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Alkaline Peroxide and Laccase Treatment for the Delignification of Ricinus Communis Biomass to Enhance Saccharification

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Abstract: ***Ricinus communis stem is one of the potential substrate for bioethanol production; however outer lignin sheath is a major bottleneck for saccharification and so to the fermentation. The conventional acid based pretreatment has major drawback as it produced inhibitors like 5-hydroxy methyl furfural (HMF) and furfural. Present investigation is attempted to evaluate delignification by treatment of alkaline peroxide and laccase. Delignification and saccharification are studied and compared on the basis of weight loss of biomass during the process, lignin loss, and reducing sugar yield after cellulase treatment. Alkaline peroxide treatment showed around 34% weigh loss of biomass and 8.26% of reduced lignin in a process, as compared to native biomass (20.60%). Saccharification yield was found as reducing sugar 774.48 mg/g dry weight pretreated biomass. In case of laccase, results were recorded as 23% weight loss, 11.50% lignin remains after treatment and 658.67 mg/g reducing sugar as saccharification yield.***

Keywords: Ricinus communis, delignification, alkaline peroxide, laccase.

I. INTRODUCTION

Increased fossil fuel consumption over the past two decades has made it necessary to search for the alternate and renewable source of energy (Saini et al., 2013). In this context, lignocellulosic bioethanol is one of the bright alternatives as it can be easily blended in the fossil fuels. However, generation one ethanol (produced from sugar and starch) raise food verses fuel crisis (Fillat et al., 2017). Therefore to fulfill the demands of fuel, lignocellulosic ethanol is more sustainable option for today's fuel crisis. The use of lignocellulosic biomass for production of ethanol is progressively gaining importance due to its qualities like abundant availability, low cost, regeneration etc (Prasad et al., 2007; Howard et al., 2003). The lignocellulosic biomass contains mainly agro waste, paper waste, food and organic municipal waste, grasses and forest foliages. Among them, grasses, unconventional crop residues and forest waste are the most underexplored lignocelluloses for fuel production (Rajak and Banerjee, 2018).

Recinus commus is an herbaceous plant belongs to member of Euphorbiaceae and is an annual crop useful for production of castor oil from its seeds (Althuri and Banerjee, 2017). After Brazil and China, India is the third major producers of castor oil; which produces tons of R. communis biomass per year (Mukhopadhyay and Banerjee, 2015). Excluding the fruits, the whole vegetative portion of the plant is treated as waste as it contains potential toxin as recin. It makes the plant completely inedible for even animals and burn or dumped in the soils. Nevertheless, fermentable sugars present in the stem and leave of R. communis makes it potential substrate for important products like ethanol.

It has been reported that, the vegetative parts of R. communis contained of more than 42% cellulose, hence can be utilized as low cost substrate for bioethanol. However, the major hurdle to utilize the cellulose is the presence of lignin sheath and low hydrolysis yield. The asymmetrical and heterogeneous nature of lignin resist to chemical and enzymatic hydrolysis of cellulose polymer (Singh et al., 2014). These physical and chemical barriers are cleared with the help of pretreatments which allow destructing the lignocellulosic bundles and enhance the accessibility of cellulose and hemicellulose for enzymatic hydrolysis.

Different physical, chemical and biological methods are been tried to facilitate and enhance saccharification viz. steam explosion, microwave irradiation, acid, alkaline, peroxide, solvents, ionic liquids, fungus and laccase etc (Morando et al., 2014). Out of these, several methods like acid, alkali or high temperature or pressure lead to produce various unwanted byproducts like 5-hydroxymethyl furfural, furfural, acetic acid etc. These byproducts act as inhibitory for enzymatic hydrolysis and subsequent fermentation which will eventually add up another step as detoxification of pretreated biomass to avoid low yield. However, certain methods like alkaline peroxide or enzymatic delignification may able to achieve high yield without detoxification process.

