

Review Article

A Review Article of Biological Pre-Treatment of Agricultural Biomass

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ABSTRACT

Pre-treatment is a key step in the production of bioethanol from lignocellulosic biomass. Current pre-treatment techniques including physical, chemical and physico-chemical methods may increase the cost of production and produce inhibitors. In addition, they are not environmentally friendly. On the other hand, biological pre-treatment is mild, less costly, eco-friendly and consumes less energy. Despite all these benefits, several factors affect the biological pre-treatment process including microbial strain, the culture and environmental conditions as well as the type of lignocellulose material. To overcome these setbacks, different forms of biological pre-treatments such as microbial and ligninolytic enzyme pre-treatments as well as processes are studied. This review presents an overview of different forms of biological pre-treatment, various processes carried out with the aim of enhancing delignification and drawbacks of this pre-treatment process.

Keywords: Agricultural residues, biological pre-treatment, fungi, lignocellulosic biomass, ligninolytic enzymes

INTRODUCTION

Growing demand for energy worldwide is negatively affecting our environment

due to the rise in fossil fuel combustion (Larran et al., 2015). The negative effects of fossil fuel combustion coupled with concerns about energy security, especially in growing economies, have resulted in the search for new sources of energy supply (Arora et al., 2016). Among the alternative sources of energy available, bioethanol produced from biomass has the potential to reduce dependence on petroleum products

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(Lopez-Abelairas et al., 2013a). Different types of biomass can be used for bioethanol production (Oke et al., 2016). Crops such as corn, sugar beets, sorghum and sugar cane are being used for the production of bioethanol (Lemée et al., 2012). However, the use of food crops to produce bioethanol contributes to high food prices globally (Larran et al., 2015).

Lignocellulosic biomass, a non-food source, can be transformed into useful products such as bioethanol, methane or other important chemicals (Cianchetta et al., 2014). Lignocellulosic biomass is rich in energy, less expensive and abundant all over the world (Okeke et al., 2015). This biomass comprises residue from crops and forest products that are not used. They are essentially made up of lignin, hemicellulose and cellulose (Horisawa et al., 2015) and are a good source for bioethanol production (Castoldi et al., 2014). However, access to the sugar component of lignocellulosic biomass is the major problem of bioethanol production from such materials (Garcia-Torreiro et al., 2016). The presence of lignin serves as a protective cover preventing enzymatic access to the hemicellulose and cellulose (Castoldi et al., 2014). A suitable pre-treatment technique which is able to eliminate and/or reduce or alter the lignin component; exposing the cellulose and hemicellulose for degradation and fermentation is, therefore, very important (Ma & Ruan, 2015).

Pre-treatment of lignocellulosic biomass can be physical, chemical, biological or a combination of these (Larran et al., 2015).

Physico-chemical pre-treatment techniques, although effective, may produce substances that may impede sugar hydrolysis and the fermentation process (Salvachúa et al., 2013). These techniques are also costly and may not be environmentally friendly (Asgher et al., 2016). Biological pre-treatment is more eco-friendly and requires less energy as well as mild conditions (Garcia-Torreiro et al., 2016). It is a promising alternative to non-environmentally friendly physical and chemical pre-treatment methods (Arora et al., 2016). This review sought to discuss different forms of biological pre-treatment, its various processes carried out with the aim of enhancing delignification and drawbacks of this pre-treatment technique.

AGRICULTURAL BIOMASS

Different stages in crop production generate materials that may be used as feedstock for bioethanol production (Serna et al., 2016). Biomass from crop cultivation is rich in energy, less expensive (Okeke et al., 2015) and abundant in supply all over the world (Placido et al., 2013). The cell wall of plants is made up of a network of polysaccharides, structural proteins and phenolic compounds (Jenkins, 2014) that make the cell wall rich in chemicals and fermentable sugars for biofuel production (Guerriero et al., 2016). Although agricultural biomass has the same major components, there is variation in its composition from one species to another (Galbe & Zacchi, 2012).

The sustainable availability of agricultural residue is very important in the quest to increase the production and

utilisation of bioethanol as an alternative to fossil fuel (Cardoen et al., 2015). Searle and Malins (2016) reported that majority of European Union (EU) member states will have more than enough feedstock in 2020 to meet the directive of 0.5% advanced biofuel blending targets for transportation. Among the EU member states, France and Germany are the largest producers of agricultural residue. Cardoen et al. (2015) stated that 611 Mton/year of agricultural field residue is generated in India, out of which 158 Mtons (25%) are deemed to be surplus. Out of this surplus agricultural residue, 41 Mton/year are from sugarcane bagasse, 28 Mton/year from paddy straw, 21 Mton/year from wheat straw and 19 Mton/year from cotton stalk. Gao et al. (2016) reported that the overall agricultural residue in China is 10,818 PJ, out of which 8,419 PJ are available for the production of energy. The energy potential of rice residue (2,418 PJ), corn residue (2,334 PJ) and wheat straw (1,232 PJ) makes up 71% of the overall available energy supply. Rice husk, corn cob, sugarcane bagasse and peanut hull make up about 12% to 15% of the total available agricultural residue in China. Saini et al. (2015) indicated that worldwide production of the main agricultural residue is 354.34 million tons of wheat straw, 731.3 million tons of rice straw, 128.02 million tons of corn straw and 180.73 million tons of sugarcane bagasse. The largest amount of rice and wheat straw are produced in Asia, while corn straw and sugarcane bagasse are generally from America. Research into

