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Comparative assessment of acids and alkali based pretreatment on sawdust for enhanced saccharification with thermophilic cellulases

Evaluación comparativa de tratamiento a base de ácidos y bases sobre el sierro para la sacarificación mejorada con celulasas termófilas

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Abstract

The present study focuses on the use of thermophilic recombinant cellulases to produce fermentable sugars for conversion to bioethanol which is an important renewable energy resource to be worked upon due to climatic change, energy insecurity and nonrenewable nature of fossil fuels. Therefore, sawdust of pinus wood was subjected to pretreatment using different acids such as phosphoric, nitric, acetic and alkalis i.e. sodium hydroxide, calcium hydroxide and ammonia in various concentrations. Maximum delignification was observed using 8% nitric acid as it resulted in to 74.02% delignification and increased the cellulose availability to 81.8%. Subsequently, pretreated biomass was assessed for improvement in hydrolysis to less complex sugars employing thermophilic recombinant cellulases cloned from *Thermotoga petrophila*. Saccharification reaction parameters such as incubation time, temperature, biomass and enzyme concentrations were optimized. The optimized conditions were revealed as 3 h incubation time, 65 ºC temperature, 0.1 % (w/v) substrate, 250, 2550 and 70140 U of Endo-1,4-β-glucanase, Exo-1,4-β-glucanase and β-1,4-Glucosidase, respectively. This optimization study resulted in 34.61% saccharification yield which is 1.82 folds increase compared to saccharification yield of untreated biomass. This study is unique in providing insight to pretreatment using Nitric acid as in literature use of nitric acid as a pretreatment agent is not well investigated.

Keywords: Green energy, renewable energy, biofuels, catalysis, bioconversion.

Resumen

El presente estudio se centra en el uso de celulasas recombinantes termófilas para producir azúcares fermentables para la conversión en bioetanol, que es un importante recurso de energía renovable para trabajar debido al cambio climático, la inseguridad energética y la naturaleza no renovable de los combustibles fósiles. Por lo tanto, el aserrín de madera de pinus fue sometido a un tratamiento previo utilizando diferentes ácidos como fosfórico, nítrico, acético y álcalis, es decir, hidróxido de sodio, hidróxido de calcio y amoníaco en diversas concentraciones. Se observó una máxima designificación usando ácido nítrico al 8%, ya que resultó en una designificación del 74.02% y aumentó la disponibilidad de celulosa al 81.8%. Posteriormente, la biomasa pretratada se evaluó para mejorar la hidrólisis a azúcares menos complejos empleando celulasas recombinantes termofílicas clonadas de *Thermotoga petrophila*. Se optimizaron los parámetros de la reacción de sacarificación, como el tiempo de incubación, la temperatura, la biomasa y las concentraciones de enzimas. Las condiciones optimizadas se revelaron como 3 h de tiempo de incubación, 65 ° C temperatura 0.1% (p / v) sustrato, 250, 2550 y 70140 U de Endo-1,4-β-glucanasa, Exo-1,4-β-glucanasa y β-1,4-Glucosidasa, respectivamente. Este estudio de optimización dio como resultado un rendimiento de sacarificación del 34,61%, que es un aumento de 1,82 veces en comparación con el rendimiento de sacarificación de la biomasa no tratada. Este estudio es único para proporcionar información sobre el pretratamiento con ácido nítrico, ya que en la literatura el uso del ácido nítrico como agente de pretratamiento no está bien investigado.

Palabras clave: energía verde, energías renovables, biocombustibles, catálisis, bioconversión.

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1 Introduction

As nonrenewable resources are depleting rapidly so, the whole world's attention has been shifted towards bioethanol, biogas, biodiesel etc which is a renewable biofuel (Haq *et al*., 2019; Kumar and Sharma, 2017). It is recommended as more efficient and safer than other fossil fuels owing to different characteristics like high octane number, greater flammability limits, more heat production, less particulate matter and NOx emission on combustion etc (Venu and Madhavan 2017). Its production requires glucose from starchy and sugar crops. However, in order to avoid conflict from food crops it is better to use lignocellulosic waste like rice straw, wheat straw, sawdust etc. Moreover, it is cheap, reliable, effective and locally available (Oropeza *et al*., 2019; Ravikumar *et al*., 2017).

