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Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol

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Abstract

Background

Enzymatic hydrolysis, the rate limiting step in the process development for biofuel, is always hampered by its low sugar concentration. High solid enzymatic saccharification could solve this problem but has several other drawbacks such as low rate of reaction. In the present study we have attempted to enhance the concentration of sugars in enzymatic hydrolysate of delignified Prosopis juliflora, using a fed-batch enzymatic hydrolysis approach.

Results

The enzymatic hydrolysis was carried out at elevated solid loading up to 20% (w/v) and a comparison kinetics of batch and fed-batch enzymatic hydrolysis was carried out using kinetic regimes. Under batch mode, the actual sugar concentration values at 20% initial substrate consistency were found deviated from the predicted values and the maximum sugar concentration obtained was 80.78 g/L, which on using fed-batch strategy was implemented to enhance the final sugar concentration to 127 g/L. The batch and fed-batch enzymatic hydrolysates were fermented with Saccharomyces cerevisiae and ethanol production of 34.78 g/L and 52.83 g/L, respectively, were achieved. Furthermore, model simulations showed that higher insoluble solids in the feed resulted in both smaller reactor volume and shorter residence time.
Conclusion

Fed-batch enzymatic hydrolysis is an efficient procedure for enhancing the sugar concentration in the hydrolysate. Restricting the process to suitable kinetic regimes could result in higher conversion rates.

Keywords

Enzymatic hydrolysis, Fed-batch, Kinetic model, Fermentation, Delignified substrate, Bioethanol

Background

Production of cellulosic ethanol from lignocellulosic biomass represents a potential alternative to the petroleum fuel due to its renewable nature and sustainable availability. Currently, the major strategy used for cellulosic ethanol production includes three main steps i.e., biomass pretreatment, enzymatic hydrolysis and ethanol fermentation [1,2]. The enzymatic hydrolysis contributes significantly to the cost of cellulosic ethanol and from the process economics perspective, the improvement in the enzymatic hydrolysis step is a prerequisite [3,4]. The main obstacles for enzymatic hydrolysis are low rate of reaction, high cost of enzyme, low product concentration and lack of understanding of cellulase kinetics on lignocellulosic substrates [5,6]. One way to overcome this problem is to operate the enzymatic hydrolysis using high insoluble solid consistency [7-9]. However, the saccharification reaction at high insoluble solid consistency will have to encounter the problems of increased viscosity, higher energy requirement for mixing, shear inactivation of cellulases, and poor heat transfer due to rheological properties of dense fibrous suspension [9,10].

Interestingly in fed-batch enzymatic hydrolysis such problems could be avoided by adding the substrate and/or enzymes gradually to maintain the low level of viscosity [11]. The fed-batch enzymatic saccharification process has several other economic advantages over conventional batch process such as lower capital cost due to reduced volume, lower operating costs and lower down-stream processing cost due to higher product concentration [6,7]. There are several reports on fed-batch enzymatic saccharification which mainly deal with the development of appropriate kinetic models for mechanistic description of the phenomena [9,12,13]. However, the reports on process operation, optimization and control for fed-batch enzymatic saccharification are scarce [14]. Till date, the strategies used for fed-batch enzymatic saccharification are categorized into three main groups i.e., (i) to recycle enzyme; (ii) fed-batch SSF to mitigate inhibitory effect and (iii) fed-batch saccharification to increase the cumulative substrate in a reactor [7]. Here, the present study falls within the third category and our main emphasis was to enhance the total solid content and sugar concentration, which eventually resulted in higher ethanol production.

The experimental data on cellulose hydrolysis by cellulases point to various bottlenecks that decrease the rate of conversion. Mathematical modeling of the enzymatic hydrolysis process is an important tool for analyzing these bottlenecks [5]. Use of mathematical modeling can lead to several advantages viz. the effect of feeding profiles on sugar conversion can be evaluated apriori, kinetics of the hydrolysis process can be studied and process simulations can be made to understand the kinetic regimes. Recently, Hodge and colleagues [7] have used
model based fed-batch approach to develop a feeding profile for the fed-batch enzymatic saccharification, while, Morales-Rodriguez and coworkers [14] used a modeling approach to reduce the amount of enzyme during the fed-batch enzymatic saccharification.

The present study deals with the development of the feeding profile and a mathematical model for the understanding of the enzymatic saccharification kinetics in a stirred tank reactor (STR). Moreover, the hydrolysates obtained after batch and fed-batch enzymatic hydrolysis has subsequently been fermented to ethanol, and an overall comparison between batch and fed-batch process has been presented.

Results

Kinetics of batch and fed-batch enzymatic hydrolysis

A series of batch experiments were performed using the initial substrate concentrations 5%, 10%, 15% and 20% (w/v). Then applying the kinetic model the rate constants of cellulose hydrolysis, $k_i$ ($i=1-4$) was calculated in each case. It was observed that the rate constant for enzymatic hydrolysis decreases with an increase in the initial insoluble solid concentration (Figure 1). However, the $k_i$ values have shown good correlation with the initial substrate consistencies used for enzymatic hydrolysis with a regression coefficient $R^2$ of~0.9.

Figure 1 Plot between substrate concentrations versus time for the enzymatic saccharification of delignified lignocellulosic biomass at different initial substrate consistencies

The maximum rate constant ($k_1$=0.0421 h$^{-1}$) was obtained when the hydrolysis was carried out with 5% initial substrate concentration. The rate constants $k_i$ were then validated using the glucose concentration measurements obtained during the hydrolysis experiments. The root mean square error (RMSE) values between the predicted and the experimental values for enzymatic saccharification carried out at 5, 10, 15 and 20% initial substrate consistency were 0.997, 0.779, 1.843 and 1.995, respectively (Figure 2 a-d). The results also indicated that the maximum deviation of the experimental data from the model prediction was observed when the enzymatic saccharification was carried out at 20% initial substrate consistency. Moreover, the experimental values also depicted that the sugar concentration increased significantly only up to 15% substrate consistency and declined thereafter at 20% substrate level (Figure 2 a-d). The maximum sugar concentration obtained at each substrate concentration were 41.10 g/L ($S_{1,0}$ =5%), 72.47 g/L ($S_{2,0}$ =10%), 90.07 g/L ($S_{3,0}$ =15%) and 80.05 g/L ($S_{4,0}$ =20%) (see Figure 2 a-d).

Figure 2 Plots between the actual and the predicted values of glucose concentration released during the enzymatic hydrolysis of delignified lignocellulosic biomass at 5% (A), 10% (B), 15% (C) and 20% (D) initial substrate consistencies

Kinetics of fed-batch enzymatic hydrolysis

The kinetic parameters determined from the batch experiments was used to simulate the hydrolysis profile during the fed-batch enzymatic hydrolysis. Fed-batch hydrolysis was performed employing discrete feeding policy. Insoluble solid substrate concentration (50 g) was added at 24, 56 and 80 h. The insoluble solid concentration was measured at 4 h
intervals. A similar pattern of pulse responses were observed in both the experimental and predicted values for every instance of addition of 50 g feed to insoluble substrate (Figure 3). However, the final insoluble substrate concentrations for both the predicted and experimental values were 58.52 and 65.59 g/L, respectively (Figure 3).

