

## EFFECT OF PHYSICAL AND CHEMICAL PRETREATMENT METHODS ON SAWDUST FOR IMPROVED SACCHARIFICATION

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### Abstract

Sawdust from sawmills being invaluable substrate possess high potential for the production of bioethanol due to many deep rooted benefits. Production of bioethanol by enzymatic hydrolysis of lignocellulosic biomass imply many steps including pretreatment, saccharification and fermentation but pretreatment is the most crucial step as structural hindrances are to be removed first for cellulases to act on lignocellulosic substrates. In the present study different pretreating agents i.e. Autohydrolysis, hydrogen peroxide, microwaves and urea: thiourea are assessed for lignin removal from saw dust. Among these, maximum delignification of 32.96% was observed using urea: thiourea in a proportion of 1:4. Furthermore, best pretreated samples of each strategy were further analyzed for saccharification using hyperthermophilic cellulases under similar conditions. Among all, urea:thiourea pretreated sample of sawdust showed maximum saccharification i.e. 37.38% as compared to control sample for which 14.56% saccharification was recorded. This pretreatment resulted in 2.56 fold increase in the saccharification of saw dust.

**Key words:** Biomass, Lignocellulose, Delignification, Pretreatment, Saccharification.

### Introduction

The fossil fuel consumption to fulfil energy needs is been steadily increasing. It is estimated that during the last century its use has been increased to 17 folds (Zabed *et al.*, 2016). The burning of fossil fuels is turned out to be snagging as it is leading to depletion of resources, emission of toxic and greenhouse gases like CO<sub>2</sub>, SO<sub>2</sub>, and NO<sub>x</sub> (Apergis & Payne, 2017). This situation has made non-conventional fuel originated from bio-renewable resources, a main focus for scientific world (Apak *et al.*, 2017). These fuels are labelled as biofuels which can be outlined as liquid or gaseous fuels produced from biomass for the transport sector, cosmetics, pharmaceuticals and alcoholic beverages (Lu *et al.*, 2016). Among them bioethanol is consumed far and wide (Ibrahim & Bugaje, 2018). The ethical concerns about using food as fuel raw materials has diverted attention towards the exploitation of inedible feedstock alternatives like municipal and industrial waste, wood and agricultural residues. Different substrates like sugars, starches and lignocellulosic substances are exploited to work out on this target (Hossain *et al.*, 2016).

Utilization of woody wastes like sawdust is more valuable which is having more cellulosic content (Shaheen & Emam, 2018). The woody material is ranked into two types as softwood and hardwood on the basis of their composition. The wood from conifers and gymnosperm trees fall into the group of softwoods and they show low density and faster growth rate. Evergreen species of different trees like cedar, douglas fir, pine, spruce etc are said to be softwood which is more recalcitrant and a difficult target for bioethanol production (Mupondwa *et al.*, 2017). Detailed study of composition of plant cell shows cellulose, hemicellulose and lignin to be major components with 4:3:3 ratio (Wang *et al.*, 2015).

Cellulose is the most abundant renewable energy resource as annually several billion tons of cellulose is produced by plants worldwide. It is the major component of plant cell in which D-glucopyranose units are linked by 1, 4- glycosidic bond linearly. Main chain and branch

chain glucans give rise to hemicellulose which is second most abundant component making 25-30% of the plant cell (French, 2017). Glucan composition may vary in different types of wood and may include xylan, xyloglucan, glucomannan, mannan, galactomannan, callose etc. Phenylpropane units nonlinearly and randomly link with each other giving rise to a complex three dimensional polymer which is named as lignin. Three major monomers in this structure are coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Huang *et al.*, 2016). Study of elemental composition shows that living plant cells are composed of carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorous, calcium, magnesium, iron, potassium, sodium, copper, zinc, manganese, chlorine and others (Ronen, 2016).

