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Recent Advances in Fermentation of Lignocellulosic Biomass Hydrolysate to Ethanol

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Abstract – During the last past decades considerably large efforts have been made to optimize the production of lignocellulose derived fuel ethanol production which is economically feasible. Lignocellulosic materials serve as abundant feedstock, to produce fuel ethanol from renewable resources at reasonable costs. Following the pretreatment, the enzymatic hydrolysis process can be run separately (SHF) or simultaneously (SSF) with fermentation. But, there are some technological barriers such as toxic inhibitors released from the pretreatment of lignocellulosic feedstock's, lower scarification rates by enzymes and simultaneous and rapid fermentation of hexoses and pentose sugars, which needs to be addressed for efficient conversion of lignocellulosic biomass to bioethanol. The review paper covers all these aspects, challenges and development in the field of fermentation.

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INTRODUCTION

The world's present economy is greatly dependent on various fossil energy sources such as oil, coal, natural gas, etc., being used for the production of fuel, electricity and other goods (Uihlein *et al.,* 2009). Rising energy consumption, diminution of fossil fuels and increased environmental concerns has shifted the focus of energy generation towards biofuel use. **Bioethanol** is considered the most potential next generation automotive fuel because it is carbon-neutral and could be produced from renewable resources like lignocellulosic biomass (Kumar *et al.,* 2009). The cost and availability of the feedstock are crucial as it contributes 65–70% to the total ethanol production costs (Balat and Balat, 2009).

Lignocellulosic biomass (such as agricultural residues, forestry wastes, waste paper, municipal solid wastes, and energy crops) has been considered as possible raw material for ethanol production due to its renewability, large quantities, low prices (relative to grain or sugar), and potential environmental benefits (Chen, 2011; Talebnia *et al.,* 2010 and Hickert *et al.,* 2013). Lignocellulose is the most plentiful renewable biomass produced from photosynthesis, and its annual production was estimated in 1×10^{10} MT worldwide (Sanchez and Cardona, 2008).

In general, lignocellulosic feed-stocks are divided into three categories: (1) agricultural residues (e.g., crop residues and sugarcane bagasse), (2) forest residues, and (3) herbaceous and woody energy crops (Carriquiry *et al.,* 2011). Usually, lignocellulosic biomass contains 35 - 50% cellulose, 25 - 30% hemicelluloses and 20 - 25% lignin. Cellulose, a polymer of glucose residues connected by β-1, 4 linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature (Saha *et al.* , 2006). Hemicellulose is a short, complex carbohydrate structure that consists of different polymers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose), and sugar acids (Hendriks and Zeeman, 2009). Lignin is the third major component, a complex polymer of phenyl propane (p-coumaryl, coniferyl and sinapyl alcohol), act as cementing agent provides plants with the structural support and an impermeable barrier against microbial attack and oxidative stress (Howard *et al*., 2003). The biological process for converting lignocellulose to fuel ethanol requires: (1) delignification to liberate cellulose and hemicellulose; (2) depolymerization of carbohydrate polymers to produce free sugars; and (3) fermentation of mixed hexose and pentose sugars to produce ethanol (Balat and Balat, 2009). The key requirements for an economical lignocellulosic ethanol process include: efficient pretreatment methods of lignocelluloses, availability of low-cost hydrolytic enzymes, and use of optimal microbial strains capable of converting hexose and pentose sugars (Chen *et al.,* 2012) to ethanol, at high rates (Chen, 2011).

Pretreatment is the first and most important step in cellulose to ethanol technology because it can remove hemicelluloses, lignin and increase the porosity of materials which improves enzymatic sac-charification (Hendricks and Zeeman, 2009). Goals of an effective pretreatment process are (i) formation of sugars directly or subsequently by hydrolysis (ii) to avoid loss and/ or degradation of sugars formed (iii) to limit formation of inhibitory products (iv) to reduce energy demands and (v) to minimize costs (Sarkar *et al.,* 2012). Pretreatment includes physical, chemical, biological and thermal methods and their combinations. Among the pretreatment methods, dilute acid pretreatment has been widely studied and has been shown to effectively solubilize and hydrolyze hemicellulose into monomeric sugars and soluble oligomers, removing it from the cellulose fibers (Lu *et al.,* 2007). **Enzymatic hydrolysis** of pretreated lignocellulosic biomass involves biochemical reactions that convert cellulose into glucose and hemicellulose into pentoses (xylose and arbinose) and hexoses (glucose, glactose and manose), catalyzed by cellulase and hemicellulase enzymes respectively. In the manufacture of bioethanol by technologies involving enzymatic hydrolysis, the cost of enzymes, low hydrolysis rate caused by product (sugar) inhibition, low productivity of the microorganisms has been identified as the limiting factors for the downstream processes (Gonzalez *et al.,* 2011).