the effective utilisation of lignocellulose materials from agricultural biomass is on the increase (Wang et al., 2013). Different types of agricultural biomass including wheat straw, corn stover, corn cob, banana stalk and sugar cane bagasse have been biologically pre-treated for bioethanol production (Table 1 and 3).

PHYSICAL AND CHEMICAL PRE-TREATMENT

Physical, chemical and physico-chemical methods have been used to pre-treat lignocellulose materials (Maurya et al., 2015). Methods including milling, chipping, grinding and/or irradiation (gamma rays and electron beam) have all been applied to pre-treat lignocellulose material. However, these processes demand high energy, making them very expensive (Zhu, 2011).

Chemical pre-treatment is the most extensively used method. Chemicals such as acids, bases, ionic liquids and organic solvents are used for pre-treatment of lignocellulose materials (Aver et al., 2014). Sulphuric, nitric, hydrochloric and phosphoric acids have all been used (Nieves et al., 2016). However, high concentration of these acids is dangerous and corrosive (Kristiani et al., 2013). Partial hydrolysis of the cellulose and hemicellulose components (He et al., 2014) as well as production of inhibitors, including furfural derivatives, acetic acid, phenolic and other aromatic compounds, may also occur (Kim et al., 2016). Dilute phosphoric acid has been

reported as less corrosive and toxic compared to dilute sulphuric acid (Siripong et al., 2016), although very expensive (Nair et al., 2015). Organic solvents such as methanol, ethanol, acetone, ethylene, glycerol, acetic acid, glycols or phenols are also sometimes used to chemically pre-treat lignocellulose material (Hideno et al., 2013). Organic acids including maleic, oxalic, fumeric and citric acids are also used for pre-treatment (Lewandowska et al., 2016). Ionic liquids produce less inhibitory compounds but are expensive and can be toxic to hydrolytic enzymes (Ninomiya et al., 2013). Alkaline solutions including sodium, potassium or ammonium hydroxides can also break the bonds linking lignin to carbohydrates (Steffien et al., 2014).

Combinations of both physical and chemical methods have also been reported as an effective pre-treatment method. Steam explosion, hydrothermolysis, wet oxidation and ammonia fibre explosion (AFEX) are all types of physico-chemical pre-treatment methods. Steam explosion may produce inhibitors, while hydrothermolysis and wet oxidation requires consumption of large amount of energy. AFEX pre-treatment, however, is not suitable for biomass containing high lignin content (Galbe & Zacchi, 2012). Generally, physical, chemical and/or physico-chemical techniques may require special equipment and machines as well as harsh conditions, and this normally results in high energy demand and production of inhibitors that impede enzyme hydrolysis and fermentation processes.

BIOLOGICAL PRE-TREATMENT

Biological pre-treatment makes use of either microorganisms or the enzymes they produce to break down the lignin content of lignocellulose material (Ishola et al., 2014). Biological pre-treatment should be able to significantly reduce carbohydrate loss. It is, therefore, very important to choose a microorganism with high delignification potential but with less ability to break down cellulose during the pre-treatment process (Lopez-Abelairas et al., 2013b).

Microbial Pre-Treatment

A number of microorganisms such as bacteria, fungi and actinomycetes have the ability to reduce the lignin content in lignocellulose materials (Ma & Ruan, 2015). However, the ability of a microorganism to degrade only lignin is very important in preventing the loss of cellulose during the pre-treatment process. The best combination is a microorganism that will degrade low amounts of sugar and high amounts of lignin within the shortest possible time (Garcia-Torreiro et al., 2016).

