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A review on current technological advancement of lignocellulosic bioethanol production

Abstract

Bioethanol production from lignocellulosic biomass has several advantages. Lignocellulosic bioethanol production process consists of three steps including; pre treatment, enzymatic hydrolysis and fermentation. But there are several technological challenges of each step for commercial bioethanol production. The ideal pre treatment characterized by reduced cellulose crystallinity, significant lignin reduction and less inhibitory compound generation. The enzymatic hydrolysis process depends on cheaper cellulose production and higher reducing sugar yield within shorter incubation time. Fermentation process depends on isolation/development of yeast strain which can ferment both pentose hexose sugars. The present review deals with recent technological advancement of each step of lignocellulosic bioethanol production. The current review mainly focused on fermentation step, genetic engineering of yeast and thermotolerant yeast for ethanol production. The current literature can be useful for further technological advancement of lignocellulosic bioethanol production.

Keywords: bioethanol, lignocellulosic biomass, fermentation, genetic engineering of yeast, thermotolerant yeast

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Abbreviations: RON, research octane number; MON, motor octane number; HMF, hydroxy methyl furfural; SHF, separate hydrolysis and fermentation; CBP, consolidated bio processing; SSF, simultaneous saccharification and fermentation; ECC, enzymatic convertibility of cellulose

Introduction

In current time most of the fuels that comes from non-renewable resources depleting at a high rate as well as their demand increasing rapidly. Mainly these fuels are mainly derived from fossil fuels of which we have a limited reserve. Moreover, along with the rapid rise in their consumption, these fuels have created many problems for our atmosphere; one of the common problems is their contribution to global warming.[1] To overcome such problems we need alternative fuels and that does not pollute the environment and can be easily produced. Bio energy resources in the form of energy crops and lignocellulosic biomass are the newly found alternatives, as they are pollution free and with this advantage, their development is also gaining high importance. Lignocellulosic biomass is now being used on a large scale as the main substrate for biofuel production.

Lignocellulosic biomass consists of biological resources, plant waste residues that produce fuels by biological procedure.[2] They consist of plant cell walls that are made up of structural carbohydrates i.e. cellulose, hemicelluloses and lignin as their major components. In these components lignin plays a role of a limiting factor in bioethanol production as it does not let enzyme contact, during the hydrolysis of cellulose. These components vary from species to species and situation to situation. The biofuels can be used as solid fuel, liquid fuel (long chain alcohols, biodiesel, methanol, ethanol, algal diesel, butanol, alkanes) and gaseous fuel (methane and hydrogen). All these kinds of fuels can be easily produced by using diverse primary energy resources like plant biomass, solar energy, as well as secondary energy like electricity and hydrogen.[2] One of the important biofuel at the present time is Bioethanol, as it has special properties that improve its octane number; research octane number (RON) and motor octane number (MON) of bioethanol are 109 and 90 respectively and averaged 99 compared to 91 for gasoline. Gasoline does not burn easily during compression ignition due to its low octane number.[3] Bioethanol can also mix with gasoline easily or can be used as clean alcohol in higher octane number engines having heat of vapourisation.[4]

Production of bioethanol from lignocellulosic biomass

The total annual production of lignocellulosic biomass is approximately 10-15 billion ton and about 50% of the world's biomass is considered as lignocellulosic.[5] The main lignocellulosic biomass that used for bioethanol production is depicted in Table 1. The production of bioethanol from lignocellulosic biomass includes the following three major steps i.e. pre-treatment, hydrolysis mainly with the help of enzymes and fermentation.[6]

Pre-treatment

It is the most crucial step and used to delignify the lignocellulosic biomass as lignin acts as a barrier in enzyme interaction with the cellulose. Among all the steps it is one of the most costly steps accounting for 33% of the total production cost. The main challenge in the bioethanol production lies in pre-treatment step as it is very expensive and time consuming.[7] Lignocellulosic biomass possesses a very complex structure consisting of cellulose, hemi-cellulose and lignin. In the process of pre-treatment the complex structure of lignocellulosic biomass is distorted so that its cellulose component can be free for enzyme action. The enzymatic action hydrolyses cellulose into sugars that are further fermented. After pre-treatment procedure the cellulose crystallinity also reduced and the porosity of the raw substrate improved, increasing the sugars formation and improving enzymatic hydrolysis, avoiding formation of any kind of inhibitor

that create problem in the hydrolysis or fermentation step. The main purpose of this step is to make cellulose more accessible to enzymatic hydrolysis. There are many ways by which this step is completed like physical pre-treatment, biological pre-treatment, chemical pre-treatment and solvent pre-treatment. There are different kinds of pre-treatment processes including chemical, physical, biological, physio-chemical etc.