Many bacteria and other microorganisms feed upon cellulose for energy and yield valuable products like bioethanol. They produce enzymes which act on cellulosic substrate but the crystallinity and polymerization of substrate hinders its (Rojas, 2016). So, bioethanol production is carried out by three processing steps like pretreatment, hydrolysis (enzymatic saccharification) and microbial fermentation. Pretreatment is incredibly crucial step as it takes off lignin that stamps out a big hurdle from cellulosic biomass thus facilitates further processing. Under ideal conditions, a catalytic agent targets glucosidic bonds through micropores (Wang *et al.*, 2015). Destruction of hydrogen bonds facilitates unzipping of cellulosic chains thus swelling and development of channels takes place but practically it is not feasible. Pretreatment triggers desegregation of inter and intra fibril structure that outcomes in the disruption of lignin. So, in this way cellulose and hemicellulose turn out to be more available for further processing (Si *et al.*, 2015). Pretreatments can be categorized into distinct sections for instance physical, chemical and biological method. Physical pretreatment entails heat, pressure, steam pretreatment etc. Chemical pretreatment occurs when different chemicals like acid, alkali, hydrogen peroxide etc are used (Guilliams *et al.*, 2016).

The second step in the stairway to bioethanol production is hydrolysis of pretreated biomass to get hold of fermentable sugars. This process can be proficiently carried out by the action of enzymes which are termed as biocatalysts. Cellulases are spotted to be very efficient for conversion of cellulosic biomass into fermentable sugars as they precisely target the cellulose chains and hydrolyze it (Sindhu *et al.*, 2016). Cellulases is an assemblage of enzymes that is produced by a numeral fungi like *Trichoderma*, *Penicillium*, *Fusarium*, *Phanerochete*, *Humicola* and bacteria like *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Erwinia*, *Acetovibrio* (Imran *et al.*, 2016). Cellulases can be labelled as exoglucanases, endoglucanases,  $\beta$ -glucosidases etc pointing specific activity for certain parts of cellulosic chains. Endoglucanase shatter cellulose chains in contrast exoglucanase go after non-reducing ends to open up new sites. Hydrolysis is accomplished by  $\beta$ -glucosidases as they hydrolyze cellodextrin and cellobiose turned out by the action of previous enzymes (Li *et al.*, 2016). It makes sure the conversion of metabolic products originated by those enzymes into glucose units (Colussi *et al.*, 2015).

*Thermotoga petrophila* is proved beneficial for the industrial processes as it is a hyperthermophile giving signs of optimum growth at 80°C. Its enzymes efficiently act at elevated temperatures by boosting substrate solubility, dropping viscosity, pumping cost and likelihood of bacterial contamination (Akram *et al.*, 2018). The optimizing a range of parameters like temperature, pH, substrate concentration, enzyme units can be valuable for escalating hydrolysis yields that ends into supplemented monosaccharide liberation for increased bioethanol production.

In the present study numerous pretreatment strategies were checked up for their competency by relating lignin content left behind after pretreatment and glucose yield obtained following saccharification.

## Materials and Methods

**Chemicals:** For accuracy and precision analytical grade chemicals purchased from authentic suppliers of Sigma, Fluka, Merk Ltd. were used.

**Raw materials:** Pinus tree softwood was collected from local furniture market of Lahore, Punjab Pakistan which is categorized as softwood. A locally manufactured sieve was used to get sawdust particles of up to 0.475mm size as sieve had 0.475mm pore size. Sawdust was made dust and impurities free by washing with distilled water. Washed sawdust was stored in airtight bottles after drying in hot air oven at 105°C (Universal Oven UN750 Plus Memmert, Germany) (Kim *et al.*, 2013).

**Cellulases:** Three different cellulases named Endo-1,4- $\beta$ -glucanase, Exo-1,4- $\beta$ -glucanase and  $\beta$ -1,4-Glucosidase were employed. Efficient recombinant thermophilic enzymes were borrowed from the principle investigator of a project under the heading "Production of bioenergy from plant biomass" enduring at the Institute of Industrial Biotechnology Government College University Lahore, Pakistan. Important characteristics of these enzymes like optimum temperature, optimum pH and enzyme activity are mentioned in the below Table 1.

