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A strategic laccase mediated lignin degradation of lignocellulosic feedstocks for ethanol production

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ABSTRACT

Lignin degradation using laccase is a cleaner, biocatalytic, and substrate specific alternative to improve holocellulose recovery from lignocellulosic feedstocks for bioethanol production. The degree of laccase mediated lignin degradation varied with the biochemical composition of the biomass. In the present article, Fourier-Transform Infra-Red (FTIR) spectroscopy and simplex centroid mixture design algorithm based studies were conducted to probe the correlation between biomass composition and laccase mediated lignin degradation. FTIR peak intensity analyses of the mixtures i.e., combination 20, 21 and 31 from 57 mixed biomass sets studied suggested that G type lignin has synergistic effect on laccase mediated delignification while presence of S type lignin showed antagonistic relationship with laccase adsorption and delignification. Simplex centroid based optimization of the selected mixture (i.e., combination 31) resulted in a concoction composed of *Saccharum spontaneum* (0.2031), *Saccharum officinarum* tops (0.1968), *Ricinus communis* (0.6000) with maximum delignification 80.13%. Enzymatic delignification of this optimized mixture was supported by FTIR studies where peak intensity at 1514 cm^{-1} and 1595 cm^{-1} corresponding to the aromatic skeletal vibrations of lignin decreased in magnitude after delignification. X-Ray Diffraction study deciphered 3.66% increase in crystallinity of the delignified sample corresponding to cellulose while Scanning Electron Microscopy revealed structural distortion in the sample. Besides, reduction in the calorific value of the biomass (2.75 kJ/g) further substantiated the delignification process. Lignin degradation products analyzed through gas chromatography mass spectroscopy unveiled the presence of various carboxylic acids, phenolic acids, heterocyclic aromatic compounds and *N*-heterocyclic compounds manifesting laccase action on lignin. The study on the effect of pretreatment on saccharification process showed that 80% delignification was sufficient for maximum reducing sugar yield (587 mg/g).

1. Introduction

Current world energy crisis due to limited fossil fuel reserves and increasing global warming concerns has intensified the worldwide focus towards development of a sustainable technology for alternative fuel production (Weldemichael and Assefa, 2016; Liu et al., 2015). Sugarcane and corn have been utilized for bioethanol production conventionally in many developed countries; but Government of India has imposed a strict restriction on utilization of food crops for fuel generation. India being a thickly populated developing country, the food vs fuel concern is one of the sensitive issues for the government that cannot be ignored. In this context, non-edible lignocellulosic biomass has been considered to be the only alternative to address food, fodder vs fuel controversy (Bhutto et al., 2015).

Lignocellulosics are the renewable plant resources (da Silva et al., 2013) that are unsuitable for human consumption are annually produced at a rate of 1×10^{10} MT worldwide (Sanchez and Cardona, 2008). These non-edible lignocellulosics include grazable (i.e., non-food but fodder type) and non-grazable (i.e., non-food and non-fodder type) varieties both rich in holocellulosic content (60–70% w/w) that can be utilized for bioethanol production. The constituents of the remaining biomass are lignin, proteins, fats, pectins, salts, minerals, ash, and fixed carbon.

Ethanol production from celluloses has been well reported but utilization of hemicelluloses for improved ethanol yield has been the major concern till date. A detailed study on the possible routes for ethanol production from hemicelluloses has been conducted by Gírio et al. (2010) which states that a suitable biomass processing

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method is essential for maximum cellulose as well as hemicellulose recovery without which the bioethanol produced cannot be commercially viable.

The substantial grazable varieties of lignocellulosics available in India are the naturally occurring virgin biomass such as *Saccharum spontaneum* (Kans grass), *Triadica sebifera* (Chinese tallow), *Crotalaria juncea* (Indian hemp) etc., and agricultural residues such as *Saccharum officinarum* (Sugarcane) tops, wheat straw, rice straw, wheat bran, rice husk, maize cobs, and corn stover.

The other potential non-grazable lignocellulosics include *Lantana camara*, *Ricinus communis, Ananas comosus* (pineapple) leaf wastes, *Bambusa bamboos, Arundo donax* (giant cane) etc., that are non-fodder in nature either due to the presence of toxins (Sharma et al., 1981; Wolfson and Solomons, 1964) such as lantadene A, B and triterpene acid in *L. camara*; ricin toxin in *R. communis,* and bufotenidine and gramine in *A. donax* respectively, or due to wax coated spiny leaves (*A. comosus* leaf wastes) and high fibre content (*B. bambos*) (Banik et al., 2011). Therefore, the non-grazable varieties (*R. communis, L. camara, B. bambos* and *A. comosus* leaf wastes) along with *S. officinarum* tops and *S. spontaneum* commonly available in the north-east regions of India were considered in the present study with the intention to convert waste to wealth.