Softwood is more resilient type of wood for bioethanol production as compared to agricultural crops waste and is composed of three biopolymers namely cellulose, hemicellulose and lignin (Bibi *et al*., 2017). Several bacteria and other microorganisms are capable to reap energy from different cellulosic substrates as they possess highly specific and proficient enzymes but high polymerization and crystallinity of the substrate is the main hindrance in their catalytic activity (Haq *et al*., 2020; Yu *et al*., 2016). Therefore, it is necessary to carry out pretreatment which targets the inter fibril and intra fibril structure of the substrate exposing the cellulose and hemicellulose for further processing (Hsu, 2018). Pretreatment is reported to cause changes in lignin structure like modification, swelling, partial decrystallization and solvation (Aguado *et al*., 2019). Pretreatment is usually carried out by physical, chemical and biological methods, creating porous cavities in lignocellulosic biomass for enzymatic action but the selection of method is dependent upon biomass used, methods efficiency and cost effectiveness (Chen *et al*., 2017). Chemical pretreatment is considered a better option as it can effectively change the native structure of lignocellulosic biomass with less production of toxic compounds (Behera *et al*., 2014). While, biological pretreatment do not provide efficient results and is also not economical at present.

After biomass pretreatment, bioconversion of cellulose and hemicellulose to monomeric subunits is carried out which can be fermented to bioethanol (Rastogi and Shrivastava 2017). Different bacteria like *Clostridium* sp, *Bacillus* sp, *Acetovibrio* sp, *Microbispora* sp, *Streptomyces* sp and fungi like *Penicillium* sp, *Fusarium* sp, *Humicola* sp, *Schizophillum* sp have been reported to produce cellulases that are able to efficiently hydrolyze cellulose (Imran *et al*., 2016). The use of over expressed recombinant enzymes has been considered more promising as, such enzymes prove to be more thermally stable (Khare *et al*., 2015). Cellulases produced by *Thermotoga petrophila* have pronounced importance since they possess stability up to 80 ºC. It is better to use these enzymes as they are adapted to tail off viscosity which can in turn drop pumping cost and risk of bacterial contamination (Colussi *et al*., 2015). So, enzymatic saccharification is preferred over other saccharification methods (Guo *et al*., 2018). Exoglucanase act on the non-reducing ends of cellulose chains to break them and making cellulose more available for the action of endoglucanase. During this process, cellobiose is produced which has an inhibitory action on above two enzymes. So, β-glucosidase is added to the reaction mixture which converts cellobiose into glucose thus other cellulases can act more without any hindrance. Therefore, it is suggested to use a cocktail of cellulases for better results (Colussi *et al*., 2015; Josefsson *et al*., 2008).

Bioethanol production can be labor saving if maximum sugars are extorted from lignocellulosic biomass which is reliant upon effectual saccharification and the optimization of various parameters like temperature, pH, time duration, substrate and enzyme concentration can be worthy (Akhtar *et al*., 2017). Based upon above mentioned facts several pretreatment schemes like a range of alkali and acids were taken up for pretreatment of biomass and the best pretreated biomass was expended for the optimization of saccharification to develop an efficient and economical process of converting biomass to fermentable sugars.

2 Materials and methods

2.1 Raw materials

Sawdust of Pinus wood was collected from local furniture market of Lahore, Punjab Pakistan and uniform sized particles up to 0.475 mm were obtained by sieving through the locally fabricated sieve having pore size of 0.475 mm. Sawdust obtained was washed with distilled water thrice to remove dust particles and

impurities. After drying in a hot air oven (Universal Oven UN750 Plus Memmert, Germany) at 105 ºC, biomass was stored in airtight bottles for further use (Kim *et al*., 2013).

2.2 Cellulases

The enzymes i.e. Endo-1,4-β-glucanase, Exo-1,4-β-glucanase and β-1,4- Glucosidase used in present study were thermophilic recombinant enzymes obtained from the principle investigator of the project entitled "Production of bioenergy from plant biomass" at the Institute of Industrial Biotechnology Government College University Lahore, Pakistan. These enzymes were cloned in mesophilic host *E. coli* by taking respective genes from *T. petrophila*. The characteristics of these enzymes such as optimum temperature, optimum pH and enzyme activity are presented in the table 01.

