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Biological Pretreatment Strategies For Enhanced Saccharification Of Lignocellulosic Biomass In 2G Ethanol Biorefineries

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ABSTRACT
Rapidly increasing demand and depleting reserves of crude oil have pushed scientific community to work towards finding alternatives of the fossil fuels. Lignocellulosics based 2G ethanol is being looked as a sustainable eco-friendly alternative of the crude oil to be utilized in transportation sector. Despite decade-ful of research on developing 2G biorefineries, pretreatment of LCB still remains one of the major bottlenecks. There are several physical and chemical pretreatment methods in practice since years but high process cost, production of fermentation inhibitors and toxic waste generation are some of the major concerns associated with them. Biological pretreatment is a good alternative in this direction which offers a greener and cleaner pathway to get rid of recalcitrant lignin fraction that obstructs the access of cellulolytic enzymes to target sites present in LCB. Although biological pretreatment strategies are being explored from past couple of decades but development of an economically viable and efficient technique is still under research. The recent developments in this field have indicated towards formulation of bacterial co-cultures, fungal co-cultures and bacteria-fungi co-cultures to attain efficient bioconversion of lignocellulosic biomass into ethanol.Through this article, an effort is made to review various biological pretreatment strategies in practice with main emphasis on the enzyme and microorganisms involved, regulation of ligninolytic enzymes, and process parameters affecting the success of the strategy adopted.
Keywords: Biological pretreatment, saccharification, lignocellulosic biomass, regulation, consortium, co-culture, laccase, lignin

1. INTRODUCTION

Pretreatment of lignocellulosic biomass (LCB) is one of the three essential steps: pretreatment, hydrolysis and fermentation, involved in the production of 2G biofuels (Xiao, Yin, Xia, & Ma, 2012). The breakdown of LCB into fermentable sugars is restricted strongly by its crystallinity, degree of recalcitrance and polymerization with lignin being the major hindering moiety(Kim & Lee, 2006; Yang & Wyman, 2006). Lignin limits the second step of biorefineries i.e., hydrolysis by binding in a non-productive manner to the hydrolytic enzymes (Esteghlalian, Hashimoto, Fenske, & Penner, 1997). Numerous pretreatment strategies such as grinding, milling, chopping, liquid hot water treatment, ammonia fiber explosion (AFEX), dilute ammonia, ionic liquids, organosolv process, alkali, acid and biological treatment have been used by the researchers to disrupt interactions of lignin with carbohydrate fraction for overall improved hydrolysis yield(Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010; Keshwani, 2009; Socha et al., 2014; Taherzadeh & Karimi, 2008).Among all the above stated strategies, biological pretreatment of LCB seems to be a potent mild and

eco-friendly method that does not involve generation of fermentation inhibitors and toxic waste products. Moreover, biological pretreatment strategies involve lesser cost and energy inputs (Sharma, Xu, & Qin, 2019).The biological pretreatment strategies rely mostly on fungi and bacteria which can efficiently degrade lignin, hemicellulose and little of the cellulosic fraction(Sánchez, 2009). The studies have established white rot fungi as the most efficient biological technique to delignify LCB through the action of lignin peroxidases, manganese peroxidases, and laccases (Kumar & Wyman, 2009; Shi, Chinn, & Sharma-Shivappa, 2008). Recent studies also indicate towards the potential of bacterial enzyme systems to efficiently treat LCB for enhanced sugar yields(Verma & Shirkot, 2014). The concept of consortia and co-culture is also into practice by many researchers which not only bring the diverse catalytic machineries on a common platform, helps in enhancing the lignocellulolytic abilities of different microorganisms as well. The platform developed therefore improves the yield of monomeric sugars resulting in improved subsequent 2G ethanol yield. However, long residence time for effective delignification and ability of the microbes to attack cellulose and hemicellulose fractions of biomass are couple of limitations associated with the biological pretreatment strategies. These biological strategies are not fully exploited yet, therefore, there is need for an extensive research in this field to develop an economically viable process. This reviewsummarizes various biological treatment methods addressing the responsible microorganisms, associated enzymes and their regulation.