The utilization of these lignocellulosics for fuel generation is challenging due to two prime reasons. Firstly, due to the lack of sufficient quantity of single type of feedstock to run a large scale ethanol refinery and secondly, due to the recalcitrance of the biomass owing to the presence of lignin which accounts for 15–20% (w/w) of total biomass in bound form to the cell wall carbohydrates (cellulose and hemicelluloses) thereby stressing on lignin removal (Narra et al., 2014). Several pretreatment techniques which include the application of dilute acid, base, ammonia fibre expansion, steam explosion etc. for delignification have been considered worldwide. A recent review by Silveira et al. (2015) provided an alternative technique for conducting pretreatment with low harmful impact on environment which involved the use of greener solvents namely ionic liquids and supercritical fluids for lignin degradation.

Although various pretreatment methods (physical/chemical/physico-chemical) have been reported in the literature but using a biocatalyst is considered environmentally significant as the entire process is conducted under mild operating conditions devoid of any harsh or toxic waste disposal streams (Lu et al., 2010). Moreover, the harmful intermediates such as furfurals and hydroxylmethyl furfurals affecting the saccharification and fermentation steps that are otherwise produced from acid pretreatment; and the use of huge quantity of water for washing the treated biomass during chemical (acid/alkali) pretreatment method are avoided in this enzymatic venture (Boruah et al., 2016; Bak et al., 2009). Owing to these advantages, in the present study laccase produced by white rot fungus (*Pleurotus djamor*) was employed for conducting pretreatment studies through greener means.

Laccases are a class of extracellular oxidoreductase group of enzymes that act on phenolic/non-phenolic moieties in lignin rich substrates (Kunamneni et al., 2007). Laccases are attractive industrially for delignification because they utilize molecular oxygen to catalyze the reaction instead of hydrogen peroxide as in the case of other lignin degrading enzymes such as manganese and lignin peroxidases.

It is apparent that, lignocellulosics from different origins have different lignin (i.e., either guaiacyl (G), syringyl (S), hydroxy phenyl (H), GS or GSH type) and carbohydrate composition, which needs to be studied in depth to understand the substrate and enzyme interaction for proper mixed biomass preparation to maximize delignification and reducing sugar production resulting in enhanced ethanol yield. Thus, in the present article the unaddressed aspect of, correlation between biomass composition and laccase action was dealt in details based on the data obtained from FTIR and simplex centroid mixture design algorithm adopted to select, and optimize the proportions of lignocellulosics for maximum delignification.

2. Materials and methods

2.1. Substrates

Six different lignocellulosics namely, *R. communis, L. camara, S. officinarum* tops, *S. spontaneum, A. comosus* leaf wastes and *B. bambos*, were collected locally from the forest area of IIT, Kharagpur, India and sun dried and milled to a final particle size of 0.2 mm for subsequent studies.

2.2. Enzymes

Laccase secreted extracellularly by *P. djamor* on lignin rich substrate was assayed for activity using 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as a substrate (Bhattacharya and Banerjee, 2008). To conduct delignification studies, laccase having activity 500 IU/mL was used while extracellular *endo*-glucanase (20 IU/mL), β-glucosidase (5.53 IU/mL) and xylanase (270.28 IU/mL) cocktail secreted by *Trichoderma reesei* RUT C30 was used to conduct the saccharification process (Das et al., 2008). The activities of *endo*-glucanase, β-glucosidase and xylanase were assayed using protocols of Zhang et al., 2007 and Jeffries et al., 1998.

2.3. Characterization of composition of lignocellulosic feedstocks

Lignin content of the substrates was measured by the titrimetric method and TAPPI method (Rajak and Banerjee, 2015; Templeton et al., 2010). Cellulose was estimated by "semi-micro determination method", and hemicellulose content was estimated by anthrone method (Updegraff, 1969; Marlett and Lee, 2006). Ash content was estimated using the NREL protocol (Sluiter et al., 2008; ASTM Standard Method Number, E1755-01). To determine the content of extractives, the weight difference (%) in the initial sample and the summation of total lignin, hemicelluloses, celluloses and the ash content was considered in the view that the biomass comprises only of cellulose, hemicelluloses, lignin, ash (minerals) and extractives (Yang et al., 2006; Sharma et al., 2016).

2.4. Selection of best combination of lignocellulosic substrates from various mixed feedstocks

Each milled substrate was mixed in 1/2, 1/3, 1/4, 1/5 and 1/6 fraction in various combinations of two, three, four, five and six component blends respectively, and subsequently used for further studies.

Various combinations of raw substrates (*Ricinus communis,* RIC; *Lantana camara,* LC; Sugarcane top, SCT; *Saccharum spontaneum,* KG, Pineapple leaf wastes, PA and *Bambusa bambos,* BB) obtained were taken in separate 50 mL Erlenmeyer flasks with final substrate concentration 30% (w/v) to which the required volume of laccase (500 IU/mL) was added i.e., to 1 g raw substrate, 3.333 mL laccase (1666.5 IU/g) was used. The reaction was carried out at 35 °C for 6 h where the initial pH of the solution was kept at 7 using 0.1 M sodium phosphate buffer. The resultant solid residues were oven dried at 60 °C to constant weight and subsequently used for residual lignin estimation.