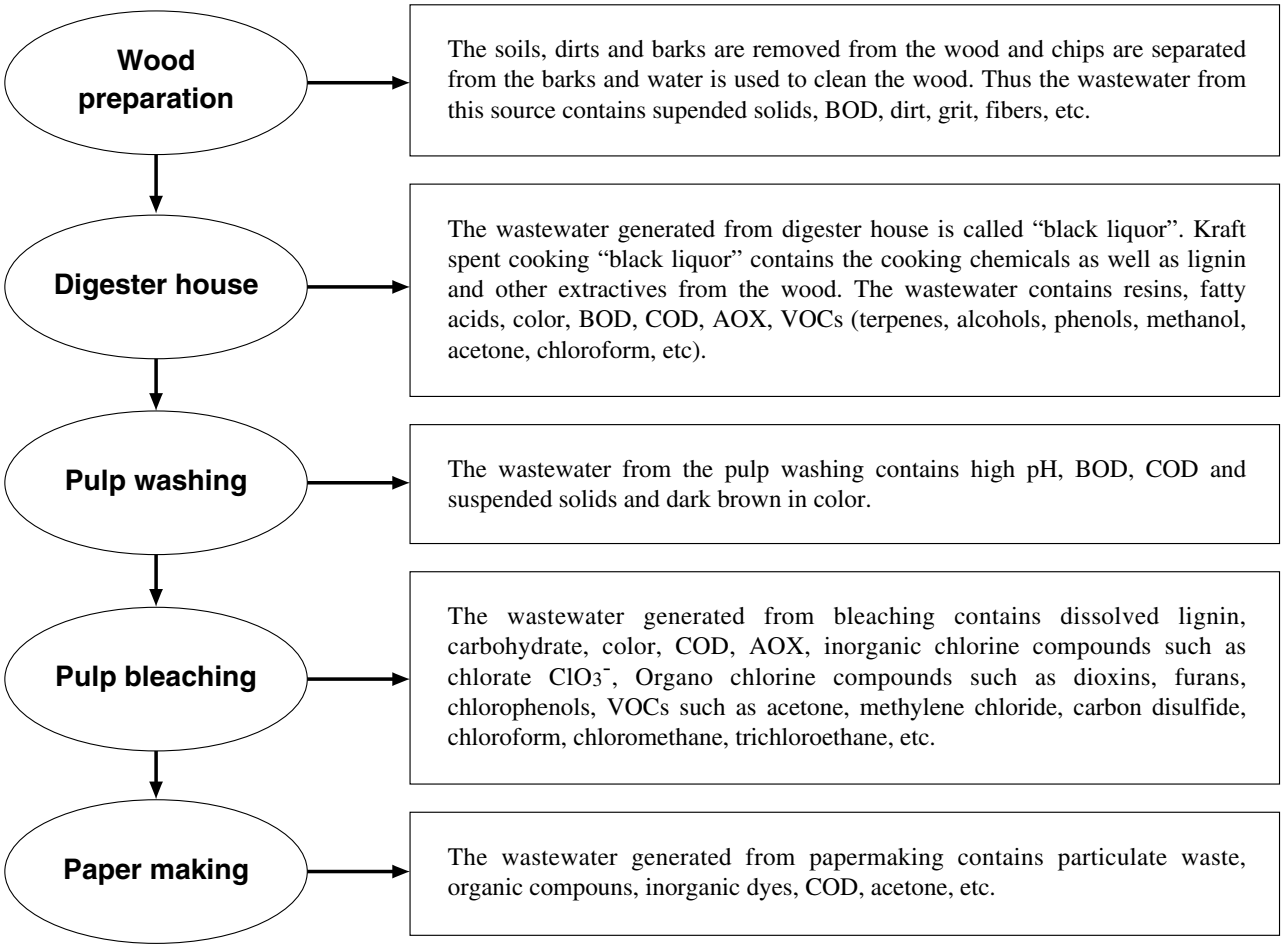

Figure 1. Process for preparation of pulp and paper from woody and non-woody plants, and generation of pollutants from various sources of pulping and paper making.

effluents have different characteristics and need specialized treatments. Almost 150m^3 tons of waste water are generated for producing 1 ton of paper (Pokhrel and Viraraghavan, 2004). The effluent is highly toxic having recalcitrant compounds that persist in the environment, adversely affecting flora and fauna (Ali and Sreekrishnan, 2001; Singhal and Thakur, 2009a).