**Figure 3** Plot between the experimental versus predicted insoluble solids during the fed-batch enzymatic hydrolysis

**Comparison between batch and fed-batch enzymatic hydrolysis**

A comparison between batch and fed-batch enzymatic hydrolysis was made to determine which had a higher cellulose conversion. The results showed that in fed-batch operation at $S_{i} \geq 20\%$, the remaining insoluble solids in the reaction slurry was 65.59 g/L. While in contrast, during the batch enzymatic hydrolysis at $S_{i,0} = 20\%$ (w/v), the concentration of remaining insoluble solid was 107.29 g/L. The time profiles of glucose concentration and cellulose conversion levels for both batch ($S_{i,0} = 20\%$) and fed-batch ($S_{c} = 20\%$) were plotted in Figure 4 a - b. The final sugar concentration for batch and fed-batch were 80.78 g/L and 127.0 g/L (Figure 4 a), while the cellulose conversion was 40.39% and 63.56%, respectively (Figure 4 b). These results showed that intermittent addition of solids in a repeated fed-batch mode, resulted in better conversion compared to the addition of an equal amount once at the beginning of a batch.

**Figure 4. Comparative profiling of batch and fed-batch enzymatic saccharification (A) glucose production, (B) cellulose conversion**

**Simulation of kinetic model**

The kinetic parameters determined from the batch experiments were used to simulate different feeding policies. These simulations provide an insight about the operational protocol that may be implemented to obtain the best hydrolysis results. The simulation of discussed kinetic model under the fed-batch optimization approach has been shown in Figure 5 and 6. The Figure 5 depicted four different feeding policies developed from simulations with the target cumulative insoluble solids in the reactor as 20%. The simulation results showed that the cumulative insoluble solid concentration increases with time and saturates at different final values depending on the initial feed concentration at dilution rates of 0.1-0.4 h$^{-1}$ (Figure 5). The results indicated that long residence times are required to reach these higher solids levels, when solids were controlled at 5% or lower (Figure 5). While, the simulation results in Figure 6 indicated that higher insoluble solids levels in the feed resulted in both smaller reactor volumes and shorter residence times to achieve a given feeding objective. However, it has been predicted from the simulation results that the feed controlled at 10% initial solid levels resulted in maximum saccharification.

**Figure 5** Kinetic simulation profile for cumulative insoluble solids feeding at different initial substrate concentration (5-15%) at varied dilution rate of (A) 0.1 h$^{-1}$, (B) 0.2 h$^{-1}$, (C) 0.3 h$^{-1}$ and (D) 0.4 h$^{-1}$

**Figure 6** Kinetic simulation profile for the volume of bioreactor at different initial substrate consistency (5-15%) using dilution rate of (A) 0.1 h$^{-1}$, (B) 0.2 h$^{-1}$, (C) 0.3 h$^{-1}$ and (D) 0.4 h$^{-1}$
Fermentation of enzymatic hydrolysate

The fermentation profiles of batch ($S_{4,0}=20\%$) and fed-batch ($S_i=20\%$) enzymatic hydrolysates containing 76.52±2.82 and 117.35±1.14 g/L initial sugars have been shown in Figure 7 and 8. The fermentation of batch enzymatic hydrolysate brought about the production of 34.78±1.10 g/L ethanol with yield and productivity of 0.45 g/g and 3.16 g/L/h, respectively, after 11 h of incubation (Figure 7). Moreover, the biomass production during the fermentation of batch enzymatic hydrolysate increased till 8 h (1.86±0.04 g/L) and then remained almost constant (Figure 7). While, the fed-batch enzymatic hydrolysate when fermented with *S. cerevisiae*, produced 52.83±1.70 g/L ethanol and 4.50±0.004 g/L biomass with an ethanol yield of 0.45 g/g and ethanol productivity of 4.40 g/L/h after 12 h of incubation (Figure 8).

**Figure 7** Fermentation profile of batch enzymatic hydrolysate

**Figure 8** Fermentation profile of fed-batch enzymatic hydrolysate

**Discussion**

The main aim of the present investigation was to achieve high ethanol concentration as the final ethanol concentration in the fermentation broth is critical to make a cost-effective ethanol production process. Since the ethanol concentration is directly proportional to the sugar concentration, hence high concentration sugar syrup is a prerequisite. In the present study, the process modeling consisting of mass balance and kinetic models were used to provide insights into the process performance and to optimize the process for enhanced enzymatic hydrolysis. During the batch saccharification at different consistencies, a regular decrease in the rate constant with increase in the substrate concentration was observed (Figure 1) and the reaction was assumed to be a first order reaction. This decrease in rate may be attributed to the product inhibition, improper heat and mass transfer and the thermal deactivation of enzymes [7, 12]. The difference between the experimental values and those predicted through simulation for our batch experiments at 20% insoluble solid consistency may be attributed to the same reasons (Figure 2d).

To overcome this problem in batch operation, fed-batch enzymatic hydrolysis was implemented. This approach exploits the property of cellulose solubilization during the enzymatic hydrolysis to increase the solid loading to the reactor, which otherwise would be difficult to handle if the entire insoluble solid was added initially. Interestingly, considering the fact that there are two phases present in the slurry, in the present study, the cellulose conversion has been mentioned in terms of g/L of actual liquid present in the slurry, which was a major pitfall in the earlier report [7], who reported the conversion in terms of g/Kg of total slurry. The later was further amended by correcting the measurement of glucose in the liquid phase (which may represent only 80-90% of the total mass of the slurry) for the content of insoluble solids in order to accurately estimate conversion [15].

The present study demonstrated that fed-batch hydrolysis resulted in higher solid saccharification with high saccharification yield. The results in the (Figures 4a and 4b) depicted a final sugar concentration of 127 g/L with~64% cellulose conversion, which was significantly higher than the cellulose conversion at batch operation ($S_{4,0}=20\%$). It is estimated that an increase in solid substrate consistency from 5 to 8% in simultaneous
saccharification and fermentation process (SSF) reduced the process cost by 19% [16]. While according to report by National Renewable Energy Laboratory (NREL), Department of Energy (DOE), US, an increase in solid consistency from 20 to 30% can reduce the minimum ethanol selling price by $0.10/gallon ethanol [17]. Therefore, the high final sugar concentrations obtained in this work may lead to an economically competitive process.

Comparison of the accuracy of the model prediction validated that a well-designed fed-batch approach could be used to allow an STR reactor capable of handling pretreated \textit{P. juliflora} at below than 10\% insoluble solids to operate at cumulative initial insoluble solids as high as the set goal of 20\% (Figure 5). Moreover such validation are also in accordance with the earlier reports of Hodge and coworkers [7], according to whom, using fed-batch strategy the STR, was able to achieve very high cumulative solid loading ($S_{c,0}=20\%$), thus improving its working capability. In addition, the model may also be used to determine a fed-batch feeding policy required to maintain proper mixing and temperature control necessary for high cumulative insoluble solids.