2.3 Pretreatment with alkali

Sodium hydroxide, calcium hydroxide and ammonia were used for the pretreatment of biomass for delignification after the modified method of Kumar *et al*. (2009). These alkalis were employed in varying concentrations (10, 20, 30, 40 and 50%). Pretreatment was carried out by taking sawdust and alkali in a screw capped bottle in 1:12 ratio. The reaction was carried out at 125 ºC for 30 minutes in a stainless steel pressurized closed chamber. After pretreatment, extensive washings with distilled water were carried out to bring biomass pH to neutral and then biomass was stored in zipper bags after drying at 105 ºC.

2.4 Pretreatment with acids

Modified methods of Lih *et al*. (2009) were adopted to carry out dilute acid pretreatment. Different acids like nitric, phosphoric and acetic acid were used with varying concentrations (2, 4, 6, 8 and 10%). Sawdust was supplemented to dilute acid in 1:12 in screw capped bottles and pretreated at 125 ºC for 30 min. Rigorous washing was done with distilled water to neutralize the biomass. After that, it was dried in hot air oven at 105 ºC and stored in zipper bags for further use.

2.5 Lignocellulosic content estimation

Lignin content left in the pretreated biomass and cellulose content available was gagged by consulting TAPPI standards (1993).

2.6 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out by adapting in the saccharification scheme used by Lim and Lee (2013). Saccharification was performed by taking 0.25% pretreated substrate along with 50 U of each cellulolytic enzyme. The reaction was carried out at 80 ºC for 6 hours in a capped conical flask. Assessment of total reducing sugars was done using DNS method by Miller (1959) and saccharification percentage was calculated by the formula given below.

$$\text{Percentage saccharification} = \frac{c \times v \times f1 \times 100}{m \times f2} \quad (1)$$

c = sugar concentration in the hydrolysates estimated as total reducing sugars (mg/mL),
v = liquid volume (mL) of the hydrolysates,
$f1$ = factor used to convert monosaccharide to polysaccharide due to water uptake during hydrolysis (0.9 for hexoses) (Hernandez *et al*., 2019),
m = amount of initial substrate (mg),
$f2$ = factor for the carbohydrate content of the substrate (total carbohydrate, mg/total substrate, mg).

2.7 Optimization of reaction conditions

Various reaction conditions such as time, temperature, biomass concentration and enzyme units were assessed to figure out ideal conditions for maximum saccharification (Haq *et al*., 2018).

2.8 Scanning electron microscopy of biomass

To probe structural changes brought about at every step i.e. pretreatment and saccharification, Scanning Electron Microscopy (SEM) (JSM-6480, Tokyo, Japan) was carried out (Selig *et al*., 2007). No processing or treatment of the samples was needed as they had polymeric nature and were sent to Center for Advance Studies in Physics (CASP) Government College University Lahore.

2.9 FTIR analysis

Actual chemical composition and the changes in that composition after every step was accurately predicted using FTIR spectroscopy. For this purpose dried sawdust samples from each step were scanned between 4000 and 40 cm^{-1}. Baseline and necessary corrections were also applied for penetration depth and frequency variations (Castoldi *et al*., 2014).

Table 1. Thermophilic recombinant cellulases catalytic profile.

Enzyme	Optimum temperature (ºC)	Optimum pH	Enzyme Activity (U/mL)
Endo-1,4-β-glucanase	90	6.0	5
Exo-1,4-β-glucanase	90	6.5	25
β-1,4- Glucosidase	80	6.0	3507

2.10 Statistical analysis

An SPSS version 16.00 was used to statistically analyze the data obtained from the all the experiments carried out in triplicates. Significant probability value (P) was calculated by subjecting replicates to one way ANOVA. Error bars were used in the figures to indicate standard deviation (±SD) among three replicates with the difference of $p<0.05$ significantly in the result section (Heck *et al*., 2013).