Fungi are very important in the biological pre-treatment process as they are able to produce ligninolytic enzymes to break down lignin (Ghorbani et al., 2015). Lignin-degrading basidiomycetes (white-rot fungi) are the major decomposers in the forest ecosystem (Kamei et al., 2012) and are the most widely used microorganisms for biological pre-treatment of lignocellulose

materials (Pinto et al., 2012). The widespread use of white-rot fungi is due to their ability to produce high levels of ligninolytic enzymes (Cianchetta et al., 2014). Unlike other fungal groups, white-rot fungi are able to degrade all the major components (lignin, hemicellulose and cellulose) of lignocellulose materials. Some white-rot fungi may degrade lignin, hemicellulose and cellulose at the same time. However, others will selectively degrade lignin over the other components (Hatakka & Hammel, 2010). The degradation rate of white-rot fungi differs from one species to another (Castoldi et al., 2014). Although the major components of lignocellulose materials are the same, different fungi will act differently when cultured on these materials (Garcia-Torreiro et al., 2016). A number of white-rot fungi have been successfully used to reduce the lignin content of different lignocellulose materials (Table 1). Microscopic analysis of biologically pre-treated *Eucalyptus grandis* sawdust by Castoldi et al. (2014) revealed extreme changes in the structure of pre-treated sawdust, including separation of fibre and pore formation in much of the surface of the cell wall, compared to untreated sawdust. These changes were reported to be clearly related to the growth of the fungi studied. Pores formed in material pre-treated with *Pleurotus pulmonarius*, *Trametes* sp. and *Ganoderma lucidum* were more visible compared to the others although pre-treatment was done under the same conditions. This resulted in differences in total cellulose composition after pre-treatment (Table 1). Variations in growth and

degradation rates of the fungi were stated as the possible cause for the structural and total cellulose differences observed (Castoldi et al., 2014). Similarly, Lopez-Abelairas et al. (2013) reported different sugar yields of $46 \pm 2\%$ and $65 \pm 2\%$ after pre-treating wheat straw with *Pleurotus erynii* and *Irpex lacteus*, respectively for 21 days under the same optimised conditions. Saha et al. (2016) observed great variations in the rate of delignification ($1.5 \pm 0.0\%$ to $51.4 \pm 2.9\%$) of corn stover by various fungi under the same pre-treatment conditions of 74% moisture level and 28°C temperature for 30 days (Table 1). Wang et al. (2014) also reported variations in delignification rate for *Trametes velutina* and *Trametes orientalis* after pre-treating *Populus tomentosa* with the two microorganisms for 12 weeks to enhance enzyme hydrolysis (Table 1).

Pre-treatment of corn stalk with *Phanerochaete chrysosporium* (Zhao et al., 2012) and *Pleurotus ostreatus* (Saha et al., 2016) under different conditions resulted in differences (35.3% and 54.7%, respectively) in the rate of delignification between the two processes. These differences may be attributed to the fungi variation and/or differences in the pre-treatment conditions. Environmental as well as nutritional conditions greatly affect microbial growth and hence, delignification. An effective lignin degrading microorganism and an efficient process of culturing are very important for ensuring high rate of delignification (Chang et al., 2014). Saha et al. (2016) reported that under optimum culture conditions of 84% moisture, 28°C

temperature and 42 days of incubation, *Phlebia brevispora* was able to increase the sugar yield of corn stover by 15.4% after pre-treatment. The metabolic activities of microorganisms may be influenced by the type of substrate they are cultured on. The composition of agricultural residues may vary from one species to the other (Galbe & Zacchi, 2012). *Irpex lacteus* pre-treatment of different agriculture residue under the same conditions resulted in differences in the rate of delignification among the various residues (Table 1). This was attributed to variations in the structure of agricultural residues with respect to species, tissue, origin and growth period (Garcia-Torreiro et al., 2016).

Brown-rot fungi have the ability to degrade wood. However, brown-rot fungi degrades mainly the hemicellulose and cellulose components of lignocellulose materials, leaving behind a chemically-modified lignin residue. They are able to degrade hemicellulose and cellulose without the removal of lignin, or remove only a very small part of it. Their ability to degrade both hemicellulose and cellulose without lignin removal has been attributed to both oxidative and hydrolytic attacks. It has been suggested that the oxidative non-enzymatic attack by these fungi is through the use of low molecular weight chemicals that are able to diffuse and degrade cellulose (Hatakka & Hammel, 2010). Several brown-rot fungi produce low molecular weight chemicals, including hydrogen peroxide and oxalic acid, which are used during the non-enzymatic degradation process (Schilling et

al., 2012). Schilling et al. (2012) reported the use of the brown-rot fungi *Gloeophyllum trabeum* and *Postia placenta* to break down aspen, spruce or corn stover for a period of 16 weeks before saccharification with enzymes. Generally, pre-treatment with the brown-rot fungi increased sugar yield threefold. *Gloeophyllum trabeum* pre-treatment of aspen for two weeks provided the best yield i.e. 72% glucose.

Ascomycetes and mitosporic fungi are responsible for soft-rot decay in wood. These soft-rot fungi predominantly degrade carbohydrates in lignocellulose material, causing extensive reduction in the carbohydrate content. Some soft-rot fungi can, however, partly degrade lignin. Compared to the white-rot fungi, not much study has been done on the degradation of lignocellulose material by soft-rot fungi (Hatakka & Hammel, 2010).