Four different families of organic compounds that can be found in pulp and paper mill waste waters and sludge sediments are biocides, resin and fatty acids, surfactants and plasticizers, and chlorinated organic compounds (Ali and Sreekrishnan, 2001). Biocides are often used for wood preservation and during paper-making to avoid problems associated with microbial, fungal and algal growth. Biocides used in paper-mills can be of different types: 2,2-dibromo-3-nitrilpropionamide (DBNPA), 2-(thiocyanomethylthio)-benzotiazole (TCMTB) and etc. Wood extractives include lipophilic (fatty and resin acids (RAs), sterols, steryl esters and triglycerides) and hydrophilic (lignans, low-molecular-mass lignins, lignin-like substances and hemicelluloses) compounds that dissolve in waters during paper production are present in effluent. Resin and fatty acids are of different types: linoleic acid, stearic acid, palmitic acid, margaric acid, isopimaric acid, dehydroabietic acid, dichlorodehydroabietic acid and etc. which are toxic to aquatic life, causing jaundice in rainbow trout. Surfactants, such as linear alkylbenzene sulfonates and alkylphenol ethoxylates, are present in effluent because of their use as cleaning agents or as additives in antifoamers, deinkers, dispersants, etc. The various chlorinated compounds that are utilized or produced during the bleaching process and are detectable in the effluents and sludge are chlorolignin compounds, chlorophenols such as pentachlorophenol (PCP: Sharma *et al.,* 2009), chlorobenzenes, chlorinated acetic acids, chlorinated thiophenes, chloroguaiacols, chlorosyringol, chlorovanillin, chlorocatechol, polychlorinated dibenzo-para-dioxin (PCDD), polychlorinated dibenzofuran (PCDF), polychlorinated biphenyl (PCB) (Singhal and Thakur, 2009b). The major natural compounds may be degraded and converted in to biofuels.

2.2 Lignocellulosic biomass in effluent

Lignocelluloses are a major renewable natural resource of the world. It is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter (Duff and Murray, 1996). The major components of lignocellulose materials are cellulose, hemicellulose and lignin (Pandey *et al.,* 2000). Cellulose is usually coated with other polymers, predominantly xylan and lignin, which hinder cellulolysis. The chemical composition of cellulose is simple, consisting of D-glucose residues linked by β-1,4-glycosidic bonds to form linear polymeric chains of over 10,000 glucose residues. Physical and chemical evidence indicates that native cellulose contains both highly crystalline and less ordered amorphous or para crystalline regions. The cellulose crystal in the fibres of higher plants associated with other cell wall components such as lignin and hemicellulose resulting in even more complex morphologies.

Hemicellulose, second most abundant non-cellulosic plant polysaccharide, includes glucose, mannan, xylan, and organic acids. Hemicellulose occurs in primary and secondary cell wall of the plant, and plays a passive role mainly by filling the voids around cellulose fibrils. They constitute 35% of total dry weight of higher plants and are an important structure in aggregation of native cellulose. Hemicellulose is a highly branched, heteropolymer, non crystalline in nature and readily hydrolysed. It is made up of pentose (D- xylose, L-arabinose) and hexoses (D-galactose, D-mannose, L-rhamnose, L-facose) and organic acids such as D-glucuronic acids (Rana *et al.,* 1996). Polymers of xylose are major component of hemicellulose and most abundant plant polysaccharides in plant materials after cellulose. In general xylan content account for 15-30% of the total dry weight in angiosperms and 7-12% in gymnosperms. Softwood xylan contains glucomannon and arabinoglucoronoxylan while hardwood hemicellulose is glucoronoxylan.

Lignin, an integral part of the secondary cell wall of plants, is a complex polyphenolic arising through dehydrogenative free radical polymerization of p-coumaryl, coniferyl and sinapyl alcohols in which phenyl propane unit forms the basic structure (Glenn *et al.,* 1986). In lignin more than two third of phenyl propane units are linked by ether bonds and the rest by carbon-carbon bonds. It contains both phenolic and alkyl hydroxyl groups available for the reaction. Lignin reactivity is limited by the accessibility, heterogeneity and stability of the polymeric linkage. Lignin plays a significant role in the carbon cycle sequestering atmospheric carbon into the living tissues of woody vegetation. Lignin is a complex enigma and structural details of the phenypropanoid units in plant are still emerging.

3. Degradation of lignocelluloses

Lignocellulose can be degraded by physical, chemical and biological procedures (Rana *et al.,* 1996). Physical methods involve milling and radiation treatment that decreases particle size, surface area and degree of polymerization. In chemical procedures, lignocelluloses are treated with acid, alkali, ammonia, steam, hypochlorite etc. to remove lignin. But the main problem is low yields because of sugar degradation products and inhibits fermentation process. Biological or enzymatic

degradation of lignocellulose yields maximum, and provides promising aspects of bioconversion product because of high specificity of enzyme substrate reaction. Crystalline structure and microstructure and porosity of cellulose are also not degraded by biological degradation. Moreover, the enzymes cleave the cellulose bond precisely and product conversion rate is higher.