2.11 Materials

All the chemicals used were of analytical grade and were purchased from authorized dealers of Sigma Fluka, Merk and Acros Ltd.

3 Results and discussion

3.1 Pretreatment

Varying concentrations of sodium hydroxide, calcium hydroxide and ammonia were analyzed for their competence regarding lignin removal from sawdust. Among these, liquid ammonia (10%) was found more proficient as it caused maximum delignification of 63.68% ($p<0.05$).

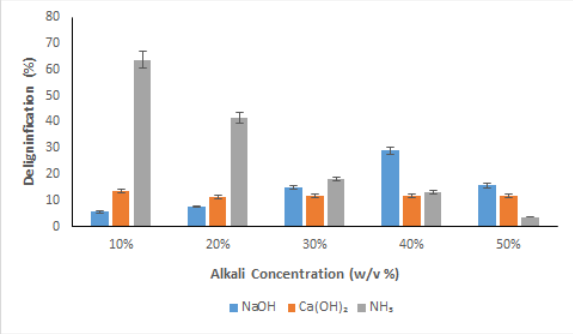

Fig. 1. Delignification comparison of sawdust after pretreatment with different alkali having variable concentrations.

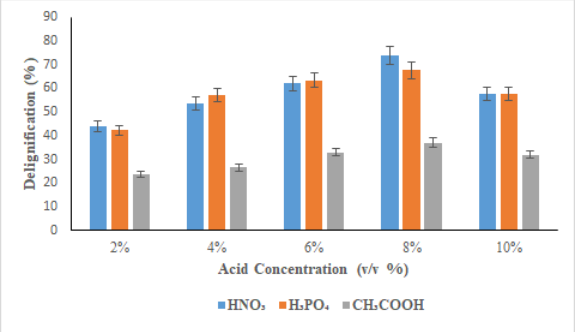

Fig. 2. Delignification comparison of sawdust after pretreatment with different acid having variable concentrations.

Second highest delignification (29.05%, $p<0.05$) was obtained using 40% sodium hydroxide while 10% calcium hydroxide pretreatment resulted in 13.45% ($p<0.05$) delignification as evident from fig 1. This might be due to a reason that ammonia has the ability to disrupt cross linking in the biomass as it forms NH4OH which hydrolyzes glucoronic acid esters and also cleaves cross linking bonds, in this way the fibrilar structure of cellulose is changed. Kim and Lee (1996) obtained 94-99% delignification by pretreating corn cobs/ stover, switch grass and hybrid poplar turning out their pretreatment to be more efficient in comparison to present study might be due to the reason that delignification was enhanced by the combine effect of ammonia and hydrogen peroxide used. Moreover, the nature of substrate was also different as they used corn cobs/ stover, switch grass and hybrid poplar while in the present study sawdust was used for the pretreatment. Li *et al*. (2004) reported 39% delignification employing 15% aqueous ammonia for pretreatment of corn stover which is less efficient to our findings where 10% aqueous ammonia was used to achieve 63.68% ($p<0.05$) delignification might be due to less complex structure of saw dust as compared to corn stover.

In dilute acid pretreatment, different concentrations of nitric, phosphoric and acetic acid were used and nitric acid was distinguished as most active agent among all to carry out delignification as it

caused 74.02% ($p<0.05$) delignification of sawdust. The pretreatment comparison abilities of different acids are shown in fig 2. Nitric acid attacks the biomass but the cellulose is left almost unaffected as it acts like a catalyst instead of an acidifier. Kim *et al*., (2014) observed 31.17% lignin content in the rice straw after pretreatment with nitric acid which was greater than lignin content obtained in the present study as higher temperature (180 ºC) was used while in the present study pretreatment was done at 125 ºC. Additionally, different nature of substrates may also have an important role in variation of results. Other dilute acids were found to be less efficient as 6% phosphoric acid caused 67.59% ($p<0.05$) delignification and 8% acetic acid caused 37.15% ($p<0.05$) delignification.

Being the most delignified substrate among all, nitric acid pretreated substrate along with control was subjected to cellulose estimation in the substrate. The cellulose availability was increased from 45% ($p<0.05$) in control to 81.8% ($p<0.05$) in pretreated substrate as given in table 2.