Hydrogen peroxide is widely used for belching of high lignin wood pulp commercially (Phan and Tan, 2014). Alkaline peroxide treatment is characterized to decrystallize cellulose where in proper condition hydrogen peroxide will react readily with lignin and

convert it to natural low molecular water soluble product (Ayeni et al., 2013; Banerjee et al., 2011). It was found to be effective for treatment for delignification of sugar cane bagasse (Rabelo et al., 2014).

Enzymatic pre- treatments of lignocellulosic biomass are advantageous over the other physio chemical pretreatment approaches due to its reaction specificity. In enzyme catalyzed reaction, no other byproducts are formed and will facilate hydrolysis to achieve highest yield of reducing sugar (Moreno et al., 2015). Laccases (E. C. 1.10.3.2) are a class of extracellular enzymes which oxidizes phenolic group in lignin liberating and water soluble small oxidized molecules (Plácido and Capareda, 2015). The main advantage of laccase catalyzed treatment is the preservation of holocellulose and no byproduct or inhibitor production. Rajak and Banerjee (2015) achieved more than 84% of delignification of Saccharum spontaneum biomass by optimization various laccase treatment parameters. In present work, alkaline peroxide and laccase treatments were evaluated for the delignification of R. communis biomass in search of elevated hydrolysis yield of reducing sugars.

II. MATERIALS AND CHEMICALS

A. *Substrate*

Ricinus communis biomass was obtained from local filed area of Jalgaon, (MS), India. After harvesting, vegetative parts were sundried and used as biomass. It was crushed to powder and milled to pass through a mesh screen 35 (~0.5 mm). Powdered samples were further dried at 60 ^{0}C and stored in air tight bottles at room temperature for further experiments.

B. *Compositional Study of r. Communis*

Physical properties like moisture, total solid, volatile solid, and ash were analyzed as per the laboratory analytical procedure developed by National Renewable Energy Laboratory, USA (Sluiter *et al*., 2008, Sluiter *et al*., 2004). Holocellulose was measured spectrophotometric ally by phenol sulfuric method. Lignin was analyzed by gravimetric analysis by 72 % H_2SO_4 digestion method (Sadasivam and Manickam, 1996).

C. *Enzymes And Chemicals*

All the chemical reagents used were of analytical grade purchased from Himedia Limited, Mumbai. Laccase was produced from indigenously isolated white rot fungi Perenniporia temphropora L-168 and crude enzymatic preparation was used for treatment of lignocellulosic biomass. Cellulase produced from Trichoderma ressei purchased from Himedia Limited Mumbai.

D. *Enzyme Assays*

Laccase activity was measured spectrophotometrically using ABTS as substrate. One international unit (IU) of laccase activity was defined as the amount of enzyme required to oxidize 1 micro mol of ABTS per minute under the assay conditions (Narkhede, 2014). Cellulase activity was measured by dinitrosalicylic acid method defined by Ghose, (1987) where filter paper unit was defined as µmol of glucose equivalent liberated per ml per minute of culture filtrate under assay conditions (Saini *et al*., 2013).

E. *Pretreatments*

A. *Alkaline peroxide pretreatment:* Two grams of dried *R. communis* biomass powder was mixed to 50 ml of alkaline peroxide solution (1% w/v hydrogen peroxide added to 1.25 N NaOH) so as to achieve a solid loading of 4% (w/v, grams dry weight per 100 ml). All the flasks were placed at 121 ^{0}C for 20 minutes in a laboratory autoclave and cooled subsequently. The solid biomass was washed with distilled water and filtrated with vacuum filtration. The solid reside was dried at 60 ^{0}C to a constant weight and weight loss of the biomass, lignin and holocelluloase were determined to evaluate pre-treatment.