Certain bacteria have been effectively used to pre-treat lignocellulose material. The bacteria *Bacillus* sp. isolated from forest soil in Japan have been used to degrade alkali lignin. Initial concentrations of 0.05-2.0 g/l cell culture were able to degrade not less than 61% alkali lignin within 48 h. Pre-treatment of rice straw by *Bacillus* sp. also resulted in 20% degradation of Klason lignin, with 3.2% cellulose degradation (Chang et al., 2014). Pourcher and Peu (2016) isolated and identified five strains of lignin-degrading bacteria from soil and sediments. The isolates included *Serratia* sp. JHT01, *Serratia liquefacien* PT01, *Stenotrophomonas maltophilia* PT03, *Mesorhizobium* sp. PT04 and *Pseudomonas*

chlororaphis PT02. All the isolates were able to significantly grow and degrade lignin. Pre-treatment of rice straw with lactic acid bacteria produced a total sugar concentration of 30% compared to 16% for untreated rice straw (Chang et al., 2014).

Unlike white-rot fungi, pre-treatment with actinomycete results in the easy recovery of degraded lignin. Saritha et al.

(2013) reported the use of *Streptomyces griseorubens* ssr 38 for degradation of lignin in paddy straw. A large amount (25%) of acid-precipitable polymeric lignins (APPLs) was recovered from pre-treated paddy straw. Delignification helped to increase the carbohydrate content, and this resulted in an increase in saccharification efficiency (97.8%).

Table 1
Delignification effect of different microorganisms on Lignocellulose material

Organism	Substrate	Incubation Time (days)	Effect of Delignification	Reference
<i>Phanerochaete chrysosporium</i>	<i>Eucalyptus grandis</i>	30	2.8% total cellulose	(Castoldi et al., 2014)
<i>Pleurotus ostreatus</i>			16.7% total cellulose	
<i>Pleurotus pulmonarius</i>			15.4% total cellulose	
<i>Trametes</i> sp			10.1% total cellulose	
<i>Irpex lacteus</i>	Wheat straw	21	42.3 ± 2.3% delignification	(Garcia-Torreiro et al., 2016)
<i>Pleurotus eryngii</i> (ATCC 90787)	Corn stover		45.8 ± 3.5% delignification	(Lopez-Abelairas et al., 2013)
<i>Irpex lacteus</i> (Fr. 238 617/93)	Corn cob		17.1 ± 5.3% delignification 46 ± 2% sugar yield	
<i>P. chrysosporium</i> NRRL-6370	Wheat straw	21	65 ± 2% sugar yield	(Saha et al., 2016)
<i>P. sanguineus</i> NRRL-FP-103506	Corn stover	30	51.4 ± 2.9% delignification	
<i>I. lacteus</i> FP-101234			51.0 ± 1.2% delignification	
<i>C. stercoreus</i> NRRL-6573			46.7 ± 1.8% delignification	
<i>P. compactus</i> NRRL-A-2351			46.2 ± 0.8% delignification	
<i>P. brevispora</i> NRRL-13108			45.4 ± 1.5% delignification	
<i>A. bisporus</i> NRRL-20762			39.6 ± 1.6% delignification	
<i>C. cinereus</i> NRRL-20638			7.1 ± 0.6% delignification	
<i>B. fumosa</i> FP-135285-T			6.6 ± 0.8% delignification	
<i>F. velutipes</i> NRRL-2367			4.4 ± 0.0% delignification	
<i>R. crocatus</i> MJL-1465			3.6 ± 0.2% delignification	
<i>Panaeolus</i> sp.FP-102035			2.4 ± 0.1% delignification	

Table 1 (continue)

<i>C. pannocincta</i> FT-100624			2.2 ± 0.0% delignification	
			1.5 ± 0.0% delignification	
<i>Trametes orientalis</i>	<i>Populus tomentosa</i>	84	47.3% delignification	(Wang et al., 2014)
<i>Trametes velutina</i>			58.1% delignification	
<i>P. chrysosporium</i>	Corn stalk	15	35.3% delignification	(Zhao et al., 2012)
<i>Pleurotus ostreatus</i>	Corn stalk	30	54.7% delignification	(Chen et al., 2016)
<i>Phlebia brevispora</i> NRRL-13108	Corn stover	42	15.4% increase in sugar yield	(Saha et al., 2016)

Process of culturing microorganisms for delignification. For effective and efficient delignification, microbial growth and activities should be optimum. The culturing process as well as the environment should lead to optimum growth and activities. Understanding the behaviour of microorganisms and the conditions under which they grow best will help to optimise the conversion of lignocellulose material into bioethanol (Shi et al., 2014). Different culturing techniques and processes have been employed with the aim of optimising microbial growth and activities for enhancement of delignification.