3.2 *Effect of incubation time*

Saccharification using pre-treated saw dust was carried for different time periods i.e. 1-9 h to find out the optimum reaction time. The increase in saccharification yield with passage of time was observed initially and maximum saccharification i.e. 16.03% ($p <0.05$) was achieved after 3 hours as depicted in fig 3. Further, increase in time duration was found to have a declining effect on percentage saccharification. Incubation time of the saccharification is dependent upon different factors like half-life of enzyme, nature of substrate, source of enzyme, production of inhibitory products etc. As soon as the enzymes make contact with the substrate, they swiftly act on the substrate to yield products. In the course of their action, different inhibitors of enzymes are generated that may adversely affect the hydrolysis resulting into less yield (Saqib *et al*., 2010). However, Cara *et al*. (2008) were successful in getting hold of 76.5% saccharification that was most probably contributed by an increased incubation time (72 hours). Enzymatic cellulose complex (Celluclast 1.5 L), β-glucosidase (Novozym 188) and more harsh conditions like 1.4% acid concentration, 210 ºC are deduced to be the reason.

3.3 *Effect of biomass concentration*

Various biomass concentrations (0.1-0.5%) were used to achieve maximum saccharification using constant concentration of cellulases. The analysis showed 0.1% substrate concentration of biomass valuable for the course of hydrolysis. An upturn in biomass concentration caused a decline in percentage saccharification. In the present study 0.1% substrate was found to yield 16.64% ($p <0.05$) hydrolysis as illustrated in fig 4. Substrate concentration (biomass) is another factor that contributes to enzyme activity as increase in substrate molecules engages more active sites of the enzymes which increases the rate of reaction.

Table 2. Lignocellulosic composition of treated and untreated sawdust.

Biomass	Cellulosic Content (%)	Delignification (%)
Sawdust (untreated)	45	0
Sawdust (pretreated)	81.8	74.02

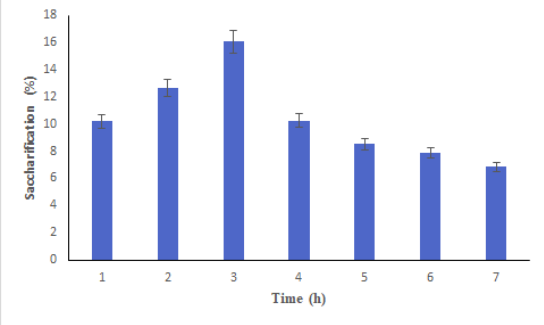

Fig. 3. Effect of reaction time on the saccharification of nitric acid treated sawdust.

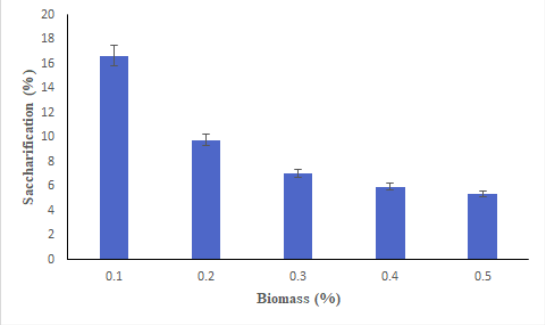

Fig. 4. Effect of substrate concentration on the saccharification of nitric acid treated sawdust.

However, it should also be considered that once

active sites are occupied further increase in the substrate molecules does not increase the rate of reaction. Increasing substrate concentration may also give rise to binding of more molecules which causes competitive inhibition of the enzymes. Moreover, poor stirring, decline in synergism among enzymes and inhibition by formation of saccharification products may also pessimistically affect the process (Xio *et al*., 2004). However, Alruman (2016) obtained 71.03% saccharification yield by saccharifying alkali treated date palm waste in 4% concentration which contrasted with the results of present study as nature of substrates is poles apart.