FERMENTATION

Glucose and xylose are the two dominant sugars in lignocellulosic hydrolyzates after saccharification, both need to be fermented efficiently into ethanol at high yield (Singh and Bishnoi, 2011) employed by several microorganisms, principally bacteria and yeasts (Almeida *et al.,* 2007). The fermentation organism must be able to ferment all mono-saccharides present and in addition, withstand potential inhibitors in the hydrolysates. Some anaerobic thermophilic bacteria are potential microorganisms for the production of ethanol due to their capability to metabolize a wide spectrum of sugars found in lignocellulose. Additionally, several advantages are associated with the production of ethanol at high temperatures, e.g. high bioconversion rates, reduced risk of contamination, and facilitated product recovery (Crespo *et al.,* 2012). *Saccharomyces cereviseae* and *Zymomonas mobilis*, commonly used microorganisms in alcohol fermentation where *Saccharomyces cerevisiae*, most prominent ethanol-producing yeast, proved to be more robust than bacteria being more tolerant to ethanol and inihibitors present in hydrolysates of lignocellulosic materials (Olson and Hahn-Hagerdal, 1996). However, simultaneous and rapid utilization of sugar mixtures is considered essential for economically feasible production of biofuel and commodity chemicals from biomass hydrolysates (Kim *et al.,* 2010). But current approaches are inefficient, since no native microorganisms can convert all sugars as most of them prefer glucose over other monomeric sugars and do not assimilate other sugars until glucose is consumed (Stulke and Hillen, 1999). *Saccharomyces cerevisiae* lacks the ability to ferment hemicellulose derived pentose (C5) sugars, which may constitute up to 45% of the raw material (Kumar *et al.,* 2009 and Sukumaran *et al.,* 2010), due to lack of the key enzymes in the xylose-metabolising pathway (Meinander *et al*., 1999).

Hemicellulose hydrolysate can be converted to xylitol by several microorganisms notably *Pachysolen tannophilus, Candida shehatae*, and *Pichia stipitis* (Wright, 1998 and Villarreal *et al.,* 2006). The xylose fermenting yeast *Pichia stipitis* has shown promise for industrial applications because it ferment xylose rapidly with a high ethanol yield and apparently produces no xylitol (Dominguez *et al.,* 1993). Also *Candida* ferments xylose to xylitol in a high yield and productivity but sometimes, due to the inhibitors in hydrolysate, it is difficult to obtain a high xylitol concentration in the fermentation broth and efficiencies are lower. In addition, they also need microaerophilic conditions and are sensitive to inhibitors, higher concentrations of ethanol and lower pH (Chandrakant and Bisaria, 1998). Therefore, worldwide, lots of R&D efforts are being directed to engineer organisms for fermenting both hexose (C6) and pentose (C5 sugars) with considerable amount of success. Numerous technologies for strain development have been employed to engineer *S. Cerevisiae* capable of fermenting xylose rapidly and efficiently. These include i) optimization of xylose-assimilating pathways, ii) perturbation of gene targets for reconfiguring yeast metabolism, and iii) simultaneous co-fermentation of xylose and cellobiose (Kim *et al.,* 2013). Successful ethanol production in xylose fermentation has been achieved using recombinant *S. cerevisiae* strains with heterologous xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis* along with over-expression of *S. cerevisiae* xylulokinase (XK) (Eliasson *et al.,* 2000 and Katahira *et al.,* 2006). Thus, the efficient utilization of xylose in hemicellulose in addition to glucose in cellulose by a recombinant xylose-fermenting *S. cerevisiae* strain would offer an opportunity to reduce the production cost of bio-ethanol significantly (Zaldivar *et al.,* 2001). A number of genetically engineered ethanol-producing strains capable of metabolizing xylose and other pentose sugars into ethanol have been developed (Yao and Mikkelsen, 2010;), but a common problem with these organisms is their sensitivity to inhibitors present in undetoxified hydrolysates (Dien *et al.,* 2003). Thus, two important requirements for an efficient ethanol-producing microorganism are to ferment a variety of sugars (pentoses and hexoses) and to tolerate stress conditions (Zaldivar *et al.,* 2005).