**Table 1. Thermophilic recombinant cellulases catalytic profile.**

| Enzyme                       | Optimum temperature (°C) | Optimum pH | Enzyme activity (U/mL) |
|------------------------------|--------------------------|------------|------------------------|
| Endo-1,4- $\beta$ -glucanase | 90                       | 6.0        | 6.8                    |
| Exo-1,4- $\beta$ -glucanase  | 90                       | 6.5        | 22                     |
| $\beta$ -1,4-Glucosidase     | 80                       | 6.0        | 4209                   |

**Steam pretreatment:** A modification in the method of Ballesteros *et al.*, 2000 was made to carry out steam pretreatment. Auto hydrolysis was carried out at 125°C with varying pretreatment time duration from 15-75 minutes (15 minutes time increment) by taking 20 g sawdust and 20 mL distilled water in different flasks.

**Hydrogen peroxide pretreatment:** Modifications in the protocol of Vasco & Zhang, (2013) were brought to carry out the pretreatment. Varying concentrations of hydrogen peroxide solutions (1-5% v/v) were taken in different flasks with 20 g biomass and processed at 125°C for 30 minutes in an autoclave. The pretreated biomass was washed with distilled water to attain neutral pH and after drying was stored in zipper bags for further processing.

**Microwave pretreatment:** Transformed procedure of Jin *et al.*, (2016) was executed by pretreating biomass along water for 1 to 5 minutes with 1 minute time increment. After pretreatment biomass was washed with distilled water and drying in hot air oven was achieved before storing in zipper bags.

**Urea: thiourea pretreatment:** A varied pretreatment method of Gholamzad *et al.*, (2014) was experimented by taking different concentrations of urea: thiourea solutions along biomass in different flasks to pretreat at 125°C for 30 minutes. Pretreated biomass was dried after washings with distilled water and stored in zipper bags for further use.

**Lignocellulosic content estimation:** A brief lignocellulosic content profile was developed after analyzing pretreated biomass consulting TAPPI standards.

**Enzymatic hydrolysis:** An adapted saccharification scheme of Lim *et al.*, 2013 was used to check saccharification ability. For this purpose 0.1% substrate was saccharified with 6.8, 22, and 4209 units of Endo-1,4- $\beta$ -glucanase, Exo-1,4- $\beta$ -glucanase and  $\beta$ -1,4-Glucosidase at 80°C and 50 rpm. Total reducing sugars produced after saccharification were analysed through DNS method by Miller (1959) and then following formula was used to find out percentage saccharification.

$$\text{Percentage saccharification} = \frac{c \times v \times f_1 \times 100}{m \times f_2}$$

c = sugar concentration in the hydrolysates estimated as total reducing sugars (mg/mL)

v = liquid volume (mL) of the hydrolysates

f<sub>1</sub> = factor used to convert monosaccharide to polysaccharide due to water uptake during hydrolysis (0.9 for hexoses)

m = amount of initial substrate (mg)

f<sub>2</sub> = factor for the carbohydrate content of the substrate (total carbohydrate, mg/total substrate, mg)

**Statistical analysis:** The data was statistically analyzed by manipulating SPSS version 16.00. Replicates were subjected to one way ANOVA to uncover significant probability value (P). Error bars in the figures point towards standard deviation ( $\pm$ SD) with the difference of  $p < 0.05$  among three replicates in the result section.

## Results and Discussion

**Steam pretreatment:** Auto-hydrolysis of sawdust was worked out by supplementing water to sawdust for different episodes of time ranging from 15 to 75 minutes with 15 minutes time laps. The chemical analysis of these time periods put on view that by the means of given conditions 60 minutes time duration was more effectual as it made decrease in the percentage lignin happen from 35.8% to 30.08% (Fig. 1). It also illustrated 13.9% delignification of cellulosic biomass after steam pretreatment. It is proposed that during steam pretreatment acetic acid is produced by the chemical and mechanical effects on acetyl groups present on hemicellulose. Moreover, at elevated temperatures water takes the action of weak acid and leads to impulsive separation of fibers thus lignin is removed instinctively. Reduction in energy and chemical requirement are in favor of this method but incomplete destruction of lignin carbohydrate matrix results into condensation and precipitation of lignin and digestibility of cellulosic content is decreased (Pan *et al.*, 2005; Alvira *et al.*, 2010). Cara *et al.*, (2006) steam pretreated olive tree wood biomass with hot alkaline hydrogen peroxide for 45 minutes and reported reduction in lignin from 20.4% to 15.7% so, present study was found to be more effective.