Table 1 Composition of various types of biomass obtained from different sources

Types of biomass	Cellulose content (%)	Hemi-cellulose (%)	Lignin (%)
Spruce and Pine	40-45	25-30	30-60
Sweet sorghum baggase, Sugarcane baggase, Barley straw, Rice hulls, Wheat straw, Rice straw etc.	35-50	25-50	May-15
Poplar and Aspen	45-50	25-40	20-25
Paper, Newspaper	40-55	25-40	20-25

Chemical pre-treatment: This is the most studied method and it involves the use of variety of chemicals like acids, alkalis, organic solvents, peroxides, inorganic solvents like hydrochloric acid, sulphuric acid. Mostly dilute-acid pre-treatment method is used; it is finished in two steps: depolymerisation of hemicelluloses at 140°C for at least 15minutes avoiding the formation of carboxylic acids or furan. In second step the treatment with dilute acid is done at 190°C for 10 minutes helping better cellulose interaction with enzyme during enzymatic hydrolysis.[8] Low temperature, usually 121°C is often used for dilute-acid pre-treatment that avoids breaking of sugars into hydroxy methyl furfural (HMF) and furfural.[9] But dilute-acid pre-treatment has disadvantage as the acid used causes corrosion of the instruments; to avoid this expensive coatings or use of stainless steel that is acid resistant is recommended. Chemical pre-treatment is also done with the help of alkalis like NaOH, lime, aqueous ammonia that result in dilute base addition, increase in crystalinity, increase in degree of polymerisation and internal surface area and degradation of lignin. Alkali pre-treatment method is more reliable as it decreases the degradation of sugars that are to be hydrolysed and requires lower temperature and pressure compared to the dilute-acid pre treatment and is cheaper.[10]

Physical pre-treatment: Its main aim is to reduce the cellulose crystallinity, degradation of lignin and hemicelluloses without affecting cellulose. Physical pre-treatment process includes steam treatment, grinding, milling, chipping etc. Radiations such as microwaves are also being used for this purpose as they can easily penetrate the biomass surface and simultaneously heat the surface. This results in an easy access of enzyme to the surface of cellulose during hydrolysis, decrease in the crystallinity and increase in the degree of polymerisation. These processes demand lot of power supply because of which the cost increases.[10]

Physio-chemical pre-treatment: It is one of the oldest, most studied processes. Ammonia fibre steam explosion is the favourite option always, as it helps in converting hemicelluloses and lignin into soluble oligomers using auto hydrolysis reaction by steam at very high pressure followed by depressurizing to disrupt the structure of lignocellulosic biomass.[11] The main factors that are considered during steam explosion include temperature, holding time, chip size and moisture content. This process is recommended for agricultural waste and hardwood but it is not good for softwood.[1]

Biological pre-treatment: While all the other methods have high energy demands and need proper equipments, biological pre-treatment methods need much less energy as compared to physical/chemical methods and are eco-friendly. Mainly white rot fungi are used in this process as they help in the proper degradation of lignin. Brown rot fungi are also used as they help in degrading lignin by attacking it with the help of enzymatic laccase and peroxides. Laccase is a copper containing compound oxidase enzyme, which can easily remove lignin, increasing the interaction of cellulase enzyme with cellulose.[10]

Hydrolysis

The cellulose that is left behind after the pre-treatment step is converted into glucose, for this we can use different catalysts such as concentrated; dilute acids or biological enzymes derived from algae or fungi. The reaction involved during hydrolysis step is following:

$$(C_5H_8O_4)n + nH_2O \rightarrow nC_5H_{10}O_5 \text{ (for pentose)} \quad (1)$$

$$(C_6H_{10}O_5)n + nH_2O \rightarrow nC_6H_{12}O_6 \text{ (for hexose)} \quad (2)$$

The cellulose and hemi-cellulose are being converted into xylan for pentose sugars and glucan for hexose sugars.[3] Saccharification can be done by acids, alkalis or enzyme among which enzymes are mostly preferred due to their low cost of processing, requirement of mild operating conditions, high sugar yield and lack of corrosion problem.[12] Cellulases are specific enzymes for celluloses and they constitute a mixture of enzymes that help in the hydrolysis of cellulose to glucose. The main 3 three enzymes present in cellulases are:

i. Endo glucanase which creates free chain ends by attacking regions of low crystallinity in celluloses.

ii. Exo glucanase/Cellobiohydrolase which removes cellobiose units from the free chain ends.

iii. β-glucosidase which produces glucose by hydrolysing cellobiose.