Cellulolytic microorganisms do not occupy an ecological niche of their own. They exist in concert with many other cellulolytic and noncellulolytic strains of bacteria and fungi (Niku-Paavola *et al.,* 2002). Nevertheless the cellulolytic strains play a vital role in the cellulosic ecosystem as the predominant polymer degrading species. Each of these protective polymers is characterized by a different intrinsic chemical and structural arrangements and consequently different groups of microorganisms and enzymes are required to degrade the different types of polymer. Since last 50 years much progress has been made for the degradation of cellulose by aerobic and anaerobic microorganisms. The enzyme system responsible for the degradation of cellulose is cellulases. There are three types of synergistic action involved in cellulolysis. The involvements are between endoglucanase and cellobiohydralase (endo-exo synergism), two cellobiohydrolase (exo-exo synergism), and between β-glucosidase and other two types of enzymes. The different modes of action of cellulolytic enzymes on the polymeric substrate are commonly described as endo- and exo- types of attack. Only a few fungi synthesize and release appreciable amounts of the cellulase enzyme into the culture medium. Important among them are *Trichoderma reesei, T. viride, Fusarium solani, Phaenerochaete chrysosporium, Humicola grisea* var. *thermoidea, Cryptococcus albidus, Emericella nidulans* var. *nidulans* and *Malenocarpus* spp. (Pandey *et al.,* 2000; Singhal *et al.,* 2009; Figure 2).

Xylanases are the enzymes that are responsible for the hydrolysis of hemicellulose. The enzymatic hydrolysis of xylans, major hemicellulose content, requires the action of several enzymes. Among these endo-1,4- β xylanases and β-xylosidase are important. Besides these other enzymes play a prominent role in the removal of side groups from polymeric xylan during hydrolysis of hemicellulose. These enzymes include arabian-furanosidase, ferulic acid esterases, $\propto$-glucuronidase, and acetylesterases. There are several microorganisms isolated for production of xylanase for environmental application including decolourization of industrial effluent and dyes. A *Bacillus* sp. isolated from sediments of pulp and paper mill effluent and degraded wood was found to produce xylanase when cultured in a synthetic media containing oat spelt xylan (1% w/v) and black liquor (10%) as inducers along with sucrose (2.5%) as additional carbon (Mishra and Thakur, 2010; Figure 3). The extracellular xylanase was purified and kinetic parameters were characterized. The purified enzyme was highly thermostable and stable over a broad pH range (pH 6-10). The molecular weight of the purified enzyme, as determined by matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF) analysis was analogous to the results obtained by SDS-PAGE, around 54 KDa. Kinetic parameters were determined by using oat spelt xylan as substrate. In

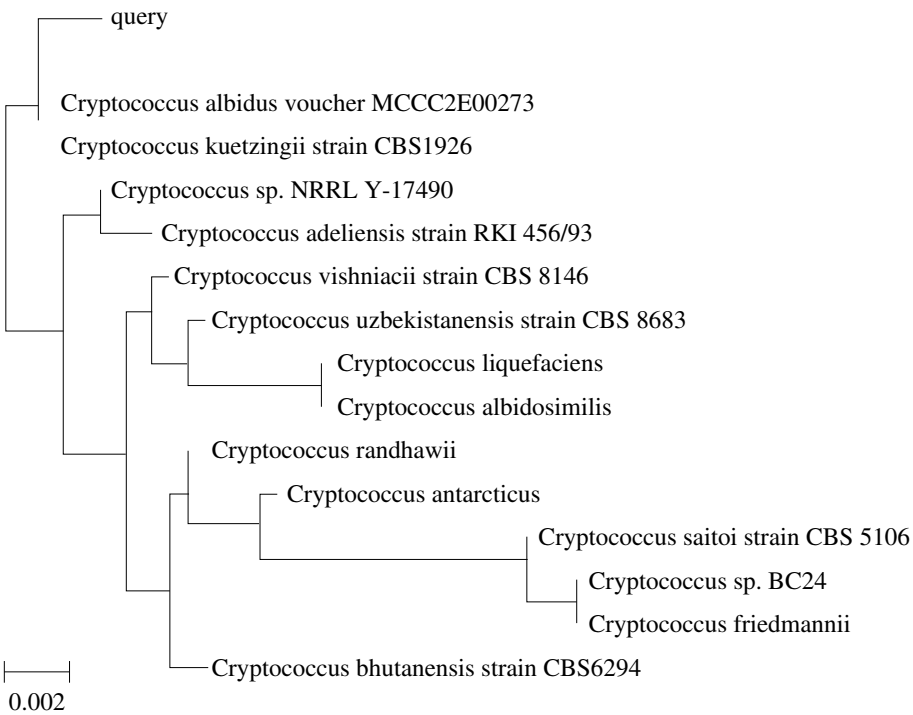

Figure 2. 18S rDNA analysis for identification of hemicelluloses degrading fungi *Cryptococcus albidus*, from effluent and degraded wood of Century pulp and paper mill, Lalkua, Uttarkhand, India (Singhal *et al.,* 2009).