The fermentation of the enzymatic hydrolysate obtained from batch and fed-batch operation also indicates the significance of the study. The fermentation of enzymatic hydrolysate from fed-batch operation brought about approximately 50\% and 40\% increment in the ethanol concentration and the ethanol productivity, respectively. As there have been estimations that by doubling the ethanol concentration from 2.5 to 5\%, the energy required to distill a fermentation broth to 93.5\% ethanol using conventional distillation techniques can be reduced by 33\% [9]. The enhanced ethanol concentration and productivity from fed-batch operation also made the process more industrially realistic.

**Conclusion**

To produce higher concentration sugar syrup and subsequently the high ethanol concentration, fed-batch enzymatic saccharification was conducted with the pretreated \textit{P. juliflora}. Through the fed-batch process, the cumulative solid loading ($S_c$) up to 20\% in a stirred tank reactor increased the sugar released by 56\% compared to the batch process with an initial insoluble solid loading of 20\%. This model used here provided additional insight into the effect of the operational conditions on productivity. This may be refined by including the degree of polymerization of substrate, accessible cellulose fraction, crystallinity of substrate and enzyme adsorption to distinguish the various causes of the decreasing rate of reaction.

**Methods**

**Raw material and chemicals**

\textit{Prosopis juliflora} wood, collected from University of Delhi South Campus, New Delhi, India, was comminuted by a combination of chipping and milling to attain a particle size of 1–2 mm using a laboratory knife mill (Metrex Scientific Instrumentation, Delhi, India). The processed wood of \textit{P. juliflora} was delignified with 4\% sodium chlorite at 120 C for 30 minutes as described earlier [18].
Commercial cellulases and 3,5-di nitro salicylic acid (DNS) were purchased from Sigma (St. Louis, Missouri, U.S.A.). Ethanol was purchased from Merck (Darmstadt, Germany). Rest of the chemicals and media components of highest purity grade were purchased locally.

Microorganism and culture conditions

The yeast Saccharomyces cerevisiae HAU procured from the culture collection of C.C.S. Haryana Agricultural University, Hisar, Haryana, India was maintained on agar slants containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; agar, 20.0 at pH 6.0±0.2 and temperature 30°C, as described earlier [1,8]. While the S. cerevisiae inoculum was grown for 24 h at 30°C in a culture medium containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; (NH₄)₂HPO₄, 0.25 at pH 6.0±0.2 [1,19]. Cells were cultured to an absorbance of 0.6–0.8 at 600 nm.

Enzymatic hydrolysis

Batch enzymatic hydrolysis

Enzymatic hydrolysis of pretreated substrate was carried out at different substrate consistency (5–20% w/v) in 0.05 M citrate phosphate buffer (pH 5.0) in a 3.0 L stirred tank bioreactor (Scigenics Pvt. Ltd, Chennai, India) fitted with Rushton impellers, heating jacket and heat exchangers for proper agitation and temperature control. Before enzyme loading, slurry was acclimatized by incubating at 50°C at 150 rpm for 2 h. Thereafter, an enzyme (lyophilized) dosage of 22 Filter paper cellulase activity (FPU) /g dry substrate (gds), 68 U β-glucosidase/gds was added to preincubated cellulose slurry, and reaction was continued for 48 h. One percent Tween 80 and 1 mM CuCl₂ were also added to facilitate the enzymatic reaction. The samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 10 min and the supernatants were used for further analysis.

Fed-batch enzymatic hydrolysis

Fed-batch enzymatic saccharification of pretreated substrate was carried out in the same bioreactor with an initial substrate consistency of 5% (w/v) in the suspension. Before enzyme loading, the slurry was acclimatized by incubating at 50°C at 150 rpm for 2 h. Thereafter, an enzyme dosage of 22 FPU/gds and 68 U β-glucosidase/gds, 1% Tween 80 and 1 mM CuCl₂ was added to preincubated cellulose slurry. The equal amount of initial substrate and half of the initial enzyme (lyophilized) was added to the enzymatic suspension thrice after 24, 56 and 80 h to get a final substrate concentration of 200 g/L. The samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 10 min and the supernatant was subjected to sugar estimation. After incubation, the hydrolysate was harvested, centrifuged to remove the un-hydrolyzed residues and the filtrate was used for fermentation studies.

Fermentation of enzymatic hydrolysate

The fermentation studies of both the enzymatic hydrolysates from batch operation (S₄₀=20%) and fed-batch operation (S₅₀=20%) were carried out. The batch and fed-batch enzymatic hydrolysates containing 37 g/L and 120 g/L sugars, respectively, supplemented with 3 g/L yeast extract and 0.25 g/L (NH₄)₂HPO₄, were inoculated with 6% (v/v) S. cerevisiae. The fermentation was carried out at 30°C, 200 rpm and initial pH 6.0±0.2. Aeration of 0.4 vvm
was maintained throughout the study. The pH was adjusted with 2 N HCl and 2 N NaOH. The samples withdrawn were centrifuged at 10,000 rpm for 10 min at 4°C and the cell free supernatant was used for the determination of ethanol produced and sugar consumed.

**Kinetics and theoretical aspects of batch and Fed-batch enzymatic hydrolysis**

Cellulose conversion is commonly used as a measure of the effectiveness of enzymatic hydrolysis of cellulose. The conversion efficiency ($\xi$) is described in terms of cellulose conversion to glucose ($G$) and the initial cellulose concentration ($S_{i,0}$), given by

$$\xi = \frac{G}{1.11} \left( \frac{1}{S_{i,0}} \right)$$

(1)

The insoluble solids level can be measured from the change in sugar concentrations relative to the initial cellulose concentration. This change in sugar levels can be related stoichiometrically to the amount of cellulose removed from the solid phase to estimate an insoluble solids level.

$$S_i = S_{i,0} - \frac{G}{1.11}$$

(2)

The equations describing the dynamic changes in $S_i$ and $G$ are

$$\frac{dS_i}{dt} = -k_i S_i$$

(3)

where $k_i$, $i = 1 - 4$, are the rate constants for different loadings, and

$$\frac{dG}{dt} = 1.11 k_i S_{i,0} - k_i G$$

(4)

These equations can be solved analytically to obtain following relation

$$G(t) = 1.11 S_{i,0} (1 - \exp(-k_i t))$$

(5)

**Mass balance equation for prediction of fed-batch capabilities**

Mass balances were performed on the reaction system to evaluate the fed-batch procedure. A key assumption was that the insoluble solids ($S_i$) are fed at a fixed flow rate ($F$). The final insoluble solid consistency obtained from the mass balance on insoluble solids at any time point is given by

$$S = S_F + S_i - k_i S_i$$

(6)

Where $S$ is the final insoluble solid concentration and $S_F$ is the concentration of solids fed. The cumulative insoluble solid ($S_c$) is the sum of the total amount of insoluble solids present initially and the amount of substrate fed to the reactor. It would represent the level of solid
Competing interests

The authors declare that they have no competing interests.

Authors’ Contributions

RG carried out the experimental work, analyzed the results and drafted the manuscript. SK helped in preparation of kinetic data and in technical checking of manuscript paper. JG designed the kinetic model and discussed the analysis of their results RCK coordinated the overall study and helped to analyze the results and finalize the paper. All authors read and approved the final manuscript.