DETOXIFICATION OF HYDROLYSATE

Physical–chemical pretreatment of lignocellulosic biomass can generate some soluble inhibitory compounds, derived from a partial sugars and lignin degradation, may be toxic to fermenting microorganisms and hinder utilization of sugars obtained from biomass (Panagiotou and Olsson, 2007). Overcoming the effects of hydrolysate toxicity towards ethanologens is a key technical barrier in the bio-chemical conversion process for biomass feedstocks to ethanol. The nature and concentration of these toxic compounds depend on the raw material and the harshness of the pre-treatment. They are classified according to their chemical structure and include furan derivates (furfural and 5-hydroxymethylfurfural derived from pentose and hexose sugars degradation, respectively), weak acids (mainly acetic acid) and phenolic compounds from lignin (aromatic acids, alcohols and aldehydes) (Palmqvist and Hahn-Hägerdal, 2000).

Biological inhibitor abatement is a probable method for eliminating inhibitory compounds from the biomass hydrolysates. In this regard a fungal isolate, *Coniochaeta ligniaria*NRRL30616, metabolizes furfural and 5-hydroxymethylfurfural (HMF) as well as aromatic and aliphatic acids and aldehydes. NRRL30616 grew in corn stover dilute-acid hydrolysate, and converted furfural to both furfuryl alcohol and furoic acid. Hydrolysate was inoculated withNRRL30616, and the fate of pretreatment side-products was followed in a time-course study. A number of aromatic and aliphatic acids, aldehydes, and phenolic compounds were quantitated by analytical extraction of corn stover hydrolysate, followed by HPLC–UV–MS/MS analysis. Compounds representing all of the classes of inhibitory side-products were removed during the course of fungal growth. Biological abatement of hydrolysates using *C. ligniaria* improved xylose utilization in subsequent ethanol fermentations (Nichols *et al.,* 2008).

With respect to lignocellulosic biomass, one of the detoxification methods, fungal laccase and peroxidase enzymes have been used experimentally to detoxify wood hydrolysates (Martin *et al.,* 2002). Laccase was expressed in recombinant *Saccharomyces* to increase resistance to phenolic compounds (Larsson *et al.,* 2001). Using laccases enzymes has been explored in which a substantial removal of phenolic compounds by laccases reduced the inhibitory effects of slurry from steam-exploded wheat straw. It led to improve the fermentation performance of thermotolerant yeast strain *Kluyveromyces marxianus* used, shortening its lag phase and enhancing the ethanol yields, and increase the substrate loadings of saccharification and fermentation broths. According to this study, detoxification by laccases could reduce costs of lignocellulosic ethanol process through the use of partially detoxified whole slurry and increasing higher fermentation rates and ethanol yields (Moreno *et al.,* 2012).

In a study, hemicellulose hydrolysate from corncobs, separated by diluted sulfuric acid and sequently detoxed by boiling, overliming and solvent extraction, was used for xylitol production by *Candida tropicalis*W103. The effect of glucose and acetate in hydrolysate on xylitol production was investigated. It was found that glucose in hydrolysate promoted growth of *Candida tropicalis* while acetate at high concentration was inhibitory. The acetate inhibition can be alleviated by adjusting pH to 6 prior to fermentation and a substrate feeding strategy. Under these optimum conditions, a maximal xylitol concentration of 68.4g $l^{-1}$was obtained after 72h of fermentation, giving a yield of 0.7gg^{-1} xylose and a productivity of 0.95gl^{-1} h^{-1} (Cheng *et al.,* 2009). Alternative methods such as ammonia/sodium hydroxide (NaOH)-neutralization to improve the efficacy of hydrolysate conditioning for ethanol production have been proposed due to no gypsum generated and reduced xylose loss (Pienkos and Zhang, 2009).