3.4 Effect of temperature

Effect of different temperatures (55-80 ºC) on the hydrolysis of sawdust was analyzed and highest saccharification (21.62%, p <0.05) was observed at 65 ºC as represented in fig 5. Increase or decrease in that temperature could not increase percentage saccharification. The optimum activity of the enzymes is also dependent upon optimum temperature. Fluctuations from the optimum temperature may change protein folding and the rate at which enzyme is adsorbed to substrate (Kocher and Kalra 2013). Cellulases employed in the present study were thermophillic in their origin with optimum activity at 90 ºC but they showed maximum saccharification at 65 ºC. This variation is temperature might be associated to the structural and compositional variation among the synthetic and natural substrates used for enzyme assay and saccahrification, respectively. A justifying hypothesis regarding their activity says that at elevated temperatures, the sugar released during the process caramelizes which can negatively influence the process.

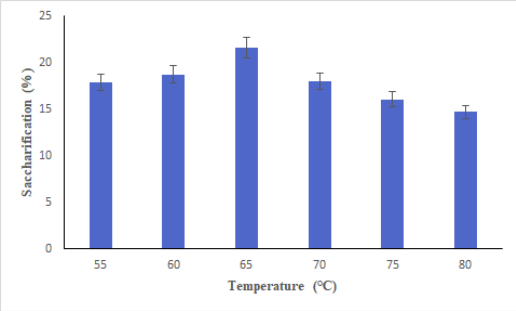

Fig. 5. Effect of incubation temperature on the saccharification of nitric acid treated sawdust.

Eklund *et al*. (1990) found 40 ºC to be the effective temperature for the saccharification of steam pretreated willow using Celluclast and Novozyme while Kim *et al*. (2008) reported 46.3 ºC as an optimum temperature for the saccharification of food waste by using Glucoamylase and Novozymes which works best in mesophilic range.

3.5 Cellulase concentration optimization

All cellulolytic enzymes i.e. endoglucanase, exoglucanase and β-glucosidase were separately optimized by experimenting with variable units of these enzymes. Highest saccharification was obtained by using 250, 2550 and 70140 U/ml of Endo-1,4-β-glucanase, Exo-1,4-β-glucanase and β-1,4-Glucosidase respectively (Fig 6). So, the optimum conditions for maximum saccharification were found to be 0.1% substrate concentration, 65 ºC temperature, time duration of 3 hours using 250, 2550 and 70140 U/ml of endoglucanase, exoglucanase and β-glucosidase, respectively. Vikari *et al*., (2007) also gave a detailed optimal ratio of consortium of enzymes from *Trichoderma reesei* as Cellobiohydrolase-I 50-60%, Cellobiohydrolase-II 10-30%, Endoglucanase 10-30%, Xylanase 5-10% and β-glucosidase less than 1% (depending on protein loading). Endoglucanase triggers hydrolysis of cellulose by acting on its non-reducing ends and cleave cellulose. Exoglucanase act on the partially degraded substrates but its activity is inhibited by cellobiose and dextrose produced by its action. This hindrance can be removed by the β-glucosidase which again relieves the action of these enzymes. It is found that during saccharification, reaction mixture is chiefly composed of glucose and cellobiose. A very active and efficient β-glucosidase is needed to convert cellobiose into glucose to prevent feedback inhibition.

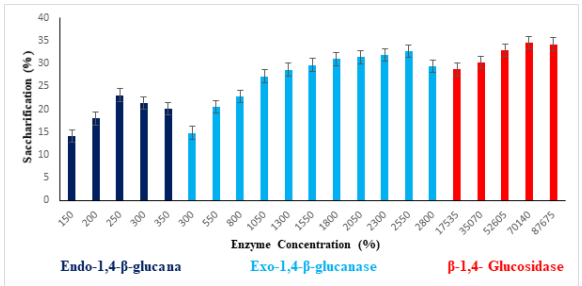

Fig. 6. Effect of cellulases loading on the saccharification of saw dust.

Therefore, it is suggested to use an optimized cocktail of cellulases for obtaining a better saccharification yield by setting aside feedback inhibition constraints (Haq *et al*., 2020). Enzyme activity can also be inhibited by the production of sugars during enzymatic action so, constant removal of sugars produced can be efficient to increase saccharification yield (Xiao *et al*., 2004). Other products having an inhibitory effect on saccharification include organic acids, furan derivatives and lignin derivatives (Chen and Jin 2006). In the present study enzymes were produced from the *Escherichia coli* having a gene from *Thermotoga petrophila*. So, these enzymes are very valuable as they are thermostable and have increased flexibility for process configurations. In this way they can be helpful in improving the process (Viikari *et al*., 2007).