2. BIOLOGICAL PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

Pretreatment of LCB is first and the most important step of 2G biofuel production since it constitutes nearly 40% of the overall process cost (Zhang, Li, Shen, Wang, & Sun, 2000). The importance of pretreatment step can be judged from the fact that yield of fermentable sugars post treatment increases upto 70% as compared to untreated biomass(Alizadeh, Teymouri, Gilbert, & Dale, 2005). The fermentable cellulose fraction in LCB is naturally protected by hemicellulose and lignin which reduces surface area accessible to cellulolytic enzymes. Therefore, proper pretreatment of LCB is necessary to increase the concentration of monomeric sugars which can be subsequently fermented into ethanol. The main enzymes involved in lignin digestion are peroxidases and laccases where former has lignin peroxidase and manganese peroxidase enzymes capable of degrading non-phenolic and phenolic lignin units, respectively. Laccases on the other hand act synergistically with peroxidases to degrade lignin completely. However, recent studies have shown that laccases can single handedlytake care of the lignin fractions of LCB (Binod, Janu, Sindhu, & Pandey, 2011). The auxiliary activity (AA) proteins including lytic polysaccharide monooxygenases (LPMOs) and cellobiose dehydrogenases (CDHs) have been reported to increase the enzymatic hydrolysis of different lignocellulosic substrates (Levasseur, Drula, Lombard, Coutinho, & Henrissat, 2013). The bacterial and fungal strains known to hydrolyze recalcitrant biopolymersare discussed in the following sections.

2.1. Bacterial strains

There are several bacteria reported for their biomass degrading potential but screening of best bacterial strain to be employed for pretreatment of LCB in the biorefineries is the most critical step for 2G ethanol production. For over several decades' fungal lignin degrading enzymes were in trend but recently the paradigm has shifted towards bacterial enzyme systems owing to their better thermal stability. The cellulolytic bacteria *Cellulomonas fimi,Thermomonosporafusca*and *Paenibacilluscampinasensis*have been showcased in past for their potential to pretreat LCB(Maki, Leung, & Qin, 2009; Sharma et al., 2019). Bacterial species such as *F. succinogenes, R. albus, R. flavefaciens,* etc. are associated withthe rumen and show enormous potential to adhere with cellulose and mediate its hydrolysis (Duff &

Murray, 1996). Some of the bacteria like *A. lipoferum*and *B. subtilis* have shown delignifying abilities because of the laccases they produce (Saritha & Arora, 2012).

2.2. Fungal strains

Fungi are the intensively explored microorganisms for their potential to produce ligninolytic enzymes. Most of the lignocellulolytic fungi belong to genus *Aspergillus, Penicillium, Trichoderma, Schizophyllum, Fomitopsis, Orpinomyces, Trametes,* etc., (Dashtban, Schraft, & Qin, 2009; Paudel & Qin, 2015; Rai, Kaur, Singh, et al., 2016). Degradation of lignin on the other hand is a complex process and its success depends largely upon the selection of fungal strain. White rot fungi with special reference to basidiomycetes have significant lignin disintegrating powers and are considered as natural degraders of lignin. A study revealing 30 wood decaying white rot fungi was conductedin which *Phellinus pni-2, Pholiotamutabilis, Phlebia brevispora-1, Phanerochaetechrysosporium* were reported as the best delignifiers (Otjen, Blanchette, Effland, & Leatham, 1987).

2.3. Other macroorganisms

Apart from microorganisms there are several macroorganisms which have enormous potential to degrade lignocelluloses. These organisms include insects, worms, gastropods and ruminant animals which possess different masticating mechanisms for physical breakdown of recalcitrant biomass and enzymatic machinery for digestion of cellulose. There are nearly 20 families of insects including beetles, termites, wasps, silverfish, cricket etc., that can degrade leaf litters, forage and wood (J. Sun, Ding, & Doran-Peterson, 2013). The enzymatic activities within the gut of earthworms such as *Perionyx excavates, Lumbricusrubellus, Eisenia fetida, etc.*, are known to mediate digestion of cellulose, chitin, lignin, starch, sugars, etc., (Pathma & Sakthivel, 2012; Vivas, Moreno, Garcia-Rodriguez, & Benítez, 2009; Zhang et al., 2000). Worm tea, the liquid leachate of vermicomposting is considered as a microbial consortium and has been used as an alternative of acid pretreatment for biofuel production (Siti, Siti, Nur, Renuka, & Norli, 2013). On the similar grounds, microbial consortia of gastropods and ruminants also have the potential to degrade lignin fraction of LCB (Fondevila & Dehority, 1994; Russell, Muck, & Weimer, 2009; Weimer, Nerdahl, & Brandl, 2015).