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References


Figure 1

$\ln \left( \frac{S_0}{S} \right)$ against Time (h) for different initial substrate concentrations:

- 5% initial substrate concentration: $y = 0.0421x$, $R^2 = 0.9284$
- 10% initial substrate concentration: $y = 0.0297x$, $R^2 = 0.9812$
- 15% initial substrate concentration: $y = 0.0216x$, $R^2 = 0.9622$
- 20% initial substrate concentration: $y = 0.012x$, $R^2 = 0.9554$
Figure 2

A

B

C

D
Figure 4

(A) Sugar concentration (g/L) over time (h) for Fed batch and Batch processes.

(B) Cellulose conversion (g/L) over time (h) for Fed batch and Batch processes.
that would be present if the entire solid were added initially and the reactor was operated in batch mode to enable comparison of fed-batch performance with the batch reactor performance on an equivalent basis.

For fed-batch operation, the model provides the rate expression for concentration of cellulose in the insoluble solid, glucose and cellulase enzyme. In addition to these variables, the dilution rate ($D$) was introduced to account for the changing mass and concentration due to a feed stream and is defined as the feed flow rate ($F$) per volume ($V$). The equations for fed-batch operations are as follows:

$$
\frac{dS_i}{dt} = -k_i S_i + D(S_{f,i} - S_i)
$$

$$
\frac{dG}{dt} = 1.11k_i S_i + D(G_{f,i} - G)
$$

$$
\frac{dE}{dt} = D(E_{f,i} - E)
$$

$$
\frac{dV}{dt} = F
$$

**Fed-batch saccharification model simulation**

Using the above kinetic model, a feeding policy was developed based upon controlling the insoluble solids below a defined critical value during the saccharification reaction. This is possible by feeding a stream of pretreated substrate at a rate that approximately matches the rate of saccharification. Using the kinetic model equations, the rate of change of insoluble solids can be determined with the set of initial operating conditions and the insoluble solids at any given time point $[S_i(t)]$ will be,

$$
S_i(t) = \frac{S_{f,i}D}{(k_i + D)} (1 - \exp(-k_i t))
$$

Using this algorithm, fed-batch feeding policies were developed by generating a set of feeding curves over various reactor solids concentration and initial conditions generated to determine within the theoretical physical limitation of the system and the potential for using a fed-batch approach.

**Analytical methods**

The cellulase activities were determined following International Union of Pure and Applied Chemistry (IUPAC) methods [20]. The hydrolysates were analysed using high performance liquid chromatography (HPLC) (Waters, USA) for the presence of carbohydrates. Carbohydrate-ZX (Agilent Technologies, USA) column (300.0 x 7.8 mm) was used with Milli-Q water as an eluent with flow rate of 1.0 mL/min keeping oven temperature at 30 C with RID detector. Ethanol was estimated by gas chromatography (GC) (Perkin Elmer, Clarus 500) with an elite-wax (cross bond-polyethylene glycol) column (30.0 m x 0.25 mm), at oven temperature 90°C and flame ionization detector (FID) at 200°C. Nitrogen with a flow rate of 0.5 mL min$^{-1}$ was used as carrier gas.
Figure 5

A

B

C

D
Figure 7

![Graph showing time (h) vs. ethanol and residual sugar concentration (g/L). The graph includes lines for sugar, biomass, and ethanol concentrations. The x-axis represents time in hours (0 to 14), and the y-axes represent concentrations in g/L for sugar (0 to 90) and biomass (0 to 3.0) and ethanol (0 to 3.0).](image-url)
Figure 8
Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials

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Abstract

The effectiveness of alkali, acid and chlorite pretreatment of lignocellulosic feedstocks for improving the enzymatic saccharification of cellulose has been evaluated. The feedstocks such as Corncob, Prosopis juliflora and Lantana camara were pretreated with varied concentration of sulfuric acid, sodium hydroxide and sodium chlorite at 121 °C for 15–60 min. Among different methods used, chlorite pretreatment removed maximum lignin with ∼90% (w/w) residual holocellulose content in all the substrates tested. Moreover, irrespective of the substrates used, the chlorite treated substrates were enzymatically saccharified from 86.4% to 92.5% (w/w). While, the alkali treated substrates containing 66.0–76.0% (w/w) holocellulose could be enzymatically saccharified up to 55% (w/w). The acid pretreated substrates were found to contain almost 54–62% (w/w) holocellulose, which on enzymatic hydrolysis could result in 39.5–48% (w/w) saccharification.

1. Introduction

Lignocelluloses, the most abundant biomass available on earth, have attracted considerable attention as an alternative feedstock for the production of various value added products due to their renewable nature and low cost availability (Kuhad & Singh, 2007). Various technological developments have improved the bioconversion of these substrates into bioethanol (Kapoor, Chandel, Kuhar, Gupta, & Kuhad, 2007). Enzymatic saccharification is one of the promising strategies to convert cellulosic biomass into sugars because of low energy requirement and less pollution. However, the primary challenge in enzymatic hydrolysis of cellulose is its low accessibility due to association with lignin. Therefore, efficient pretreatment of lignocellulosic substrates has become a pre-requisite to improve enzymatic saccharification (Zhao, Zhang, & Liu, 2008). The main focus of different pretreatment methods is to remove the lignin content and to decrease the cellulose crystallinity (Mosier et al., 2005). Although various physical (communion, hydrothermolysis), chemical (acid, alkali, solvents, ozone), and biological pretreatment methods have been investigated over the years (Gupta, Mehta, Khasa, & Kuhad, 2010; Kuhar, Nair, & Kuhad, 2008; Kumar, Barrett, Delwiche, & Stroeve, 2009), thermo-chemical pretreatment of biomass has been a pretreatment of choice to enhance substrate accessibility for efficient enzymatic hydrolysis (Himmel et al., 2007).

The thermo-chemical pretreatment strategies such as acid, alkali and oxidation are commonly used for lignocellulosic biomass. The dilute mineral acids have been reported to remove the hemicellulosic fraction from substrates to improve enzymatic saccharification of cellulose (Gupta, Sharma, & Kuhad, 2009; Schell, Farmer, Newman, & McMillan, 2003). It has dual advantage of solubilizing hemicellulose and subsequently converting it into fermentable sugars. Whereas, the alkali pretreatment removes lignin and various uronic acid substitutions responsible for inhibiting the cellulose accessibility for enzymatic saccharification (Chang & Holtzapple, 2000). Moreover, alkali treatment is also reported to increase the biodegradability of the cell walls due to cleavage of the lignin bonds with hemicellulose and cellulose (Spencer & Akin, 1980). In contrast to acid and alkali treatments, sodium chlorite, a powerful oxidizing agent has been used frequently to delignify wood for cellulose isolation (Sun, Sun, Zhao, & Sin, 2004). The chlorine dioxide produced in this pretreatment method oxidizes lignin to the phenolic compounds and in turn makes cellulose accessible.