3.6 Scanning electron microscopy (SEM)

The effect of nitric acid pretreatment and further saccharification was observed by comparing SEM results of sawdust at different stages. There were long and ordered fibers arranged in compact form before pretreatment as depicted in fig 7(a). Fragmentation and shattering of the fibers was clear from the SEM of pretreated sample which is shown in fig 7(b). More hydrolysis of the fibers was confirmed in saccharified sample as shorter fibers with more shattering were seen and more hollow spaces among them were observed that is clear from fig 7(c).

3.7 FTIR Results

Untreated and pretreated samples of sawdust were characterized by FTIR for functional group analysis through signal peaks recorded in the form of absorbance in the range of 4000 - 400 cm^{-1}. Peaks in the region 1390 - 1510 cm^{-1} are mainly due to the asymmetric deformation of C-H bonding represents presence of lignin (Altavilla *et al*., 2015). As evident from Fig 8 (a), there are present multiple peaks in the same region so it confirms the presence of lignin content in untreated sawdust. While peaks in the region 1016 - 1140 cm^{-1} represents the presence of cellulose (Castoldi *et al*., 2014). Pretreated samples FT-IR graph (fig 8 b) shows one prominent peak at 1028.19 cm^{-1} and decrease in the peaks present in the region 1390 - 1510 cm^{-1}, which confirms the removal of lignin content and availability of cellulosic content.

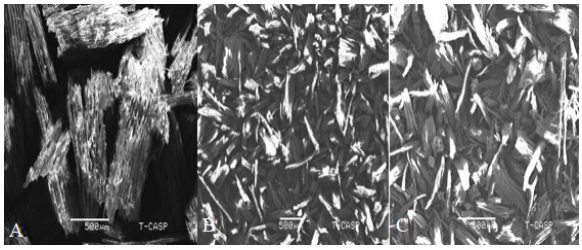

Fig. 7. Micrographs attained through scanning electron microscopy (a) before pretreatment, (b) after pretreatment and (c) after saccharification.

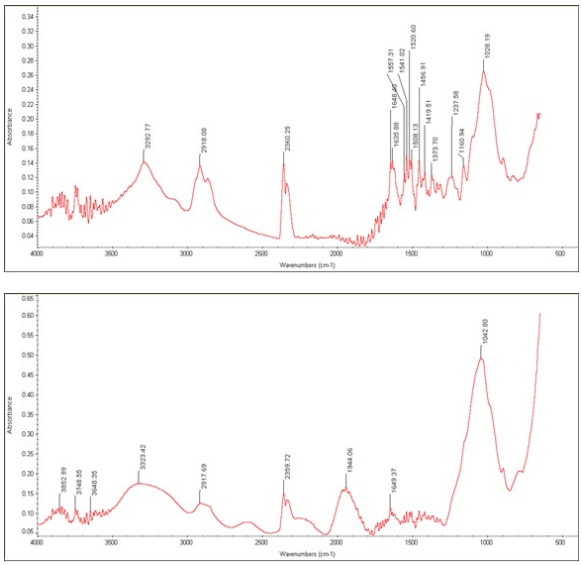

Fig. 8. (a) FT-IR graph for the untreated sample of sawdust, (b) FT-IR graph for the pretreated sample of sawdust.

Conclusions

The present study showed efficiency of the pretreating agents against the recalcitrance of softwood sawdust. All the agents were successful to remove a part of the lignin however nitric acid proved to be more efficient by removing maximum lignin. The removal of lignin made more cellulose available for the cellulolytic action. Saccharification was found to be proficient as the recombinant cellulases were very active to hydrolyse the cellulose. Further optimization of the saccharification resulted into a 1.82 folds increase in the yield. The results of present study can be utilized towards making the process of lignocellulosic based bioethanol production more economical.

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