Solid state fermentation (SSF) process has been reported to offer several benefits compared to submerged fermentation (SmF) including higher yields, lower cost, easy recovery of products and absence of froth in medium (Asgher et al., 2016). Pinto et al. (2012) reported that fungi pre-treatment under solid state fermentation resulted in higher saccharification compared to when using a liquid medium. Addition of a source of metabolic energy to the culture medium such as glucose was reported by Castoldi et

al. (2014) to improve microbial growth. The glucose helped to decrease the breakdown of the carbohydrate content of lignocellulose materials. However, Ghorbani et al. (2015) reported that a lower amount of glucose supplement is more effective for improving the process of delignification compared to larger amounts. Surfactants have also been reported to enhance delignification. Ghorbani et al. (2015) revealed that addition of a surfactant (Tween 80) to the culture medium used in their study increased the rate of delignification from 31% to 42%. Salvachúa et al. (2013) reported that adding 0.3 mM Mn(II) increased glucose yield up to 68% compared to 62% and 33% for pre-treated biomass without Mn(II) and the non-treated biomass, respectively. Saha et al. (2016) indicated that enhancement of pre-treatment conditions including the incubation period (42 days) and moisture content (84%) led to a higher sugar yield of 442 ± 5 mg/g from 383 ± 13 mg/g of *Phlebia brevispora* NRRL-13018 pre-treated corn stover, an increase of 15.4%. Ghorbani et al. (2015) revealed that low biomass-to-liquid ratio increases delignification efficiency.

However, very low ratios result in a decrease in the efficiency of delignification. The optimum biomass-to-liquid ratio in their study was 0.041 g/L.

Naturally, different microorganisms form associations and depend on each other for growth and survival. On the other hand, the presence of some microorganisms might inhibit the growth and survival of others (Wang et al., 2014). Effective and efficient breakdown of lignin in lignocellulose materials may be achieved using consortia of microorganisms. Investigations into the biological pre-treatment of Napier grass by three different groups of microorganisms designated, WSD-5 (*Coprinus cinereus* and *Ochrobactrum* sp.), MC1 (thermophilic bacteria) and XDC-2 (mesophilic bacteria in the genera *Clostridium*, *Bacteroides*, *Alcaligenes* and *Pseudomonas*) revealed that the lignin degradation efficiency of all the microbial groups was above 30% after 21 days (Wen et al., 2015). However, the nutritional as well as environmental conditions might not be favourable to all the microorganisms carrying out the processes (Berlowska et al., 2016). Wang et al. (2014) reported that monoculture of *Lenzites betulinus*, *Trametes orientalis* and *Trametes velutina* generally showed higher delignification efficiency compared to their respective co-cultures. The poor performance of co-culturing in their study was attributed to the inability of these microorganisms to co-exist. After microscopic observation, it was revealed that the hyphae of different microbial species do not enter each other's zone. For

effective and efficient delignification, the best combination of microorganisms and biomass is very significant (Cianchetta et al., 2014). It is very important therefore to screen for species that can co-exist and support each other's growth.

Enzymatic Pre-Treatment

Biological pre-treatment with lignin degrading enzymes might help to prevent the loss of hemicellulose and cellulose that occurs during microbial pre-treatment processes (Wang et al., 2013). The use of ligninolytic enzymes is on the increase because of their ability to act on specific reactions (Chen et al., 2012). The enzyme set-up for delignification is determined by the type of microorganism, substrate as well as environmental conditions during culturing. Different microbial strains produce different types of these enzymes at varying rates (Ma & Ruan, 2015).

Ligninolytic enzymes. Different types of enzymes including laccases, peroxidases and oxidases that produce hydrogen peroxide are involved in the breakdown of lignin in lignocellulose materials. However, laccase (EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13) and lignin peroxidase (LiP, EC 1.11.1.14) are the most widely used ligninolytic enzymes (Daâssi et al., 2016). The type of ligninolytic enzyme(s) responsible for delignification of a particular process is dependent on the fungi species, substrate and culture conditions. Among these enzymes, laccase is the most widely studied. Laccase is a copper-containing

enzyme that uses molecular oxygen as oxidant. Majority of white-rot fungi have the ability to synthesise laccase (Ma & Ruan, 2015). The production of laccase by white-rot fungi can be induced by adding copper, xenobiotic compounds or dyes. Laccase has the ability to degrade numerous compounds that have a phenolic structure because of its low substrate specificity. This has resulted in the use of laccase in a wide range of areas including biomass delignification and degradation of xenobiotic compounds (Wong, 2009). MnP and LiP are heme-proteins and they require hydrogen peroxide as an oxidant. They also have low substrate specificity, hence they are capable of oxidising various nonphenolic lignin model compounds as well as phenolic aromatic substrates. They were first identified in cultures of *Phanerochaete chrysosporium*. MnP is the most common lignin degrading peroxidase found in ligninolytic fungi (Hatakka & Hammel, 2010). Despite being a peroxidase, LiP is able to oxidise substrate at high redox potential (Wong, 2009).