2.4. Microbial co-cultures

In biological pretreatment strategies, high enzyme activity is always desired, however, it is not always possible to obtain such higher titers of lignocellulolytic enzymes from a single bacterial or fungal strain. Therefore, concept of co-culture of two or more microbial strains can be very useful in achieving significantly higher activities of all the desired enzymes. There could be three possible combinations of bacterial and fungal strains for performing pretreatment of LCB.

Bacterial co-cultures involving two or more bacterial species can be useful in biofuel production. Many bacterial genera such as *Cellulomonas, Clostridium, Thermomonospora, Bacillus, Streptomyces and Ruminococcus*produce various cellulases that can work together to carry out cellulolytic hydrolysis (Y. Sun & Cheng, 2002; Zhou & Ingram, 2000). A study showed significantly higher hydrolytic potential when *Paenibacillus*sp., *Bacillus* sp., and *Aneurinibacillusaneurunilticus*were cultured together in comparison to their pure cultures(Chandra & Chowdhary, 2015). The study on co-cultures of *Clostridium thermocellum* and other *Clostridia* has exhibited significant increase in cellulolytic/hemicellulolytic hydrolysis into fermentable sugars(Maki et al., 2009).

Fungal co-culture has been in practice for pretreatment of LCB from past couple of decades. A study on co-culture of *T. reesei* and *A. phoenicis*exhibited high levels of total cellulase and β-glucosidase activities whereas themonoculture of two fungi showed high total cellulase and low β-glucosidase activity, and low total cellulase and high β-glucosidase activity, respectively (Wen, Liao, & Chen, 2005). Further, a study showed that lignin

degradation improved significantly in a co-culture of *C. subvermispora*and *P. ostreatus*when compared their monocultures(Chi, Hatakka, & Maijala, 2007).

There is a recent shift in the paradigm from bacterial or fungal co-cultures to co-cultures involving both bacterial and fungal strains. This type of co-culture mimics nature where different microbial communities co-exist and communicate via interconnected networks to derive their nourishment, breaking down complex substrates into simpler ones. Examples of such techniques can be found in literature where *T. reesei* and *E. coli* were co-cultured to produce isobutanol and *Z. mobilis*and *P. stipites* were co-cultured to produce ethanol (Fu, Peiris, Markham, & Bavor, 2009; Minty et al., 2013)

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3. REGULATION OF ENZYMES INVOLVED IN BIOLOGICAL PRETREATMENT

Lignin degradation potential of microorganisms can be improved significantly by employing molecular techniques. The regulatory elements present in promoter region play an important role in regulating expression of ligninolytic enzymes. The expression of *P. chrysosporium*genes has been reported to be positively influenced through carbon and nitrogen limitation(Cohen, Hadar, & Yarden, 2001). In a protein expression study, researchers have shown the effect of different carbon sources on the expression of lignocellulolytic enzymes(Rai, Kaur, & Chadha, 2016). The effect of different substrates on the expression of genes involved in lignin degradation has also been reported in literature(Salame, Yarden, & Hadar, 2010).

4. FACTORS AFFECTING BIOLOGICAL PRETREATMENT

Although biological pretreatment strategies are greener and cleaner approaches which do not generate any fermentation inhibitor, long treatment time involved is a major limiting factor. However, screening of the most efficient microbial strains and optimization of the culture conditions can increase efficiency of the overall process. Apart from the composition of LCB, several process parameters like treatment temperature, pH, incubation time, inoculum age and concentration, moisture content and rate of aeration are the important factors governing the success of designed biological pretreatment strategy (Du et al., 2011; Isroi et al., 2011; Patel, Gupte, & Gupte, 2009).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Lignocellulosics based (2G) ethanol is being sorted as an alternative source of renewable energy, however, development of an economically viable and efficient pretreatment technology is still needed. Since biological methods have several advantages over the other physical and chemical methods of pretreatment, therefore, addressing challenges associated with the former to reduce cost and time should be the main focus of future research.

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Conflict of interest

The authors declare no conflict of interest.

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