**Hydrogen peroxide pretreatment:** Hydrogen peroxide pretreatment was conducted with several concentrations of hydrogen peroxide and 8% hydrogen peroxide solution effectively decreased to 24% showing evidence of 32.9% delignification (Fig. 2). At some point in the course of action  $H_2O_2$  decomposes and formulates mild oxidant termed as perhydroxy anion ( $HO_2^-$ ) for delignification. In the course of action, perhydroxy anion further reacts with other molecules of hydrogen peroxide and sets up hydroxyl radical. This hydroxyl radical being potent lignin oxidant triggers polymerization of carbohydrates (Kim & Lee, 1996). Park *et al.*, (2015) acquired same results as their biomass was left with 23.6-25.8% lignin which was in accordance with present study showing 24% lignin in the end. However, present study evidenced to be more fruitful since Park *et al.*, employed two pretreating agents NaOH and  $H_2O_2$  on the contrary only  $H_2O_2$  was used in present study.

**Microwave pretreatment:** Microwave pretreatment was accessed for its efficacy by pretreating at different time durations for 1 to 5 minutes as shown in figure 3. Results showed 26.6% delignification by carrying out the process for 4 minutes because only 26.6% lignin was left in the pretreated biomass which was the minimum amount of lignin left among all types of microwave pretreated biomass. Microwave pretreatment is catching more attention as it requires less energy and selectively targets the biomass. The fact that it can be started and blocked promptly makes it more valuable (Datta, 2001). In spite of

all this significant results can only be attained if by adding polar molecules and ions to biomass. In this way, exposure to microwave can hasten chemical and biological processing (Sridar, 1998). Jin *et al.*, (2016) found this method to be more efficient by getting only 16.77% lignin by pretreating Catalpa sawdust as they used a combination method in which they used microwave and  $Ca(OH)_2$  as the pretreating agents to get synergistic effects. Utilization of only one pretreating agent, microwave contributed to be the root cause of difference in results.

**Urea and thiourea pretreatment:** A new combination of urea and thiourea was used for the first time to pretreat sawdust with different combining ratios. The results showed urea: thiourea to be more effective in 1:4 ratio by giving 32.96% delignification (Fig. 4). Urea and thiourea were reported to cut short hydrogen bonding present in the cellulose (Laszkiewicz & Cuculo, 1993). In the past combinations of different pretreating agents had been used including urea and thiourea separately and those studies ended into a conclusion that urea, thiourea and NaOH were effective. It was found that 6% (w/v) NaOH and 4% (w/v) urea can effectively dissolve cellulose (Zhang *et al.*, 2002). Urea behaves as hydrogen bond donor and receptor between solvent molecules and inhibits the reassociation of cellulose molecules which causes molecular dissolution of cellulose (Zhou *et al.*, 2006).

**Saccharification studies:** Enzymatic hydrolysis was assessed for best pretreated sample of each strategy i.e. Autohydrolyzed for 60 min, 8% Hydrogen peroxide pretreated, microwave pretreated for 4 min and Urea: thiourea (1:4) pretreated saw dust. Comparison of different pretreated samples for the delignification along with control sample showed that the sawdust pretreated with urea and thiourea in 1:4 showed maximum saccharification i.e. 37.38% after 4 hours of incubation as evident from figure 5. Escalation to hydrolysis rate is imparted by dexterosus pretreatment of lignocellulosic biomass as matrix is stripped off which initiates opening of cell wall structure thus enhancing the accessibility of cellulase to the cellulose (Chandra *et al.*, 2007). Sridevi *et al.*, (2015) made out 23% saccharification of sawdust in contrast the present study was successful to get hold of 37.38% saccharification. The root cause of the lifted result may be hidden in the fact that in the former study cellulases from *Aspergillus niger* were employed while in the present study cloned cellulases were availed which were over expressed and had amplified activity due higher units. Likewise, nature and type of pretreatment may also have an impact as Sridevi *et al.*, consumed NaOH pretreated sawdust while presently a novel combination of urea and thiourea were expended to pretreat sawdust. Harun & Danquah (2011) were triumphed in getting 64.2% saccharification of microalgal biomass ascertaining their method to be on the top as only 37.38% saccharification yield was exhibited in the present study. Contributory factors of high result in the former study are supposed to be diverse nature of substrate to be saccharified (micoralgae), marked pretreatment method (sonication and acid pretreatment). However, present study can be considered as financially viable as a waste product sawdust was consumed as a substrate.