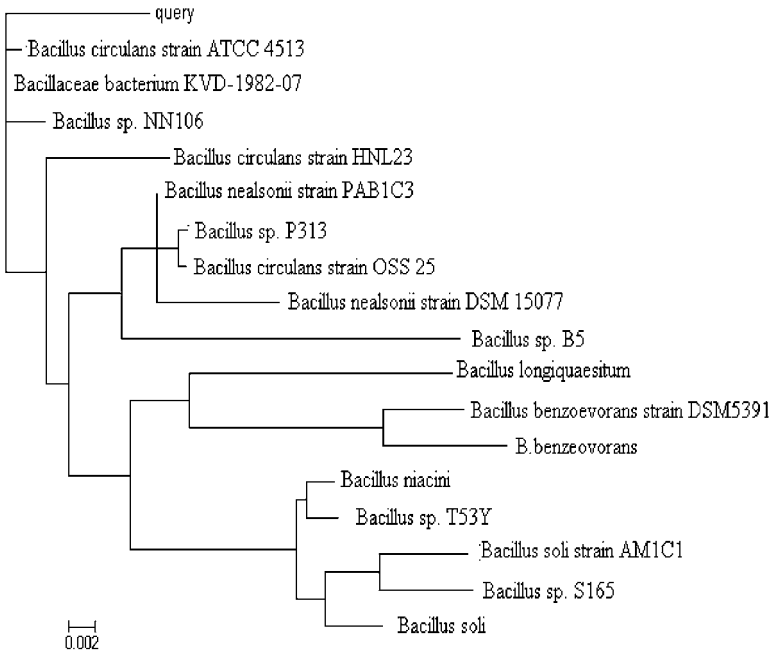

Figure 3. Phylogenetic tree of hemicellulose-degrading bacterial strain, *Bacillus* sp., based on 16S rDNA gene sequence isolated from. Boot strap consensus tree was drawn by multiple sequence alignment with Neigbour joining method using software MEGA 3.1 (Mishra and Thakur, 2010).

addition, crude xylanase showed enormous potential for decolorization of various recalcitrant dyes (Mishra and Thakur, 2010). Hence the higher stability of this xylanase makes it a good candidate for further research into its possible application in biotechnology.

Complex but non-specific enzyme systems are needed for lignin to be degraded by ligninase. The C-C and ether bonds joining subunits together must be cleaved via an oxidative mechanism. Both peroxidases and oxidases have been reported for degradation of lignin (Glenn *et al.,* 1986). White-rot basidiomycetes (Niku-Paavola *et al.,*1988) tend to harbor gene families encoding the key enzymes responsible for deploymerization of lignin: manganese peroxidase, lignin peroxidase (LiP) and laccase (Sergio, 2006). The LiP system from *Phanerochaete chrysoporium* has been the most thoroughly studied is a heme-containing glycoprotein secreted during secondary metabolism as a response to nitrogen limitation (Kirk *et al.,* 1989). Manganese peroxidase (MnP) occurs in most white-rot fungi. Similar to LiPs, MnPs are gycosylated, heme-containing enzymes that functionally require H_2O_2. The first report on MnP was in *P. chrysoporium*. Veratryl (VA) and glyoxal oxidase (GLOX) are components of the peroxidase enzyme systems (Kirk *et al.,* 1989). VA is a secondary product of fungal metabolism that has been reported to stimulate lignin degradation. A number of laccases have been found in a diverse variety of fungi, including not only wood-rotting basidiomycetes but also well-studied strains of non-ligninolytic ascomycetes from the genera *Aspergillus* and *Neurospora*. Laccases also had been found in plants where they play a role in lignin synthesis. Four fungal strains, *Cryptococcus albidus, Emericella nidulans* var. *nidulans, Aspergillus terreus* and *A.* sp., isolated from decomposed wood and effluent of pulp and paper mill in our laboratory in which *Cryptococcus albidus* had high capacity for production of laccase, a copper containing oxidases that are capable of degrading environmental pollutants (Singhal *et al.,* 2009). The study aimed to remove inhibitory products during fermentation from which ethanol may be made from sugar cane bagasse of paper mill effluent which is used primarily for preparation of pulp and paper.

The use of xylanases in pulp and paper industry has increased appreciably. The previously described xylanase producing bacteria include *Bacillus amyloliquefaciens, Micrococcus* sp., *Streptomyces roseiscleroticus* NRRL-B-11019, *Cellulomonas uda, Staphylococcus* sp. (Breccia *et al.,* 1998). Various compounds have been identified in lignocellulosic waste by GC-MS such as Acetic acid, Propanoic acid, Ethanedioic acid, Furan carboxylic acid, Glyoxylic acid, Butanoic acid, 3-Acetoxy butyric acid, Guaiacol, Valeric acid, Hexanoic acid, Benzene acetic acid, Propanedioic acid, Acetoguiacone, Phenyl propionyl glycine, t-cinnamic acid, 3,4,5-trimethoxy benzaldehyde, Tetradecanoic acid, Dibutyl phthalate, Hexadecanoic acid, Bis-(2-methoxy ethyl) phthalate, Gallic acid, Ferulic acid, Octadecanoic acid, Benzyl butyl phthalate, 1-phenanthrene carboxylic acid, Bis (2-ethylhexyl) phthalate, Di-octyl phthalate (Herna'ndez *et al.,* 2001; Gupta *et al.,* 2009). We were able to detect few compounds such as Acetic acid, 9-octadeneamide, 1,8 diphenyl 4,5 octanediol, propanedioic acid, benzoic acid, 4-ethoxy-ethyl ester,

phenyl propionyl glycine and octadecanoic acid. GC–MS is considered because it has been proven to be a very suitable technique to analyze low molecular weight compounds released from lignin due to degradation (Kaya *et al.,* 2000; Mishra and Thakur, 2011).