A new yeast strain of *Clavispora* NRRL Y-50464 has been reported that is able to utilize cellobiose as sole source of carbon and produce sufficient native β-glucosidase enzyme activity for cellulosic ethanol production using SSF. In addition, this yeast is tolerant to the major inhibitors derived from lignocellulosic biomass pre-treatment such as 2-furaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (HMF), and converted furfural into furan methanol in less than 12 h and HMF into furan-2,5-dimethanol within 24 h in the presence of 15 mM each of furfural and HMF (Liu *et al.,* 2012).

FERMENTATIVE TECHNIQUES

In the process of ethanol production from lignocellulosic materials, enzymatic hydrolysis and fermentation can be carried out by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) (Romani *et al.,* 2012). SHF allows the fermentation and hydrolysis to be performed at separate conditions; hence the fermenting organism and the enzymes can be used at independent optimum temperature and pH. However, SHF results in enzyme inhibition by the hydrolysis products, i.e. as the hydrolysis progresses, the sugar concentration in the hydrolysis bioreactor increases which can reduce the efficiency of the cellulase enzymes used (Soderstrom *et al.,* 2005).

Compared with separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) is more favoured because in SSF glucose released by the action of cellulase is converted quickly to ethanol by the fermenting microorganism, thus minimizing end-product inhibition to cellulase caused by glucose and cellobiose accumulation (Sassner *et al.,* 2008; Zhao and Xia, 2009 and Jang *et al.,* 2012). In this option, the cellulose hydrolysis and glucose fermentation steps are combined in a single vessel (Ghosh *et al.,* 1982). Since cellulase is inhibited by glucose as it is formed, rapid conversion of the glucose into ethanol by yeast results in faster rates, higher yields, and greater ethanol concentrations than possible for SHF (Sasikumar and Viruthagiri, 2010). Combining the saccharification and fermentation processes in one vessel is found to be better alternative to separate hydrolysis and fermentation (SHF) in terms of cost (De Bari *et al.,* 2002), perhaps due to reduced process time, lower energy requirement and high bioethanol yields at high solid loading (Ohgren *et al.,* 2007; Nikolic *et al.,* 2009; Lee *et al.,* 2013 and Ofori-Boateng and Lee, 2014). Simultaneous saccharification and fermentation (SSF) is considered an appropriate process that presents significant advantages for conversion of lignocellulosic biomass to ethanol (Olofsson *et al.,* 2008).

In spite of the economic advantage of SSF over separate hydrolysis and fermentation (SHF), the critical problem associated with SSF of cellulose is the difference in temperature optima for saccharification (45–50◦C) and fermentation (25–35◦C). *Saccharomyces* strains are well known as good ethanol producing microorganisms; however they require an operating temperature of 35◦C. Fungal cellulases, which are most frequently applied in the cellulose hydrolysis, have an optimum temperature of 50◦ C. At lower temperatures, the substantially lower hydrolysis rates would be unfavorable in terms of increased processing time. The fermentation efficiency of *S. cerevisiae* at high temperatures is very low due to increased fluidity in membranes to which the yeast responds by changing its fatty acids composition (Suutari *et al.,* 1990). A possible solution to solve this problem is using thermotolerant yeast strains instead of *Saccharomyces*, which would allow higher processing temperatures, thus increased rates of the hydrolysis (Kadar *et al.,* 2004). In another study, the thermal treatments made it possible to have one strain, IR2-9a, with greater ethanol yield in SSF process than the control strains (*Saccharomyces cerevisiae*). With this strain it was possible to convert pretreated lignocellulosic material into ethanol at temperatures closer to the optimal for enzymatic hydrolysis making the SSF process more efficient (Edgardo *et al.,* 2008). Genetic engineering has been employed to develop the various aspects of fermentation from higher yield to better and wide substrate utilization to increased recovery rate (Sarkar *et al.,* 2012). Researchers routinely use HPLC method for monitoring ethanol production during fermentation of biomass pretreatment hydrolysate; ethanol production during simultaneous saccharification and fermentation (SSF); and measuring acetic acid and furans formed during pretreatment (Mohagheghi *et al.,* 2006).