B. *Laccase treatment:* Two grams of dried *R. communis* biomass powder was mixed to 50 ml citrate buffer (pH 5.0) along with 10 U/ml laccase and kept at 50 ^{0}C for 6 h at 120 rpm. After this treatment, solid residue was collected by vacuum filtration, dried at 60 ^{0}C to constant weight and weigh loss of the biomass, lignin and holocelluloase were determined for treatment.

F. *Enzymatic Saccharification of R. Communis Biomass*

Enzymatic saccharification of biomass recovered from both earlier mentioned treatments was carried out using commercial grade cellulase. Twenty five ml of 50 mM citrate buffer of pH 5 was added to the Erlenmeyer flak of 100 ml along with 1.0 g of prior treated biomass. The amount of enzyme used for saccharification was 60 FPU/g of dry weight of biomass. All the mixtures were incubated for 48 h at 50 ^{0}C with agitation speed 120 rpm. After reaction, all samples were filtered and centrifuged for 10 min to remove solid residue and aliquot were used to analyze reducing sugar by DNS assay. Results were expressed as mg reducing sugar/g dry biomass calculated by the formula given by Wang *et al.*, (2016).

G. Scanning Electron Microscopy Analysis

The pre-treated and control R. communis biomass samples were pre dried at 60 ^{0}C for 6 hrs to remove the excess moisture and submitted for scanning electron micrographs (SEM). SEMs were recorded in field emission scanning electron microscope (FESEM, S-4800, Hitachi; Japan) by the method described by Sharma et al., (2017).

III. RESULTS

A. Compositional Study

Evaluation of proximate and biochemical composition is the initial step for selection of R. communis biomass for bioconversion. The untreated R. communis biomass used for present investigation, contained 72.18 ± 0.92 % total solid and 26.94 ± 0.60% moisture. The total solid content reflected actual biomass available for bioconversion after drying. The volatile solid content of R. communis was observed as 84.45 ± 0.30%. The Holocellulose and lignin content was recorded as 53.20 ± 0.48 % and 20.60 ± 0.45%, respectively (Table I). Mukhopadhyay and Banerjee (2015), reported Ricinus communis biomass, growing in parched conditions contributes major biomass containing 44.4 % cellulose, 22.8 % hemicelluloses and 19.8 % lignin.

Table I: Compositional profile of *R. communis* biomass

Characteristics	Values (in per cent)
Total Solid	72.18% ±0.92%
Moisture	26.94% ± 0. 60%
Volatile Solid	84.45% ±0.3%
Ash	15.25% ± 0.05%
Holocellulose	53.20% ± 0. 48%
Lignin	20.60% ± 0.45%

B. Pretreatment and saccharification of r. Communis biomass by alkaline peroxide and laccase treatment

The compositional and dry weight analysis has revealed pretreatment effectiveness of alkaline peroxide and laccase on the saccharification of R. communis biomass. The holocellulose content increased after both the treatments from 53.20 ± 0.48% to 84.67 ± 0.35% for alkaline peroxide treatment and 79.24 ± 0.34% for laccase treatment (Table II). The notable rise in the holocellulose was mainly due to depolymerization of lignocelluloses sheath. The lignin content decreased remarkably in comparison with untreated material. Lignin percentage for laccase treatment was rerecorded as 20.6 ± 0.45% for untreated R. communis biomass and for alkaline peroxide and laccase treatment 8.26 ± 0.38, 11.50 ± 0.23, respectively (Table II). The biomass weight loss recorded as 34.12 ± 0.65% and 23.48 ± 0.52% for similar treatment, respectively. It was mainly due to solubilization of lignin in both the cases and some extent of hemicellulose in alkaline peroxide treatment. The saccharification studies enlighten the changes in the enzymatic digestibility lignocelluloses due to the delignification efforts. The reducing sugar yield increased by around 2.9 folds and 2.46 folds for alkaline peroxide and laccase treatment, respectively.