4. Optimization of processes for lignocelluloses degradation

The biotransformation of lignocelluloses is a very complex process influenced by different factors, among which microbial activity is one of the most important (Singhal *et al.,* 2009). The success of a microbial process depends on having the right microbes in the right place with the right physiochemical environment. Properly optimized culture media for particular microorganisms not only maximize biomass production but also stimulate synthesis of enzymes. The C, N and C:N ratio need to be adjusted if metabolic activities and microbial growth are to be optimized. Significant enhancement of biological activities at optimum levels of carbon sources and initial substrate pH in colour removal of pulp mill effluent by a white rot fungusand bacteria are reported (Mishra and Thakur, 2010). The contributions of bacteria have also been reported to the utilization of low-molecular weight lignin oligomers as the sole source of carbon and energy that produce enzymes catalyzing cleavage of intermonomeric linkages. But the complete biodegradation of lignin by bacteria in natural environment where fungi are also present is not much known. However, bacteria seem to play a leading role in decomposing lignin in aquatic ecosystem because wood degrading bacteria have a wider tolerance of temperature, pH and oxygen limitations than fungi (Adsul *et al.,* 2004). *Cryptococcus albidus* yeast isolated from the sediments of Century pulp and paper mill, Lalkuan, Uttaranchal, India, had good potential for decolourization and detoxification of effluent. It was able to reduce 27% colour and 24% lignin content of the effluent on 5th day. The process of delignification and decolourization was optimized by Taguchi approach using L-8 orthogonal array (Dasu *et al.,* 2003; Roy, 2007). Seven parameters at two levels were optimized. The optimum conditions were temperature (30-35℃), rpm (125), dextrose (1%), tryptone (0.1%), inoculum size (7.5%), pH (5) and duration (24 hr). After optimization of the process parameters, the delignification and decolourization improved by 28% with 50-53% reduction in colour and 35-40% reduction in lignin. Variation in pH from 5 to 6 had most significant effect, 72%, while variation in temperature from 30 to 35℃ had no effect on the process of decolourization (Figure 4a). Most significant interaction was in between temperature and dextrose (S.I., 89%) and least significant was in between pH and duration (S.I., 2.7%) (Singhal *et al.,* 2009). *Bacillus* sp. strain LP1 was applied for the evaluation of decolorization and delignification in presence of various parameters (Figure 4b). Initially various C sources (1.5%) were tested in which removed color and lignin in presence of sucrose (46.8%, 37.1%) followed by dextrose (23%, 25.8%), sodium citrate (23.7%, 25.9%) and sodium acetate (21.2%, 18.6%) respectively (Figure 4b). Effluent was further treated in presence of inorganic and organic N source (0.05%) like urea, yeast extract, sodium nitrate and ammonium nitrate, and the results indicated that N has negative impact on growth and decolorization (Mishra and Thakur, 2010). Screening experiments were performed in advance, in presence of C and N sources to optimize the remaining process parameters by Taguchi approach (Thakur, 2004).

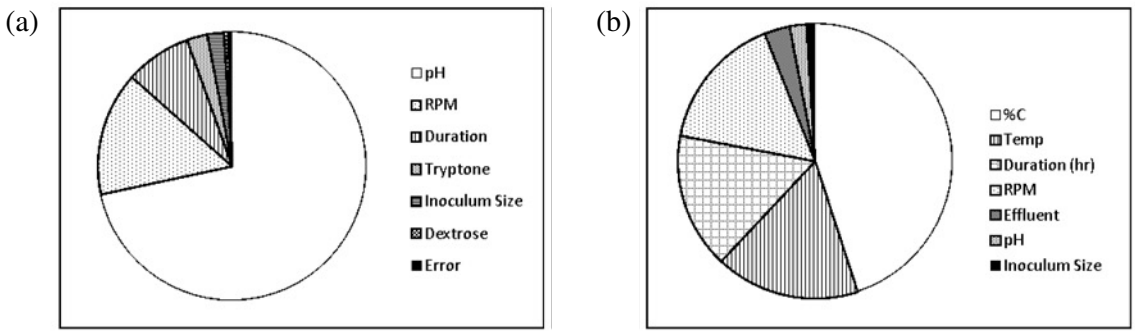

Figure 4. Percent contribution of different factors in effluent treatment of pulp and paper industry by *Cryptococcus albidus* (a) and *Bacillus* sp. (b) in presence of different factors (Mishra and Thakur, 2010).