Since there is no universal and economically viable pretreatment method available, which could be used to pretreat varied cellulosic biomass, in the present study, it has been attempted to evaluate the suitably used three pretreatment methods (acid, alkali and chlorite treatment) for lignocellulosic feedstocks viz., Prosopis juliflora (PJ, a woody biomass), Lantana camara (LC, a shrub and...
weed) and Corncob (CC, agricultural residue). The pretreatments of the substrates were attempted at varied chemical dosage and pretreatment time. The pretreated plant materials were enzymatically hydrolysed and the cellulose saccharification efficiency was determined to evaluate the efficacy of these methods. Irrespective of the substrates used, the chlorite treatment was found to be an efficient method for delignification and producing cellulose rich plant material, which was almost 90% hydrolysable by cellulases into glucose.

2. Materials and methods

2.1. Raw materials

The lignocellulosic substrates: Corncob (CC), *P. juliflora* (PJ) and *L. camara* (LC) were collected locally, dried in sunlight and then cut into small pieces. The dried material was ground and passed through a 40–60 mesh size screen using a laboratory knife mill (Metrex Scientific Instrumentation, Delhi, India). The processed substrate was thoroughly washed, dried at 60 °C and stored in sealed plastic bags at room temperature.

2.2. Pretreatments

2.2.1. Acid pretreatment

The dilute sulfuric acid pretreatment of lignocellulosic substrate (100.0 g) was carried out using varied acid concentration (1–5% (w/v)) and incubation time (15–60 min) at 121 °C. The hydrolysates after treatment were separated by filtering the contents through double-layered muslin cloth. The residual biomass was washed with tap water till neutral pH and dried in a hot air oven at 60 °C.

2.2.2. Alkali pretreatment

The substrate (100.0 g) was presoaked in different concentrations of alkali (NaOH) ranging from 1% to 5% (w/v) for 2 h and thereafter thermally pretreated at 121 °C for 15, 30, 45 and 60 min. The pretreated material was filtered through double-layered muslin cloth, washed extensively with tap water until neutral pH and dried at 60 °C.

2.2.3. Chlorite pretreatment

The lignocellulosic substrate (100.0 g) was treated with different concentrations of sodium chlorite (1–5% (w/v)) at 121 °C for 15, 30, 45 and 60 min. The pretreated material was filtered through double-layered muslin cloth, washed extensively with tap water till neutral pH and dried in a hot air oven at 60 °C.

3. Enzymatic saccharification of pretreated substrates

Cellulase from *Trichoderma reesei* (ATCC 26921) with an activity of 6.5 FPU/g, supplemented with β-glucosidase from *Aspergillus niger* (Novozyme 188) having 250 U/g was used for saccharifying the cellulosic material obtained after each pretreatment type.

Enzymatic hydrolysis of each type of pretreated plant materials (10.0 g each) was carried out with cellulase (3 FPU/ml) and β-glucosidase (8 U/ml). Tween 80 (1% (v/v)) was also added to the reaction mixture and the reaction continued up to 36 h. Samples of enzymatic hydrolysate were withdrawn at regular intervals and analysed for amount of glucose released.

4. Analytical methods

The chemical composition (α-cellulose, klon lignin, pentosans, moisture and ash) of all the three substrates and their residual solid fraction post pretreatment were determined following standard TAPPI (1992) protocols. The reducing sugars released were estimated using the DNS method (Miller, 1959) and the yield of reducing sugars in enzymatic hydrolysate (YRSEH) was calculated as follows:

\[
\text{YRSEH} = \frac{\text{Sugars released in enzymatic hydrolysate}}{\text{Total carbohydrate content in pretreated substrates}} \times 100
\]

where \(M_i\) is the amount of component in the untreated substrate and \(M_f\) is the amount of the component in the substrate after pretreatment.

5. Statistical analysis

All the experiments were performed in triplicate and the results are presented as mean ± standard deviation.

6. Results and discussion

6.1. Compositional analysis of different lignocellulosic substrates

The chemical composition analysis of different lignocellulosic biomass revealed that the holocellulose content was in the range of 61.1–71.6% (w/w), where *CC* contained maximum cellulosic material (47.5 ± 3.27%) followed by *PJ* (44.1 ± 1.72%) and *CC* (37.4 ± 4.18%). The lignin contents observed in *CC* contained maximum cellulosic material (47.5 ± 3.27%) followed by *CC* (44.1 ± 1.72%) and *PJ* (34.2 ± 4.1%). The lignocellulosic substrates had almost equal pentosans, moisture and ash content in all the three substrates and their proximate chemical composition analysis of Corncob, *P. juliflora* and *L. camara* using TAPPI (1992) protocols are presented as mean ± standard deviation.

Table 1

<table>
<thead>
<tr>
<th>Component (% (w/w))</th>
<th>Corncob</th>
<th><em>P. juliflora</em></th>
<th><em>L. camara</em></th>
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<tr>
<td>Cellulose</td>
<td>37.4 ± 4.18</td>
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<td>Pentosans</td>
<td>34.2 ± 1.02</td>
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<td>Holocellulose</td>
<td>71.6 ± 3.21</td>
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<td>Klason lignin</td>
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<td>Moisture</td>
<td>7.4 ± 0.42</td>
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<td>Ash</td>
<td>1.8 ± 0.17</td>
<td>2.0 ± 0.12</td>
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</table>

6.2. Effect of chemical pretreatment

6.2.1. Acid treatment

The dilute acid treatment is the most commonly used method to pretreat the lignocellulosic biomass to hydrolyse hemicellulosic
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Acid (%) (v/v)</th>
<th>Corncob RL (% (w/w))</th>
<th>RH (% (w/w))</th>
<th>Hemicellulose removal (%) (w/w)</th>
<th>P. juliflora RL (% (w/w))</th>
<th>RH (% (w/w))</th>
<th>Hemicellulose removal (%) (w/w)</th>
<th>L. camara RL (% (w/w))</th>
<th>RH (% (w/w))</th>
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<td>25.7 ± 1.1</td>
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<td>61.5 ± 4.0</td>
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<td>84.7 ± 2.1</td>
<td>37.9 ± 3.4</td>
<td>54.1 ± 3.5</td>
<td>90.9 ± 2.5</td>
</tr>
</tbody>
</table>

RL, residual lignin; RH, residual holocellulose.