Ligninolytic enzyme production.

Inadequate production of ligninolytic enzymes by white-rot fungi has been reported as an important factor preventing the use of ligninolytic enzymes in biotechnology (Asgher et al., 2016). The slow activity of synthesised enzymes also hinders the commercial use of these biological agents for pre-treating lignocellulose material (Hyeon et al., 2014). Optimisation of the nutritional and environmental conditions of the production medium helps to increase

enzyme production and activity. However, extreme nutritional and environmental conditions might inactivate enzymes, leading to a decrease in their activity (Asgher et al., 2016). Changing the fermentation medium or culture conditions can help to increase the production of these enzymes. Lignocellulose material containing large amount of lignin may be ideal in enhancing the production of ligninolytic enzymes (Rastogi et al., 2016). Mediators such as MnSO₄ also influence ligninolytic enzyme production by increasing the surface area for microbial growth (Asgher et al., 2016).

Different microorganisms have been used for the production of ligninolytic enzymes under different nutritional and environmental conditions (Table 2). Under optimum conditions of 50% moisture, 5 g substrate, pH 5.5, 30°C temperature, 2% fructose as carbon source, 0.02% yeast extract as nitrogen source, 25:1 carbon-to-nitrogen ratio, and 5 ml fungal spore suspension for four days, *Ganoderma lucidum* produced higher MnP activity followed by LiP and laccase (Asgher et al., 2014). Asgber et al. (2016) reported that culturing *Schizophyllum commune* on rice straw under solid state fermentation recorded the highest ligninolytic enzyme production after 144 hours. Saritha et al. (2012) revealed that *Trametes hirsute* cultured on Reese's mineral medium with 1% paddy straw as the sole carbon source in a submerged culture yielded higher activity of laccase compared to LiP and MnP after seven days. However, enzyme activities decreased with further incubation beyond

seven days. Ma and Ruan (2015) indicated that laccase was the key enzyme produced when *Coprinus comatus* was cultured in a liquid fermentation medium comprising agricultural waste materials. The highest activity of laccase was recorded on Day 6 (1520 U/ml). Cultivation of *Trichoderma reesei*, on the other hand, showed very low laccase activity (<10 U/ml) after seven days of incubation. Co-culturing of the two fungi, however, resulted in the highest laccase activity (2180 U/ml) after five days. Rastogi et al. (2016) cultivated *Pyrenophora phaeocomes* on different types of agricultural residue moistened with five parts of salt solution for the production of a ligno-hemicellulolytic enzyme cocktail. Wheat straw had the highest (25413.23 ±

35.06 IU/gds) laccase activity compared to all the other material (Table 2). Production of ligninolytic enzymes can be significantly enhanced by changing the fermentation medium. The presence of lignin and/or lignin-related compounds in the substrate can activate the production of these enzymes (Mann et al., 2015). Culturing of *Coridus versicolor* CV-1 at 28°C to 30°C on a shaker at 150 rpm for seven days resulted in laccase, Lip and MnP activities of 2066 ± 15, 0.21 ± 0.05 and 0.25 ± 0.03 U/ml, respectively. *Phanerochaete chrysosporium* PC-1 also cultured at 37°C to 39°C statically for a period of nine days yielded 0.54 ± 0.07 (LiP) and 20.52 ± 1.36 (MnP) U/ml enzyme activity (Wang et al., 2013).

Table 2
Microbial production of Ligninolytic enzymes under different conditions

Microorganism	Amt. of Culture	Substrate	Incubation Time (Days)	Ligninolytic Enzyme	Enzyme Activity	Reference
<i>Ganoderma lucidum</i>	5 ml	Wheat straw	4	LiP	532 ± 4.2 U/ml	(Asgher et al., 2014)
				MnP	882 ± 13.3 U/ml	
				Laccase	340 ± 6.4 U/ml	
<i>Schizophyllum commune</i> IBL-06	4 ml	Rice straw	6	LiP	1347.2 U/gds	(Asgher et al., 2016)
				MnP	1846.7 U/gds	
				Laccase	316.28 U/gds	
<i>Coprinus comatus</i>	5 ml	LEPBM	6	LiP	1347.2 U/gds	(Ma & Ruan, 2015)
				MnP	1846.7 U/gds	
				Laccase	316.28 U/gds	
<i>Trichoderma reesei</i>	5 ml	LEPBM	7	Lip	0 U/ml	
				MnP	0 U/ml	
				Laccase	1520 U/ml	

Table 2 (continue)