5. Production of ethanol from lignocellulosic biomass of effluent

Productions of ethanol using lignocelluloses are well established, however, utilization of a cheaper substrate such as sugarcane bagasse from effluent of pulp and paper industries could make bioethanol production more competitive with fossil

fuel and also in mitigation of green house gases (Lynd *et al.,* 1991; Lee, 1997). One important requirement for ethanol production is an efficient microorganism which can able to degrade lignocellulose and ferment a variety of sugars (pentoses and hexoses) as well as to tolerate stress conditions (Duff and Murray, 1996; Pandey *et al.,* 2000; Neureiter *et al.,* 2002). Conversion of sugarcane bagasse into fermentable sugars is possible through thermal, chemical, or enzymatic hydrolysis (Laser *et al.,* 2002). Pretreatment technology is important steps for improving the conversion of cellulose to glucose in following enzymatic hydrolysis (Eggeman and Elander, 2005). Prior to ethanol fermentation by a microorganism, the feed stock needs to be processed by saccharification technology in order to release fermentable sugars. Lignocellulose as a feedstock presents two major challenges for ethanol production due to recalcitrant to biodegradation and presence of pentose and hexose sugars (Laser *et al.,* 2002; Chen *et al.,* 2009). Industrial biocatalysts, such as the common yeast, *S. cerevisiae*, rarely possess native pathways able to efficiently ferment both hexoses (such as glucose) and pentoses (such as xylose). Metabolic engineering through genetic modification may be an effective means of manipulating the capabilities of the microorganisms (Chen *et al.,* 2009).

The cellulosic and hemicelluloses sugars obtained through acid and enzymatic hydrolysis can efficiently be used for ethanol fermentation either by separate fermentation of individual hydrolysate or fermentation of mixed hydrolysate using co-culture. However, in co-culture cultivation, optimum growth conditions of the yeasts would be different and might result in lower efficiency and lower product yield. Therefore, proper optimization of fermentation processes is significant for ethanol preparation. Tian et al. (2009) reported *S. cerevisiae* efficiently converts both glucose and mannose into ethanol, but is unable to convert xylose into ethanol. Other fungal yeast-like species, e.g. *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus*, have been found to be highly efficient xylose-fermenting strains that can be used in ethanol production (Agbogbo et al., 2006). However, these yeasts have a relatively low ethanol yield and inhibitor tolerance. For enhanced production of sugar and ethanol it is essential to optimize the composition of culture media and process conditions. There are several factors that affect enzymatic hydrolysis of cellulose including substrates, cellulase activity, and reaction conditions (temperature, pH, etc.). To improve yield and rate of the enzymatic hydrolysis, research has focused on the optimization of the hydrolysis process and enhancement of cellulase activity. Taguchi methods have been widely used to optimize the reaction variable by devising minimum number of experiments (Mishra and Thakur, 2010). This approach also facilitates to identify the impact of individual factor and find out the link between variables and operational conditions. Analysis of the experimental data using the ANOVA and factors effect, gives the output that is statistically significant (Kackar, 1985; Phadke and Dehnad, 1988). Hence, for better efficiency of ethanol production, the approach of separate hydrolysis and fermentation was preferred (Olsson and Hahn-Hagerdal, 1993).

In our Indian laboratory *Cryptococcus albidus* together with *Saccharomyces cerevisiae* was used for production of bioethanol from the bagasse residue in pulp and paper mill effluent (Thakur, 2009). Lignocellulose degrading potency of *C. albidus* was evaluated by estimation of lignocellulolytic enzymes, CMCase, FPase, β-glucosidase, laccase, xylanase and sugar (Singhal *et al*., 2009). Sugar obtained by hydrolysis of lignocellulose was fermented by *S. cerevisiae* to produce ethanol. Lignocellulolytic enzymes, CMCase (34 U/ml), FPase (3 U/ml), β-glucosidase (2.3 U/ml), laccase (32 U/ml), and xylanase (12 U/ml) were determined by *C. albidus*, however, after optimization of process parameters, temperature (30–35^{0}C); shaking condition (125 rpm); dextrose (1.0% w/v); tryptone (0.1%); inoculum size (7.5%); pH (5.0) and duration time (24 h) indicated production of CMCase (60 U/ml), FPase (6), β-glucosidase (4.61), laccase (192) and xylanase (24), and an increase in 1.5 fold sugar (Figure 5). The sugar cane bagasse treated initially by *C. albidus* in presence of optimized growth condition subsequently treated by *S. cerevisiae* indicated production of ethanol (38.4g/l). However, an increase in 1.82 fold (70.0g/l) ethanol was measured in presence of carbon (2%), nitrogen (0.24%), phosphate (0.24%) temperature (35^{0C}), stirring (150 rpm) and pH (5.5), indicated use of sugarcane bagasse of pulp and paper mill effluent for production of bioethanol (Thakur, 2009).

Krishna *et al.* (1998) used *Trichoderma reesei* cellulase and cellobiase to hydrolyze sugarcane leaves after alkaline delignification. Martin *et al.* (2008) used a mixture of endo-glucanases and cellobiases to saccharify steam pretreated sugarcane bagasse. Adsul *et al.* (2004) treated sugarcane bagasse chemically with varying quantities of lignin and hemicelluoses for the production of cellulase and xylanase enzymes by *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051 in the production medium and recovered higher xylanase and β-glucosidase activities.