The factors that affecting the rate of enzymatic hydrolysis are activity of cellulase, pH, temperature and concentration of the substrate, cellulases for hydrolysis of celluloses can be produced by a variety of organisms like fungi and bacteria; these microorganisms can be either anaerobic or aerobic, thermophillic or mesophillic. Bacteria from families of *Bacillus, Clostridium, Cellulomonas, Thermomonospora, Erwinia, Ruminococcus, Bacteriods, Acetovibrio, Streptomyces* are being used, among which *Cellulomonas fimi* and *Thermospora fusca* are mostly used.[13] Among fungi *Aspergillus, Trichoderma*, *Schizophyllum and Penicillium* families are known to produce cellulases and in all of them *Trichoderma* is the most potential producer of cellulases.[14]

Fermentation

This process converts hexoses and pentoses into ethanol by using various microorganisms like yeast, fungi and bacteria. *Saccharomyces cerevisiae* is the most commonly utilised microorganism but it cannot metabolise xylose. To deal with this problem genetic engineering is nowadays preferred for the development of recombinant strains that can utilize both pentose and hexose sugars.[3] Bioethanol fermentation step can be carried out in three different ways such as

i. Separate Hydrolysis and Fermentation (SHF)

ii. Consolidated Bio Processing (CBP)

iii. Simultaneous Saccharification And Fermentation (SSF) processes

In separate hydrolysis and fermentation (SHF), the hydrolysis is completed in one reactor and fermented in other reactor. This transfer that is being done after hydrolysis increases the cost of reaction and consumes a lot of time. In addition to this there is a risk of inhibitor formation that would eventually degrade the ethanol yield. There is another procedure called consolidated bio processing (CBP) in which cellulase production, enzymatic hydrolysis and fermentation take place in a single bioreactor, but efficiency of the process is very limited. Hence to avoid the above problem, simultaneous saccharification and fermentation (SSF) is being used as both saccharification and fermentation take place in a single bioreactor where the cellulose along with the fermenting micro organisms is also added. Moreover the glucose which produced immediately converted to ethanol. This leads to higher ethanol yields and lower energy consumption and hence lower costs. Due to its several advantages this is the most preferred method for lignocellulosic bioethanol production till date. The following sections describe further details about SSF.

Simultaneous Saccharification and Fermentation (SSF)

Ethanol production from lignocellulosic biomass requires saccharification as well as fermentation after the pre-treatment step for proper ethanol yields from fermenting sugars obtained from hydrolysis of lignocellulosic biomass. The saccharification and fermentation are now often conducted simultaneously and called simultaneous saccharification and fermentation (SSF). During SSF process, glucose produced is simultaneously getting converted to ethanol by the fermenting microorganisms; with this the inhibition of cellulose by the reaction end products is reduced and further as this whole process can take place in a single bioreactor; it decreases the cost of production. The repeated-batch operations with the cell recycling system can also help in reducing the cost of ethanol production associated with ethanol production. The SSF and repeated batch fermentation with a cell recycling system has a large potential to improve the cost of the process. When SSF is combined with repeated batch fermentation it is known as repeated batch SSF fermentation.[15] Enzymatic Convertibility of Cellulose (ECC) can be calculated on ethanol concentration basis Martín Medina et al.[16]

ECC=Ef-Ei/Ci* 0.57

Where,

Ef=final ethanol concentration in g/L

Ei=initial ethanol concentration in g/L

Ci=initial cellulose concentration in g/L

0.57=stiochiometric yield of ethanol from cellulose.