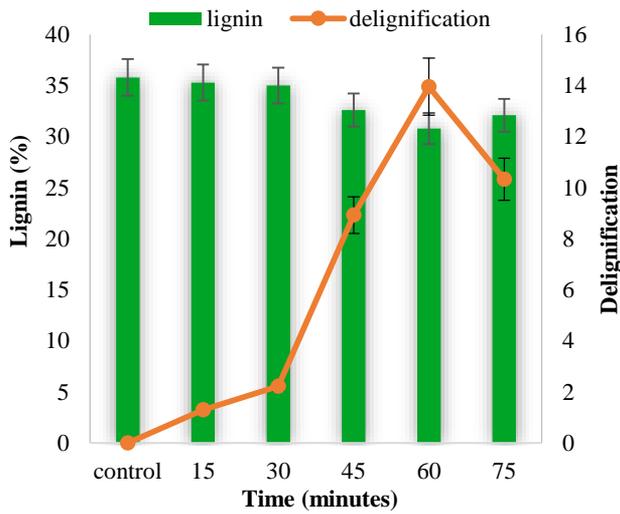


Fig. 1. Effect of time period on the lignin removal from saw dust undergone auto hydrolysis.

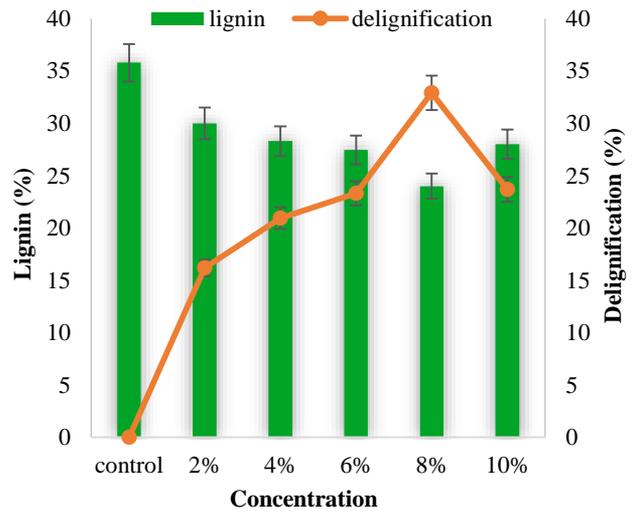


Fig. 2. Effect of hydrogen peroxide varying concentrations on lignin removal from saw dust.

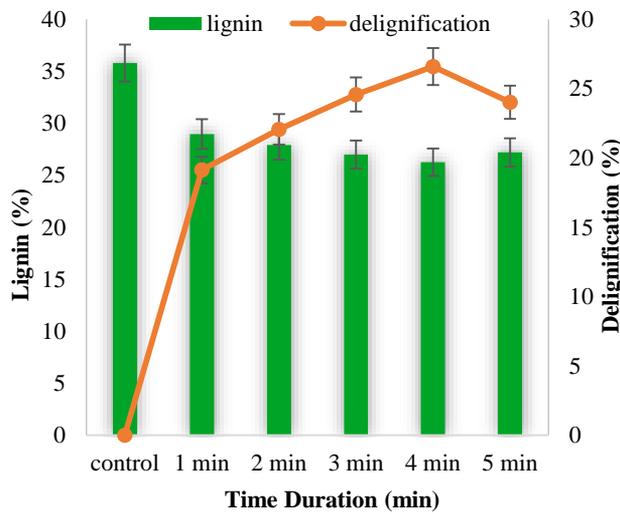


Fig. 3. Effect of incubation time on lignin removal from saw dust by microwaves.