Microorganism	Amt. of Culture	Substrate	Incubation Time (Days)	Ligninolytic Enzyme	Enzyme Activity	Reference
<i>C. comatus</i> & <i>T. reesei</i> (1:1)	7 ml	LEPBM	5	Lip	0 U/ml	
				MnP	0 U/ml	
				Laccase	<10 U/ml	
				Lip	0 U/ml	
				MnP	0 U/ml	
<i>Pyrenophora phaeocomes</i> S-1	5 discs (7 mm)	Wheat straw	4	LiP	25413.23 ± 35.06 IU/gds	(Rastogi et al., 2016)
				MnP	22305.79 ± 128.55 IU/gds	
		Dried grasses		laccase	16669.42 ± 3.00 IU/gds	
		Rice straw			10859.51 ± 46.74 IU/gds	
<i>Phanerochaete chrysosporium</i> PC-1	3 loops of spores	Peroxidase medium	9	LiP	0.54 ± 0.07 U/ml	(Wang et al., 2013)
				MnP	20.52 ± 1.36 U/ml	
				Laccase	0 U/ml	
<i>Coridus versicolor</i> CV-1	3 discs (12 mm)	Laccase medium	7	LiP	0.21 ± 0.05 U/ml	
				MnP	0.25 ± 0.03 U/ml	
				Laccase	2066 ± 15 U/ml	
<i>Trametes hirsute</i>	5 discs (6 mm)	Reese's medium	7	LiP	35.2 ± 0.86 IU/ml	(Saritha et al., 2012)
				MnP	6.67 ± 0.69 IU/ml	
				Laccase	57.9 ± 1.08 IU/ml	

Note: Lignocellulolytic enzyme production basal medium (LEPBM)

Delignification of lignocellulose material with enzymes. Compared to purified ligninolytic enzymes, crude ligninolytic enzymes used for delignification offer several benefits in the existence of factors such as protein and mediators in the medium

that helps to improve the activities of the enzyme (Asgher et al., 2016). The amount of crude enzyme extract as well as length of pre-treatment can affect the process of delignification. Different ligninolytic enzymes or combinations of enzymes play

a key role in the process of delignification depending on the type of microorganism they are produced from (Ma & Ruan, 2015).

Various studies have been conducted on delignification of lignocellulose materials from agricultural residues using crude enzyme extract (Table 3). The lignin content of wheat straw was decreased by 39.6% after pre-treatment with 25 ml of ligninolytic enzyme extract (Asgher et al., 2014). Asgher et al. (2016) reported a significant reduction (72.3%) in the lignin content of sugarcane bagasse after pre-treatment of different agricultural residue under the same conditions with 25 ml of crude ligninolytic enzyme extract from *Schizophyllum commune* for 48 h. Delignification rates in the other residue were also encouraging (Table 3). The differences in the rate of delignification may be attributed to variations in the structure and composition of the various agricultural residue. After ligninolytic enzyme pre-treatment of sugarcane bagasse for 48 hours at 35°C, maximum delignification of 33.5% was recorded with 25 ml enzyme extract (Asgher et al., 2013). As stated earlier, different microorganisms will produce different types of ligninolytic enzymes (Ma & Ruan, 2015). Crude ligninolytic enzymes from co-culturing of different microorganisms may enhance the delignification process. Delignification of corn stover with crude enzyme extract from the co-culturing of *Coprinus comatus* and *T. reesei* resulted in the highest lignin removal (45.1%) compared to the extract from monocultures of *C. comatus* and *T.*

reesei after 72 h (Ma & Ruan, 2015). Using complex enzyme systems is also another way of biologically breaking down the lignin component in lignocellulosic biomass. Hyeon et al. (2014) genetically engineered an efficient laccase complex by combining the laccase CueO of *E. coli* and the dockerin domain of a cellulosome system. The system was then fused with the scaffoldin miniCbpA to form a laccase-miniCbpA complex, which was used to pre-treat barley straw. The pre-treated barley straw was effectively fermented by cellulase, allowing *S. cerevisiae* to produce 2.34 g/L ethanol after 72 h, 2.1-fold higher than without laccase complexes.

CHALLENGES IN BIOLOGICAL PRE-TREATMENT

Although biological pre-treatment yields less waste and uses small amount of energy in addition to being eco-friendly, there are major setbacks hindering the success of this technique (Aver et al., 2014). Several factors affect the biological pre-treatment process, including the microbial strain, culture and environmental conditions as well as the type of lignocellulose material (Gai et al., 2014). Biological pre-treatment has been described as a very slow process that needs an aseptic environment. Problems associated with contamination can hinder the biological pre-treatment process; hence, the need for aseptic conditions. It requires a longer incubation time, normally several weeks to months. Various microbial strains differ with the rate at which they are able to carry out the delignification process (Tian et

al., 2012). Insufficient ligninolytic enzyme production by white-rot fungi is a major problem contributing to the long incubation time (Asgher et al., 2016). In addition, the slow activities of enzymes produced prolong the delignification process (Hyeon et al., 2014). Biological pre-treatment may produce low sugar yield as some fungi break down and utilise sugars from cellulose (Larran et al., 2015). Chen et al. (2012) reported that microbial pre-treatment may result in significant loss in the amount of dry matter. Furthermore, the large amount of space required for this process makes it industrially unfeasible.