Among all processes, SSF processes and repeated batch fed with a cell recycle process are cost effective and have improved ethanol production.[17]

Yeast genetic engineering

Yeast (*Saccharomyces cerevisiae*) is the most effective ethanol producing microorganism for the hexose sugars like mannose, glucose and galactose obtained after saccharification of cellulose. It has some specific features like high tolerance to ethanol, improved ethanol productivity and greater tolerance to the hydrolysate inhibitors. But yeast cannot ferment xylose that is the major dominant pentose sugar present in the hydrolysates and it is very important for the utilisation of lignocellulosic biomass as a raw material. To overcome this problem genetic engineering is being employed for the formation of recombinant yeast strains that have better xylose consumption rates and high ethanol yields. The use of genetic engineering for the development of fermentative and cellulolytic microorganisms like yeast has been carried out to improve their known activity of fermenting pentose and hexose sugars at once; along with this the modified yeast has very low generation of inhibitors like furfural. Genetic engineering acts as a powerful tool that helps to design a way for good ethanol production by upgrading its fermentation performance. With the help of recombinant DNA technology stress tolerant genes were incorporated in *Saccharomyces cerevisiae*.[18] Table 2 summarizes ethanol yield from genetically engineered *S. cerevisiae*. The genetically engineered *Saccharomyces* strains undergoing genetic modification were faster in converting cellulose into ethanol as compared to wild strains. However the microorganisms that are used for yeast genetic engineering must have the potential to tolerate the increase in temperature during SSF/CBP/SSCF processes. One yeast strain (*K. marxianus*) that can co-ferment pentose and hexose sugars and tolerate the temperature up to 50°C has been used for genetic engineering.[29] It was genetically modified with *Trichoderma ressei* and *Aspergillus aculeatus* to incorporate their cellulolytic activity allowing direct/continuous conversion of cellulolytic β-glucan to ethanol at temperature near about 50°C with an ethanol yield of about 0.47g/g.[30] *Zymomonas mobilis* was also used for genetic engineering as it gives high ethanol yields and can easily tolerate temperature up to 40°C. It is also being genetically modified by incorporating a number of genes and various heterologous expressions resulting in an increase in its effectiveness towards arabinose and Xylose.[31] Using genetic engineering over expression of xylulokinase and xylose isomerise were carried out to help in xylose consumption.[32] Metabolic as well as evolutionary genetic approaches were also applied to improve ethanol yields and increase stability of the engineered strains.[33]

Table 2 Genetically engineered enzymes in *Saccharomyces cerevisiae* and their sources

Gene source	Engineered enzyme	Ethanol yield (g/L/h)	Cultivation time(h)	References
Aspergillus awamori	Glucoamylase	0.23	50	[19]
Lipomyces kononenkoae	α-amylase	0.05	90	[20]
Saccharomyces diastaticus	Glucoamylase	0.66	200	[21]
Streptococcus bovis	α-amylase	0.85	72	[22]
Saccharomyces fibuligera	Glucoamylase	0.178	120	[23]

Table Continued..

Gene source	Engineered enzyme	Ethanol yield (g/L/h)	Cultivation time(h)	References
Rhizopus oryzae	Glucoamylase	0.74	120	[24]
Rhizopus oryzae	Glucoamylase	1.2	24	[25]
Streptococcus bovis	α-amylase	0.74	120	[24]
Aspergillus awamori	Glucoamylase	0.45	168	[26]
Pseudomonas amyloderamosa	Isoamylase	0.137	140	[27]
Debaromyces occidentalis	Glucoamylase with debranching activity	0.45	168	[26]
Rhizopus oryzae	Glucoamylase	0.77	168	[28]

Ethanol tolerance was developed in yeast strains in different ways, for instance by involving new yeast strains isolated from the natural environment that build their resistance for stress causing factors like adaptive evolution, i.e. the mechanism by which the cells adapt to the environment such as living over long periods by natural selection. Adaptive evolution have nowadays been used to create mutant yeast strains that can tolerate various stress causing factors like[34] high temperature,[35] freeze thawing,[36] high salt and acetic acid content.[37,38] Apart from this genomic shuffling, chemical mutangenesis, ultraviolet exposure have also helped in developing mutant strains. Transposon mediated mutant selection and deletion mutant library screening has helped to select genetically modified strains that are ethanol resistant.[39]