![Graph A](image1.png) ![Graph B](image2.png) ![Graph C](image3.png)

**Fig. 1.** Lignin removal from Corncob (A), *P. juliflora* (B) and *L. camara* (C) at different concentrations of alkali (1% NaOH, 2% NaOH, 3% NaOH, 4% NaOH and 5% NaOH).
fraction of lignocellulosic substrates, which is a critical parameter for process efficacy (Ishizawa, Davis, Schell, & Johnson, 2007). The hemicellulose fraction in CC was optimally hydrolysed to 82.2 ± 3.5% (w/w) with 2.0% (v/v) H2SO4 for 30 min. While, the optimum acid saccharification in PJ (91.2 ± 1.1% (w/w)) and LC (92.6 ± 2.9% (w/w)) was achieved with 3.0% (v/v) sulphuric acid for 45 min (Table 2). The higher acid concentration and pretreatment time requirement for optimal hydrolysis of PJ and LC may be attributed to the woody nature of these substrates. Irrespective of the substrates, increase in the acid dosage or pretreatment time beyond optimal conditions resulted in decrease of sugar yield, which may be because of the formation of sugar degradation products such as furfurals and hydroxymethyl furfurals (Chandel, Kapoor, Sigh, & Kuhad, 2007; Gupta et al., 2009). However, the acid hydrolysed CC contained 62.4 ± 2.6% (w/w) holocellulose and 27.3 ± 1.5% (w/w) lignin, while the acid treated PJ and LC were found to have 59.7 ± 4.1% (w/w) and 54.1 ± 3.4% (w/w) holocellulose with 35.9 ± 0.6% (w/w) and 38.0 ± 2.6% (w/w) lignin, respectively (Table 2). As compared to untreated substrates (control), the higher residual lignin and lower residual holocellulose in the pretreated substrates may be due to the removal of acid soluble carbohydrate fraction (hemicellulose). Our results are well in accordance with the previous reports. Chen, Zhao, and Xia (2009) reported an increase in lignin content from 19.3% to 28.4% (w/w) in acid hydrolysed corn stover. While, an increase in lignin content from 21.8% to 28.5% (w/w) was observed when switch grass was hydrolysed with 1.2% sulphuric acid at 160 °C for 20 min (Li et al., 2010).

### 6.2.2. Alkali treatment

During the alkali pretreatment, an increase in alkali concentration up to 5.0% (w/v) caused a regular increase in removal of lignin and in turn holocellulose gain in PJ and LC, while a 4.0% (w/v) alkali concentration was found to be optimum for CC (Figs. 1 and 2). In all three substrates, the lignin removal increased with increase in pretreatment time till 30 min and remained almost constant thereafter. Irrespective of the substrates, lignin content was observed to be reduced in a range of 28–36% (w/w) (Fig. 1) with a concomitant enrichment in holocellulose content (5.2–7.1% (w/w)) (Fig. 2). The residual lignin in alkali treated CC, PJ and LC were 12.4%, 20.6% and 19.4% (w/w), while the holocellulose content were 75.8%, 71.3% and 65.8% (w/w), respectively. The increased lignin removal and enrichment of holocellulose in alkali treated substrates may be due to the cleavage of the ester bonds between hydroxycinnamic acids, and the α-benzyl ether linkages of the cell wall of plant materials by alkali (Mosier et al., 2005; Silverstein, Chen, Sharma-Shivappa, Boyette, & Osborne, 2007; Zhao et al., 2008). Similarly, other studies on alkali pretreatment have also reported approximately 40–60% delignification in crofton weed stem (Zhao et al., 2008), rice straw (Jeya, Zhang, Kim, & Lee, 2009) and switch grass (Nlewem & Thrash, 2010).
Table 3
Enzymatic hydrolysis of pretreated (under optimized conditions) lignocellulosic substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pretreatment type</th>
<th>Reagent concentration</th>
<th>Pretreatment time (min)</th>
<th>RL (% (w/w))</th>
<th>RH (% (w/w))</th>
<th>YRSEH (% (w/w))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corncob</td>
<td>Untreated</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>38.5 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Alkali treatment</td>
<td>4% (w/v)</td>
<td>30</td>
<td>12.4 ± 1.0</td>
<td>75.8 ± 4.8</td>
<td>55.4 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>Chlorite treatment</td>
<td>4% (w/v)</td>
<td>30</td>
<td>5.4 ± 0.5</td>
<td>90.3 ± 6.7</td>
<td>91.5 ± 3.1</td>
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<td>Acid treatment</td>
<td>2% (v/v)</td>
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<td>27.3 ± 1.5</td>
<td>62.4 ± 2.6</td>
<td>39.5 ± 4.2</td>
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<tr>
<td>P. juliflora</td>
<td>Untreated</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>33.2 ± 2.1</td>
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<td></td>
<td>Alkali treatment</td>
<td>5% (w/v)</td>
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<td>Chlorite treatment</td>
<td>4% (w/v)</td>
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<td>Acid treatment</td>
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<td>L. camara</td>
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<td>–</td>
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<td>31.2 ± 0.9</td>
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<td>48.0 ± 3.4</td>
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</table>

RL, residual lignin; RH, residual holocellulose; YRSEH, yield of reducing sugars in enzymatic hydrolysate.

6.2.3. Chlorite treatment
All the three substrates when pretreated with 4% (w/v) sodium chlorite for 30 min, maximum lignin removal as well as gain in holocellulose content were observed. Thereafter, any further increase, either in the sodium chlorite concentration or pretreatment time did not cause any significant improvement in delignification (Fig. 3). Moreover, irrespective of the substrates tested, approximately 80–90% (w/w) delignification was obtained when pretreated under optimal conditions (Fig. 3). The pretreated substrates were found to have an increase in their holocellulose content by 26.2 ± 0.8%, 37.2 ± 5.2% and 47.3 ± 2.6% (w/w) in CC, PJ and LC, respectively, as compared to control, with almost 90% (w/w) holocellulose recovery (Fig. 4). Earlier reports with sodium chlorite treatment for the preparation of cellulose rich residue also showed similar trends (Sevenson, Cheng, Jameel, & Kadla, 2005; Sun et al., 2004; Wi, Kim, Mahadevan, Yang, & Bae, 2009). The extensive delignification of lignocellulosic substrates with chlorite treatment was due to the generation of chlorine dioxide (ClO2).
Fig. 4. Holocellulose enrichment in Corncob (A), P. juliflora (B) and L. camara (C), after treatment with different concentrations of Na-chlorite ( ■ 1% Na-chlorite, □ 2% Na-chlorite, △ 3% Na-chlorite, ▼ 4% Na-chlorite and ▼ ▼ ▼ 5% Na-chlorite).

an oxidation product of chlorous acid (HClO₂) and hypochlorous acids (HOCl) produced during the thermal degradation of sodium chlorite, which degraded the lignin either by side-chain displacement or hydroxylation/de-alkylation reaction (Hamzeh, Mortha, & Lachenal, 2006).

6.3. Comparison of different pretreatments for improving enzymatic saccharification of cellulosic substrates

The enzymatic saccharification of all the three pretreated substrates showed an improved conversion of cellulose to glucose because of lignin and/or hemicellulose removal during pretreatments. The acid pretreated samples with minimum lignin removal showed lowest enzymatic hydrolysis (39.5–48.0% (w/w)), while, the alkali and sodium chlorite pretreated substrates caused higher enzymatic saccharification, which could be because of comparatively lower lignin content in the pretreated substrates (Table 3). Similar observations on pretreatment of lignocellulosic substrates followed by enzymatic hydrolysis have also been reported by Chen et al. (2009), Gupta et al. (2009) and Kuhad et al. (2010). The limited enzymatic saccharification in presence of higher lignin may be due to the high affinity of cellulases towards lignin, which resulted in unavailability of cellulase to cellulose moieties and led to poor enzymatic saccharification yields (Yang & Wyman, 2004).