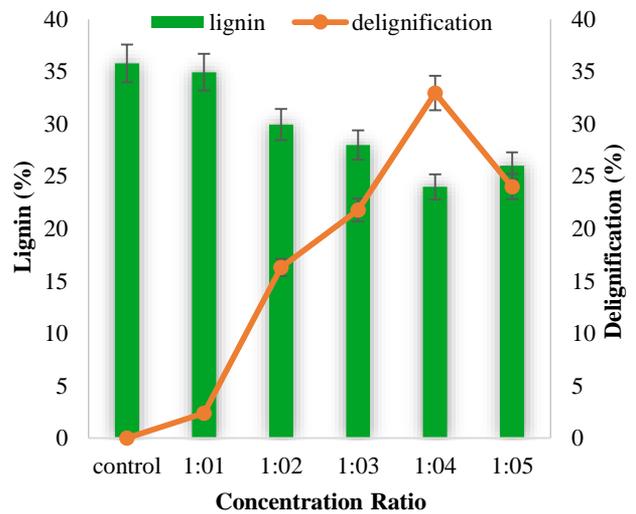


Fig. 4. Optimization of Urea and Thiourea proportion for efficient lignin removal from saw dust.

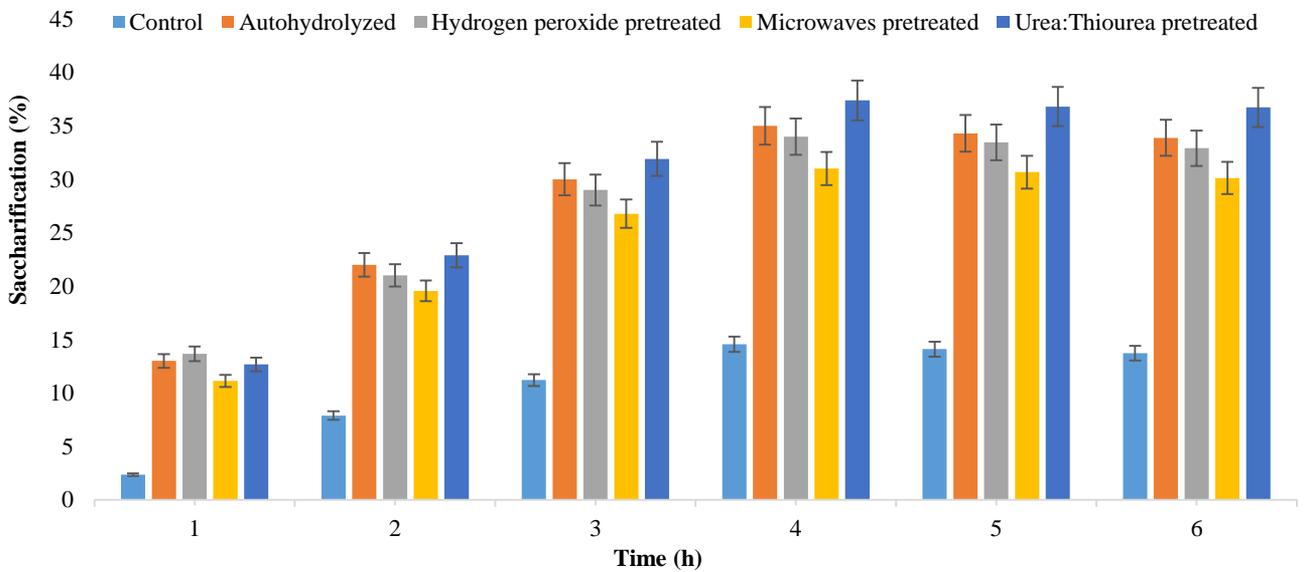


Fig. 5. Saccharification profile of differently pretreated and control samples of saw dust.

## Conclusion

It is concluded from the current study that different pretreatment strategies vary in their efficiency and action on saw dust. Among all the strategies used, urea and thiourea combination was recorded best. This observation was further supported by saccharification studies as well. This study will deepen our knowledge about the pretreatment methods about lignocellulosic substrate saw dust.

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