Table II: Comparative account of *R. communis* biomass composition before and after treatments

Pretreatments	Composition (%)		Weight loss (%)	Reducing sugar (mg/g of dry weigh of biomass)
	Holocellulose	Lignin		
Untreated	53.20 ± 0. 48	20.6 ± 0.45	-	266.78 ± 12.90
Alkaline peroxide	84.67 ± 0.35	8.26 ± 0.38	34.12 ± 0.65	774.48 ± 09.46
Laccase	79.24± 0.34	11.50 ± 0.23	23.48 ± 0.52	658.6710 .20

C. Scanning electro microscopy (SEM) analysis

The SEM images showed that the surface of native biomass (Figure 1) observed intact, bound and smooth where cellulose content was enclosed intact with the outer surface of lignin and hemicellulose. The laccase (Figure 2) and alkaline peroxide (Figure 3) treated biomass showed more destructed and disoriented structures. In case of both the treatments, morphology of outer surface changed to rough with the increase accessibility of hydrolyzing enzymes to the cellulose portion. The lignocellulosic sheath was disturbed due to both the treatments. Similar patterns of micrographs were also recorded by Mukhopadhyay *et al.*, (2011) in case of

enzymatic depolymeristation of R. communis where sequential doses of laccase and cellulases were experimented. Saini et al., (2013) also explained the effects of alkaline peroxide treatment on sweet sorghum baggase. It showed comparable similarity in morphology changes and outer surface destruction between sweet sorghum baggase and R. communis biomass.

Fig. 1: Native *R. communis* biomass

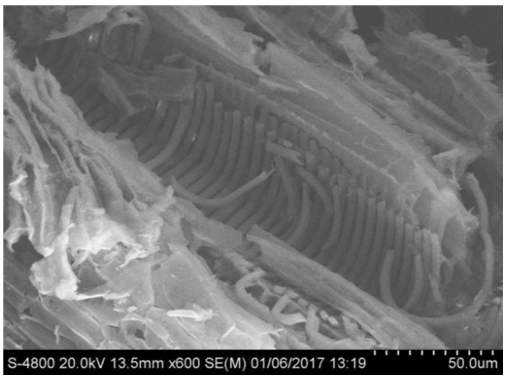

Fig. 2: Laccase treated *R. communis* biomass

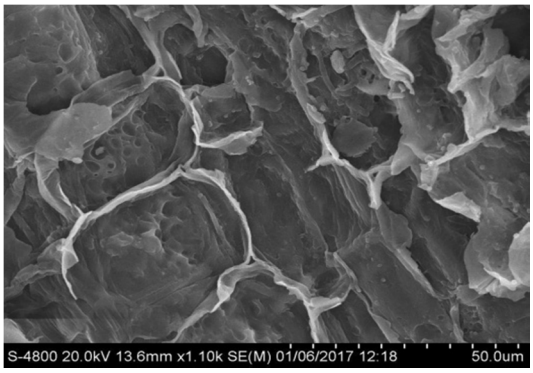

Fig. 3: Alkaline peroxide treated *R. communis* Biomass

IV. CONCLUSION

Ricinus communis produce significant amount of inedible lignocellulosic biomass with high concentration of fermentable sugar fraction. The biochemical composition of R. communis enlightens the potential of substrate for bioethanol production. The carbohydrate concentration (53.20 ± 0. 48%) of the dried biomass is found comparable to other conventional agro waste samples which are attempted earlier for bioethanol production like rice straw, wheat straw, cotton stalks etc. This study employed to resolve bottleneck of cellulase hydrolysis i.e. delignification of biomass through evaluation of alkaline peroxide and laccase treatment. Both Alkaline and laccase treatments were found potential and suitable with more than 2.9 and 2.46 fold increase in saccharification as compared to hydrolysis of untreated biomass. The SEM micrographs of untreated and treated biomass samples were also showed the destruction of outer surface of biomass. However, further investigation regarding optimization of both treatments and application in fermentation by yeast is necessary.

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