The production of ethanol (32.6%) is higher than earlier report of sugarcane leaves and from the hydrolyzate of sugarcane depithed bagasse (Krishna *et al.,* 1998). Huang *et al.* (2009) reported ethanol production by fermentation of NaOH-neutralized hydrolysate without detoxification using the adapted *Pichia stipitis*, and compared to fermentation of detoxified hydrolysate. The bioethanol yield using the adapted *P. stipitis* with both types of hydrolysate at pH 5.0 achieved 87% of the maximum possible ethanol conversion. Jonathan *et al.* (2009) used soybean hulls for production of ethanol by the simultaneous saccharification and fermentation (SSF) process with *Saccharomyces cerevisiae* D5A and recovered 31.2$\pm$0.3 (flask B) g/l ethanol after thirteen days. Even insertion and expression of *Zymomonas mobilis* genes encoding essential enzymes involved

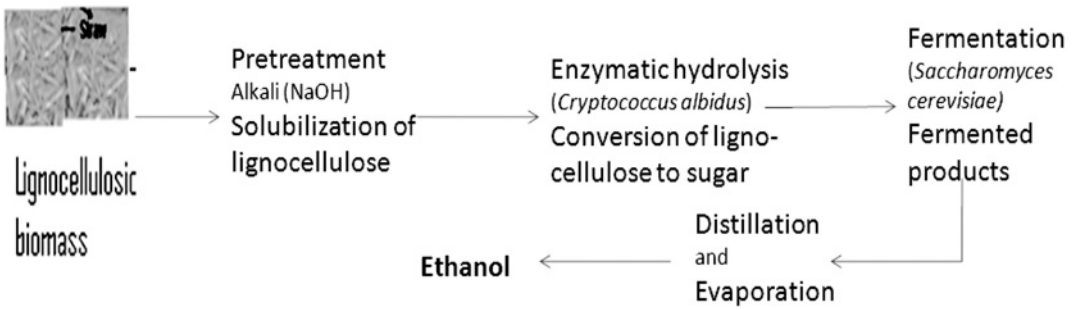

Figure 5. Production of ethanol by degaradation and bioconversion of bagasse of pulp and paper mill effluent using cellulosic hydrolysis by *Cryptococcus albidus* and *Saccharomyces cerevisiae* (Thakur, 2009).

in the fermentation pathway, alcohol dehydrogenase II (adh II) and pyruvate decarboxylase (pdc), into *E. coli*, resulting in increased cell growth and ethanol production of 30 g/l (Chen *et al.,* 2009). Oleskowicz-Popiel *et al.,* (2008) pretreatment anaerobically digested (AD) manure for the simultaneous saccharification and fermentation (SSF) recovered 30.8 kg ethanol per 100 kg dry mass of maize silage.

Gupta *et al.* (2009) used *Prosopis juliflora* as raw material for acid pretreatment, delignification and enzymatic hydrolysis, and recovered 18.24 g/l and 37.47 g/l sugars and 7.13 g/l and 18.52 g/l of ethanol with *Pichia stipitis* and *Saccharomyces cerevisiae*, respectively. Martín *et al.* (2008) used clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) mixtures as raw materials for ethanol production. The simultaneous saccharification and fermentation of the pretreated material yielded cellulose conversions of 87.5 and 86.6%, respectively, with *Saccharomyces cerevisiae* and the filamentous fungus *Mucor indicus*. Bermudagrass, reed and rapeseed were used for ethanol production by means of simultaneous saccharification and fermentation (SSF) with a batch and fed-batch mode. When the batch SSF experiments were conducted in 3% low effective cellulose, about 16 g/l of ethanol were obtained after 96 h of fermentation (Sun and Cheng, 2005). In this study production of ethanol was more due to removal of lignin from the culture medium which play inhibitory role in fermentation processes.

6. Production of biodiesels from lignocellulosic biomass of effluent

Lignocellulosic biomass of coniferous trees may be converted into toll oil subsequently to biodiesel. Tall oil is a viscous yellow-black odorous liquid obtained as a co-product of the Kraft pulping and the yield of crude tall oil is in the range of 30–50 kg/ton pulp. The word “kraft” means “strong” is “soda cook” improves the selectivity of the process - dissolving the lignin with less damage to the cellulose. The kraft process (also known as kraft pulping or sulfate process) describes a technology for conversion of wood into wood pulp consisting of almost pure cellulose fibres (Malaviya and Rathore, 2007). In the process, wood chips are treated with a mixture of sodium hydroxide and sodium sulfide, known as white liquor that break the bonds that link lignin to the cellulose. In the kraft process high alkalinity and temperature enable the conversion of soluble sodium soaps of lignin, rosin and fatty acids present as esters in the wood. The spent cooking liquor is called weak black liquor has about 15 % dry content.