In conventional SSF procedures, after pretreatment, cellulolytic enzymes are applied to hydrolyze the cellulose polymers into short oligosaccharides such as cellobiose. Since the commonly used ethanologenic yeast *Saccharomyces cerevisiaeis* unable to utilize cellobiose, an additional enzyme, β-glucosidase, is required to digest cellobiose into glucose in order to be utilized by the fermentation yeast. This cellobiose/xylose co-fermentation strategy provides an opportunity to efficiently utilize lignocellulosic biomass for microbial lipid production. A oleaginous yeast, *Lipomyces starkeyi*, was shown to consume cellobiose and xylose simultaneously and to produce intracellular lipids from cellobiose, xylose and glucose (Gong *et al.,* 2012). This cellobiose/xylose co-fermentation strategy by passes glucose repression and is expected to improve the economics of lipid production when lignocellulosic biomass is employed as raw material. The reducing sugar produced during hydrolysis were concentrated and used for ethanol production *by S. Cerevisiae* and *S. Stipitis* and their co-culture. Highest ethanol production with co-culture was 20.8 g/L and co-culture of S. Cerevisiae and *S. Stipitis* produced 32% more ethanol than *S. Cerevisiae* alone and 41% more ethanol than *S. Stipitis* alone (Singh *et al.,* 2014). Coupling of SSF process with ultrasound can accelerate the production rate of bioethanol at shorter time. In a study, SSF of OPFs was combined with ultrasound irradiation to assess the efficiency of the process on bioethanol yield (Ofori-Boateng and Lee, 2014). Many different types of processes for ethanol fermentation have been proposed, including batch fermentation, continuous fermentation, continuous fermentation with cell recycling, fed-batch cultures and repeated-batch cultures. Batch fermentation process is used extensively to convert sugars to ethanol for the production of beverages and biofuels. As for fed-batch fermentation, the intermittent addition of glucose, without the removal of the fermentation broth, into the fed-batch culture is one of the most common methods for the production of ethanol in the industry (Chang *et al.,* 2012)

EXTRACTION OF ETHANOL

Ethanol recovery from fermentation broth is traditionally done by distillation. For lignocellulose-based ethanol production to be economically viable on an industrial scale, the ethanol produced must be above 4% (v/v) in the fermentation broth (Wingren *et al.,* 2003). For most types of lignocellulosic materials, this requires operating at dry mass (DM) concentrations about 15% to achieve sufficiently high cellulose levels (Jorgensen *et al.,* 2007). However, high substrate concentration in the form of fibrous, solid materials poses two problems: (1) the increased concentrations of inhibitors such as acetic acid, furfural, and ethanol hamper the performance of yeast and enzymes and (2) high viscosity results in more power consumption in the fermentor and lowered mixing and heat transfer efficiency (Georgieva *et al.,* 2008). In order to increase the final substrate concentration while avoiding increases in viscosity, fed-batch culture was used in the SSF process (Zhang *et al.,* 2010). To recover low concentrations of ethanol from fermentation, pervaporation may be economically more feasible than distillation (Vane, 2005) as for dilute ethanol streams (less than 5 wt.%), the high energy requirements in distillation (Madson and Lococo, 2000). The ethanol from fermentation broth can be concentrated, depending on the membrane selectivity, by using hydro-phobic pervaporation before feeding it to distillation. This should reduce the energy load on the distillation. Similarly, the remaining 5 wt. % of water from the top product of distillation can be removed by hydrophilic pervaporation to achieve fuel grade (anhydrous) ethanol (>99.5 wt. %). Here we focus on the ethanol recovery from lignocellulosic fermentation broth by hydrophobic pervaporation (Gaykawad *et al.,* 2013). The breakdown of lignocellulosic biomass by pretreatment and the fermentation of the resulting sugars leads to a variety of by-products mainly divided into carboxylic acids, furans and phenolics (Almeida *et al.,* 2007), which may threat the pervaporation membrane performance.

CONCLUDING REMARKS

Efficient and rapid fermentation of all sugars present in cellulosic hydrolysates is essential for economic conversion of renewable biomass into fuels and chemicals. Simultaneous co-fermentation would allow a continuous fermentation process, which is the most effective way to reduce capital expenditures. During past years a lot of research and development has been done in the genetic improvement of strains but still more research have been established to further improve and optimize the fermentation methods and microorganisms for industrial applications.

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