Thermotolerant yeast for ethanol fermentation

SSF has different benefits for lignocellulosic bioethanol production. But in this process temperature requirements are different for saccharification and fermentation, while enzymatic hydrolysis is carried out at 45-50°C and fermentation has to be carried out at 28-30°C as microorganisms like *Saccharomyces cerevisiae* loose their activity at high temperature above 30°C. So to make SSF a success we either need cold adaptive enzymes for hydrolysis or thermo tolerant yeast strains that can tolerate high range of temperatures and ferment the sugars easily.[40] Because thermo tolerant yeast strains nowadays are isolated and identified on a large scale as they possess a lot of advantage such as they produce higher ethanol, tolerate high inhibitory compound and reaction can be performed in a single bioreactor as in consolidated bio processing (CBP).

Thermo tolerant yeast strains are mainly found in the tropical regions, hot water springs, silage and waste water from sugar and starch industries. They can easily tolerate temperature above 40°C. The microorganism mainly used for fermentation purpose is yeast it has many thermo tolerant strains, but they need to be isolated and identified. Different thermo tolerant strains grow in different conditions, as every strain has a different temperature range. *Saccharomyces* is commonly used but it cannot resist the increase in temperature because as increasing in temperature both its morphology and physiology of which start to change; moreover growth and metabolism is also affected. Different strains show different behaviour with increasing temperature as collection of metabolites inside and outside the cell membrane is highly affected. Thermo tolerance is also affected by various factors like osmotic dehydration, chemical interaction, decreasing external pH, phase of growth, constituents of the culture media etc.[41] Table 3 showed ethanol yield from different thermo tolerant yeasts.

Table 3 Ethanol production using different thermotolerant yeasts

S. No	Themotolerant yeast strains	Temperature	Ethanol yield (g/L)	References
1	*Blastobotrys adeninivorans RCKP 2012*	50°C	14.05	[42]
2	*Kluyveromyces marxianus IMB3*	45°C	22.5	[43]
3	*Pichia kudriavzevii HOP-1*	45°C	24.25	[44]
4	*Saccharomyces cervisiae TJ14*	42°C	40	[45]
5	*Kluyveromyces marxianus DBKKU-Y102*	40°C	97.46	[46]
6	*Kluyveromyces marxianus OT-1*	40°C	73.6	[47]
7	*Saccharomyces cervisiae TZ1C*	40°C	65.2	[47]
8	*Kluyveromyces marxianus TISTR-5925*	40°C	45.4	[48]
9	*Saccharomyces cervisiae ZM15*	40°C	18.79	[49]
10	*Saccharomyces cervisiaeD5A*	37°C	21.9	[50]

Candida glabrata is one of the best known yeast strains that have high thermo tolerance and good ethanol production, plus it is resistant to high concentration of acid.[15] Then *Kluyveromyces marxianus* has high tolerance to temperature of about 45-50°C and produces high ethanol yield at 38-45°C and it also consumes wide range of sugar substrate.[51,52] *Kluyveromyces marxianus* IMB3 is the most effective microorganism used for fermentation. Among the saccharomyces strains TJ14 is one of the most popular thermotolerant strains that can easily resist the temperature up to 41°C and produces about 40g/L of ethanol with the help of filamentous fungus *Acremonium cellulolyticus*.[45] In some of the thermo tolerant yeast isolated strains, it was observed that despite their good thermo tolerance and good ethanol yields were not obtained. Hence to incorporate both features in one organism genetic engineering has been carried out. There are several methods by which a gene expressing a certain feature can be inserted in a strain that lacks it; but has a gene that expresses some other beneficial feature. Methods like gene shuffling, mutagenesis, cell encapsulation, site directed mutagenesis, evolutionary engineering are practised on a large scale.[29]

Conclusion

The present review summarizes current technological advancement of Lignocellulosic bioethanol production processes; pre-treatment, enzymatic hydrolysis and fermentation. The major step in lignocellulosic bioethanol production is fermentation. For cost effective bioethanol production, there are needs for utilization of both pentose and hexose sugars and both saccharification and fermentation take place in a single bioreactor. The present review summarizes different genetic engineering strategies for utilization of both pentose and hexose sugars. The present article also focused on technological advancement of different other procedures such as Simultaneous saccharification and fermentation (SSF), Yeast genetic engineering and Thermotolerant yeast for ethanol fermentation.

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Conflict of interest

The author declares no conflict of interest.

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