Among different pretreated substrates evaluated here for the enzymatic hydrolysis, the chlorite pretreated substrates were observed to be more vulnerable to the enzymatic hydrolysis and resulted in maximum saccharification efficiency i.e., from 86.4% to 92.5% (w/w) (Table 3). The higher enzymatic saccharification in chlorite pretreated substrates may be attributed to the presence of higher holocellulose content (~90.0% (w/w)) with minimum amount of residual lignin (3.1–6.5% (w/w)). It has been shown by several workers that the delignification treatments not only remove the lignin but also act as a swelling agent, which in turn enhances the surface area of the substrate and make the substrate more amenable for enzymatic action (Gupta et al., 2009; Kuhad, Manchanda, & Singh, 1999; Kuhad et al., 2010). In contrast, the alkali treated substrates when subjected to enzymatic saccharification brought about approximately 51–55% (w/w) substrate hydrolysis (Table 3). As compared to chlorite pretreated substrates, the lower enzymatic saccharification efficiency in alkali treated substrates might be due to the lower delignification of the substrates by alkali treatment, which could be explained as the resistance of lignin removal due to strong lignin–cellulose attraction in the cell wall (Zhao et al., 2008). Our results of higher enzymatic saccharification with chlorite pretreated substrates strongly agree with the previous reports of achieving more than 65–67% saccharification in chlorite pretreated sugarcane bagasse (Adsul et al., 2005) and approximately 70% (w/w) hydrolysis from chlorite pretreated seaweed Ceylon moss (Gelidium amansii) (Wi et al., 2009).
7. Conclusion

Among different chemical pretreatment studies, the sodium chlorite pretreatment was found to be the most effective in lignin removal and led to the enrichment of the holocellulose content in treated substrates. This method offers the possibility of producing cellulosic material largely free from lignin, which eventually would be a good substrate for bioethanol production. However, there is a need to develop efficient biological delignification methods to make the process environmentally safe.

Acknowledgement

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TAPP (1992), Technical Association of Pulp and Paper Industry, Atlanta, GA, USA.


Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics

Rishi Gupta · Girija Mehta · Yogender Pal Khasa · Ramesh Chander Kuhad

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Abstract The biological delignification of lignocellulosic feedstocks, Prosopis juliflora and Lantana camara was carried out with Pycnoporus cinnabarinus, a white rot fungus, at different scales under solid-state fermentation (SSF) and the fungal treated substrates were evaluated for their acid and enzymatic saccharification. The fungal fermentation at 10.0 g substrate level optimally delignified the P. juliflora by 11.89% and L. camara by 8.36%, and enriched their holocellulose content by 3.32 and 4.87%, respectively, after 15 days. The fungal delignification when scaled up from 10.0 g to 75.0, 200.0 and 500.0 g substrate level, the fungus degraded about 7.69–10.08% lignin in P. juliflora and 6.89–7.31% in L. camara, and eventually enhanced the holocellulose content by 2.90–3.97 and 4.25–4.61%, respectively. Furthermore, when the fungal fermented L. camara and P. juliflora was hydrolysed with dilute sulphuric acid, the sugar release was increased by 21.4–42.4% and the phenolics content in hydrolysate was decreased by 18.46 and 19.88%, as compared to the unfermented substrate acid hydrolysis, respectively. The reduction of phenolics in acid hydrolysates of fungal treated substrates decreased the amount of detoxifying material (activated charcoal) by 25.0–33.0% as compared to the amount required to reduce almost the same level of phenolics from unfermented substrate hydrolysates. Moreover, an increment of 21.1–25.1% sugar release was obtained when fungal treated substrates were enzymatically hydrolysed as compared to the hydrolysis of unfermented substrates. This study clearly shows that fungal delignification holds potential in utilizing plant residues for the production of sugars and biofuels.

Keywords Bioethanol · Lignocellulose · Fungal delignification · Solid-state fermentation · Pycnoporus cinnabarinus · Saccharification

Introduction

Lignocellulosic biomass is a potential source of carbohydrate polymers for fermentation but the conversion of its structural polysaccharides into simple sugars is highly problematic due to the recalcitrancy of lignin. Lignin acts as a cementing material, which together with hemicelluloses forms an amorphous matrix in which the cellulosics fibrils are embedded and protected against chemical or enzymatic degradation (Kuhad et al. 1997; Himmel et al. 2007). Therefore, removal of lignin has become a prerequisite for the efficient utilization of carbohydrate from lignocellulosics.
Pretreatment of lignocellulosics is the most important step required to remove lignin and disrupt the crystallinity of carbohydrate fraction without losing too much of the structural sugars (Mosier et al. 2005; Wyman 2007; Kumar et al. 2009). Physicochemical pretreatments such as acid treatment (Gupta et al. 2009), alkali treatment (Carrillo et al. 2005), steam explosion (Öhgren et al. 2006) and ammonia fiber explosion (Teymouri et al. 2005) are well recognized for enhancing the conversion of cellulosic biomass into monomeric sugars. However, these pretreatment methods require high energy and often generate toxic compounds, which makes the process economically unviable and environment unfriendly (Teymouri et al. 2005; Silverstein et al. 2007). Therefore, environmentally benign pretreatment methods are required to reduce release of pollutants, costs and improve cellulose saccharification.

The biological pretreatments of plant residues to improve the accessibility of cellulosic fraction have been attracting the extensive interest of researchers (Taniguchi et al. 2005; Zhang et al. 2007a; Yu et al. 2009b). The potential of biological pretreatments has been explained by the ability of certain microbes to disrupt the plant cell wall by partial breakdown of the lignin/carbohydrate complex (Keller et al. 2003). The most promising microorganisms for biological pretreatment are basidomycetes, and among them the selective lignin degrading white rot fungi holds immense importance (Akhtar et al. 1997; Kuhad et al. 1997; Kuhad and Singh 2007; Yu et al. 2009a). Pycnoporus cinnabarinus, a selective lignin degrading white rot fungus, has been reported to produce laccase, degrade lignin and to transform lignin derived compounds (Eggert et al. 1996; Falconiner et al. 1994; Galhaup et al. 2002). Recently, P. cinnabarinus, Phanerochaete chrysorium and Crinepellis sp. RCK-1 have been tested for their lignin degradation and improvement in acid hydrolysis of the delignified substrates (Kuhar et al. 2008). However, the application of P. cinnabarinus for delignifying the lignocellulosic materials and subsequently its effect on enzymatic hydrolysis of the fermented substrate (mycosubstrate) has scarcely been studied.

In the present study, biological delignification of two different weeds (lignocellulosic substrate) viz. Lantana camara and Prosopis juliflora using P. cinnabarinus was attempted under solid-state fermentation (SSF) conditions with pretreatment scalability up to 500.0 g substrate level. The pretreated substrates were further evaluated for acid as well as enzymatic hydrolysis. The acid and enzymatic hydrolysis of fungal fermented material resulted in significant increase in the release of sugars as compared to unfermented ones. Interestingly, the acid hydrolysates of fungal fermented substrates were found to have lesser amount of toxic compounds as compared to hydrolysate from unfermented material.

Materials and methods

Raw material and chemicals

The woods of L. camara and P. juliflora were collected locally and processed through a combination of chipping and milling to attain a particle size of 1–2 mm using a laboratory knife mill (Metrex Scientific Instrumentation, Delhi, India). The grounded materials washed thoroughly with water and dried overnight at 60°C were used throughout the study.