The black liquor is concentrated in a multiple effect evaporator and after the first stage the black liquor is about 20-30%. At this stage it is called intermediate liquor (Ali and Sreekrishnan, 2001; Singhal and Thakur, 2009a). Normally the soaps start to float in the storage tank for the weak or intermediate liquors and is skimmed off and collected. A good soap skimming operation reduces the soap content of the black liquor down to 0.2-0.4 % w/w of the dry residue. The collected soap is called raw rosin soap. The raw rosin soap is then allowed to settle to release as much as possible of the entrained black liquor. The soap goes then to the acidulator where it is heated and acidified with sulfuric acid to produce Crude Toll Oil (CTO). Normally CTO contains rosin, unsaponifiable sterol, resin acids (mainly abietic acid its isomers), fatty acids (mainly palmitic acid, oleic acid and linoleic acid), fatty alcohols, sterols and other alkyl hydrocarbon derivates (Figure 6).

The principal components of CTO contain saturated and unsaturated C18 free fatty acids, free resin acids, and other non acidic components like sterols and bulky alcohols referred to as neutral or unsaponifiables. Tall oil fatty acids mainly consist of unsaturated fatty acids like oleic (25–45%), linoleic/linolenic acid (45–65%), and 1–3% saturated fatty acids (Ali and Sreekrishnan, 2001). The resin acids in CTO are bulky molecules different from the straight chain free fatty acids. Numerous production techniques and procedures have been used to convert fatty acids into alky or methy esters that make up biodiesels. Biodiesel is synthesized by chemical, enzymatic, or in vivo catalysis mainly from renewable resources. Chemical catalysis is of

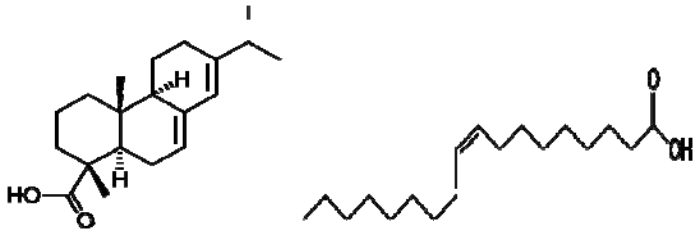

Figure 6. The most common chemicals, Abietic and Oleic acid, in Crude Toll Oil of pulp and paper mill effluent.

prime importance where alkaline or acidic- homogenous or heterogeneous chemical catalysts can be used. The most common homogenous alkaline catalysts are sodium and potassium hydroxide, due to their low cost, as well as sodium methoxide and ethoxide, which are more reactive. The optimal catalyst concentration is between 0.5 and 1%, and the final fatty acid alkyl ester (FAAE) yields between 94 and 99% can be obtained. The major drawback of alkaline catalysts is that they lead to saponification, if free fatty acid (FFA) is present in the reaction medium. The advantages of acidic catalysts are their lower sensitivity to FFA and their ability to catalyze the esterification of FFA. Due to the low reaction rate, the acidic process itself is not of great importance for biodiesel production, but can be applied as pretreatment of FFA-containing feedstock followed by alkaline catalyzed transesterification. Furthermore, enzymatic processes can be linked to chemical transesterification to esterify remaining FFA. Although the chemical catalysts are comparatively cheap and offer high reaction rates, this technique exhibits several drawbacks. It is energy consuming, requires a neutralization of the catalyst, as well as several final purification steps due to the complicated removal of the catalyst and glycerol. Furthermore, the industrial alkaline catalysis necessitates high quality, water and FFA free feedstock. There are several processes involved in biodiesel production from CTO. The methyl ester of the main resin acids which is the multi ring structure has a high melting point (25^{O}C) and boiling point (365^{0}C). The properties are highly undesirable for use in biodiesel considering cold flow behaviors. However, properties of methyl ester derived from one of the main fatty acids (oleic acid) are very similar to that of No.2 diesel fuel. However, degradation of multi ring structure of lignin molecules by enzymes may enhance production of biodiesel.

Lipases have been widely used for the enzymatic modifications of oils and fats and ester synthesis. The lipase activity increases with increase in water content to 15% w/w of oils (Shah *et al.,* 2004). Molar ratio increases with increasing methanol concentration up to methanol to oil ratio of 3:1 and then decreases. Lipases showed a higher activity at higher temperatures (30 to 50℃) in non-continuous batch operation. The highest lipase enzyme activity was obtained at pH 7.4 when the pH was varied from pH 2.0 to 12.0. For the product and process development of biodiesel, enzymatic transesterification has been suggested to produce a high purity product with an economic, environment friendly process at mild reaction conditions. The enzyme cost being the main hurdle can be overcome by immobilization. Immobilized enzyme, which has been successfully used in various fields over the soluble counterpart, could be employed in biodiesel production with the aim of reducing the production cost by reusing the enzyme (Shah *et al.,* 2004). Solar energy-to-biofuels conversion combines innovative metabolic engineering with state-of-the-art, large-scale bioprocess engineering, efficient cell harvesting, cost-effective conversion of lipid to biodiesel, and generation of other valuable byproducts are required.

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