Optimisation of biological pre-treatment processes can, however, help to increase the microbial and enzymatic activities. Culture conditions such as incubation time, pH and temperature should be varied to obtain the optimum conditions for microbial growth. This will help to enhance the growth and metabolic activities of ligninolytic fungi. Supplementing the culture medium with sources of carbon and nitrogen in addition to mediators, can also stimulate enzymatic activities and improve yield (Castoldi et al., 2014). Carbon (glucose, starch, molasses) and nitrogen (peptone, yeast extract, urea) sources as well as mediators such as veratryl alcohol, oxalate, hydrogen peroxide and manganese (II) sulphate can be added to the culture medium (Asgher et al., 2016).

Various ligninolytic fungi species have been studied and recommended for the production of ligninolytic enzymes (Duangporn & Siripong, 2015). *P. chrysosporium* is an efficient producer

of LiP and MnP, while *Phlebia radiate* secretes laccase, LiP and MnP. *Ceriporiopsis subvermispora*, *Phlebia tremelosa*, *Phellinus pini* and *Pleurotus ostreatus* have all been reported as having high delignification efficiency (Hatakka & Hammel, 2010). Under optimum culture conditions and with a suitable substrate, these fungi species can effectively degrade lignin. Isolation and study of new ligninolytic fungi species from the natural environment will also help to solve the problems associated with biological pre-treatment.

Ligninolytic enzyme production can be induced by the presence of a wide range of substrates. Choosing a suitable substrate is, therefore, very important to ensure efficient production of ligninolytic enzymes. Lignocellulose materials containing a large amount of lignin may be ideal for the production of ligninolytic enzymes (Rastogi et al., 2016). Agricultural, forestry and agro-industrial wastes are all potential substrates for the production of ligninolytic enzymes. Spent mushroom substrate (SMS) is an organic medium obtained from the cultivation of mushrooms. It is made up of agricultural residue and mushroom mycelium that remains after harvesting of mushrooms. It is very rich in extracellular hydrolytic and oxidative enzymes synthesised from the mushrooms that were growing on it (Phan & Sabaratnam, 2012). SMS may be a potential source of ligninolytic enzymes for delignification of lignocellulose materials. The Solid-State Fermentation (SSF) process provides an environment similar to the natural habitat

of fungi and helps to enhance fungi growth and increase their metabolic activities (Asgher et al., 2016). Culturing ligninolytic fungi by SSF process will, therefore, help to increase the rate of delignification and enzyme production.

Table 3
The effect of enzyme delignification under different conditions

Microorganism	Amount of Crude Enzyme (ml)	Substrate	Incubation Time (h)	Delignification Effect	Reference
<i>Ganoderma lucidum</i>	25	Wheat straw	48	39.6% delignification	(Asgher et al., 2014)
<i>Schizophyllum commune</i> IBL-06	25	Banana stalk	48	61.7% delignification	(Asgher et al., 2016)
		Corn cob		47.5% delignification	
		Sugarcane bagasse		72.3% delignification	
		Wheat straw		67.2% delignification	
<i>Coprinus comatus</i>	100	Corn stover	72	41 ± 1.6% delignification	(Ma & Ruan, 2015)
<i>Trichoderma reesei</i>	100			0.5 ± 0.2% delignification	
<i>C. comatus</i> & <i>T. reesei</i> (1:1)	100			45.1 ± 0.7% delignification	
<i>P. ostreatus</i> IBL-02	25	Sugarcane bagasse	48	33.5% delignification	(Asgher et al., 2013)

CONCLUSION

Biological pre-treatment is a mild process that is less costly and eco-friendly and it consumes less energy compared to other pre-treatment processes. Ligninolytic fungi or enzymes are used for biological pre-treatment. Ligninolytic enzyme pre-treatment has been identified as a potential alternative process to overcome the drawbacks of microbial pre-treatment including long pre-treatment time as well as utilisation of sugars in some cases. However, inadequate production of

ligninolytic enzymes coupled with low activity has been reported as an important factor impeding the use of ligninolytic enzymes in biotechnology. The biological pre-treatment processes are continuously optimised by varying the culture conditions to enhance microbial growth as well as enzyme production and activities. Despite the different optimisation processes of biological pre-treatment, an extremely effective biological pre-treatment technique with satisfactory level of delignification is yet to be established.

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