Cellulase from Trichoderma reesei (ATCC 26921), β-glucosidase (Novozyme 188) from Aspergillus niger, and 3,5-dinitrosalicylic acid (DNS) were brought from Sigma, St. Louis, Missouri, USA, while, all other chemicals were purchased locally.

Microorganism and inoculum preparation

Pycnoporus cinnabarinus ATCC 2004378, a kind gift from Late Dr. K. E. L. Eriksson, Professor Emeritus, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, USA, was grown at 30°C on malt extract agar (MEA, Dhawan and Kuhad 2003). The medium contained (g l−1): malt extract 20.0, KH₂PO₄ 0.5, MgSO₄ 0.5, Ca(NO₃)₂ 0.5 and agar 20.0 (pH 5.5), while the stock cultures were maintained on MEA slants at 4°C with periodic transfer.

The fungal cultures were grown statically at 30°C in 250 ml Erlenmeyer flasks with 50 ml of malt extract broth (MEB, Dhawan and Kuhad 2003), which were inoculated with 2 fungal discs (8 mm diameter each) from a 6 day old colony grown on MEA. The cultures were harvested and the aseptically collected fungal mat was homogenized and transferred into a fresh 50 ml MEB in 250 ml Erlenmeyer flasks. They were grown at 30°C and 150 rpm in a rotatory incubator shaker (Innova-40, New Brunswick Scientific, USA) for...
another 6 days to develop the inoculum for solid state fermentation of lignocellulosic materials.

Fungal treatment of *L. camara* and *P. juliflora* under SSF

The pretreatment was carried out in 500 ml Erlenmeyer flasks with 10.0 g of the oven dried lignocellulosic substrate (*L. camara* or *P. juliflora*), moistened with mineral salt solution (MSS) containing (g l\(^{-1}\)):\n\[\text{KH}_2\text{PO}_4 0.5, \text{MgSO}_4 0.5, \text{Ca(NO}_3)_2 0.5\text{ and pH 5.5 to obtain a substrate to moisture ratio of 1:2.5 and autoclaved (121°C for 30 min). Each flask was then inoculated with 7.5 mg fungal mass (dry weight) per gram dry substrate (gds) and incubated at 30°C for 25 days. The solid state fungal fermentation of both the substrates was also studied at 75.0, 200.0 and 500.0 g substrate levels in enamel trays of different sizes: 21.5 cm × 16.5 cm × 5.0 cm, 31.5 cm × 26.5 cm 6.0 cm and 41.5 cm × 34.5 cm × 8.0 cm, respectively. The samples (mycosubstrate) were harvested periodically, washed thoroughly and dried overnight at 60°C. The dried mycosubstrates were then analysed for biochemical changes, acid and enzymatic saccharification. The flasks containing sterilized uninoculated substrates served as control.

Evaluation of delignified substrates for acid hydrolysis

The fungal pretreated and control (non-fungal treated) substrates were hydrolyzed at 10% (w/v) substrate consistency with dilute sulfuric acid (3.0%, v/v) at 120°C for 45 min (Gupta et al. 2009). The hydrolysate was vacuum filtered and analyzed for reducing sugars and total phenolics. The residual substrate after hydrolysis was washed thoroughly to a neutral pH, dried at 60°C in a hot air oven till constant weight was achieved.

Detoxification of acid hydrolysates

Acid hydrolysates of unfermented and fungal treated substrates were detoxified by adding varied dosages of activated charcoal (0–2.5%, w/v) under constant stirring at room temperature for 30 min. After incubation, the hydrolysate was vacuum filtered and analyzed for total sugars and phenolics.

Enzymatic hydrolysis of fermented and non-fermented plant residues

Enzymatic hydrolysis of fermented and control (non-fermented) substrate samples was carried out at 5.0% (w/v) substrate consistency in 50 mM citrate phosphate buffer (pH 5.0). Prior to the enzyme loading, the slurry was incubated at 50°C for 2 h at 150 rpm. Thereafter, cellulase (24 FPU gds\(^{-1}\)), \(\beta\)-glucosidase (120 U gds\(^{-1}\)) and 1.0% (v/v) Tween 80 were added to the preincubated substrate suspension and the saccharification was carried out at 50°C and 150 rpm for 48 h. Samples were withdrawn after every 6 h, centrifuged at 10,000 rpm for 15 min and the supernatants were analysed for total reducing sugars.

Analytical methods

The total phenolics present in hydrolysates were determined by the method of Singleton et al. (1999) using Vanillin as standard, while, the reducing sugars were estimated by DNS method (Miller 1959). The plant material was extracted with alcohol-benzene (1:2 v/v) to remove wax and resins etc. The extractive free, oven dried plant material was processed for biochemical analysis following the TAPPI (1992) protocols. The holocellulose to lignin ratio (H/L) was calculated as follows:
\[
\frac{\text{Amount of holocellulose (H) present in the substrate}}{\text{Amount of lignin (L) present in the substrate}} \times 100
\]

The percent loss and gain of different components such as total organic matter (TOM) loss, lignin and holocellulose in fungal treated substrates were calculated using Eqs. 1 and 2, respectively.
\[
\text{Loss(\%)} = \frac{M_i - M_f}{M_i} \times 100 \tag{1}
\]
\[
\text{Gain(\%)} = \frac{M_f - M_i}{M_i} \times 100 \tag{2}
\]
where \(M_i\) is the amount of component in the control (non-fermented) substrate and \(M_f\) is the amount of the component left in the mycosubstrate.
All the experiments and the analysis were carried out in triplicates and the data presented is the mean.
value of the triplicates. The standard deviation was calculated using the mean values and remained within the range of ±10.0%.

Results and discussion

Solid state fermentation (SSF) of lignocellulosic substrates

Irrespective of the substrates used, the increase in loss of total organic matter (TOM) correlated positively with the increase in lignin degradation, and holocellulose to lignin (H/L) ratio. While, the holocellulose content increased till 15 days of fungal fermentation and marginally declined thereafter (Table 2). The SSF of *L. camara* and *P. juliflora* by *P. cinnabarinus* after 25 days caused 18.87 and 15.40% loss of TOM, respectively (Table 1). The loss in TOM may be attributed to the CO₂ evolution due to the metabolic activities of fungus on the substrates, however, the weight loss in substrates includes the loss of lignin and holocellulose. The fungus degraded more amount of lignin in *P. juliflora* (13.13%) than in *L. camara* (8.87%). The fungal delignification in both the substrates was higher during the first 15 days, and thereafter no significant improvement in lignin degradation was observed (Table 1). Our results are in accordance with the earlier reports on biological delignification of lignocellulosic materials with white rot fungi (Bustamente et al. 1999; Taniguchi et al. 2005; Meza et al. 2006). *P. cinnabarinus* was reported to degrade 8.5% lignin in sugarcane bagasse (Meza et al. 2006), whereas, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* degraded 9.0 and 17.0% lignin in depithed sugarcane bagasse, respectively (Bustamente et al. 1999). The fungal cultures have been observed to degrade more lignin in wheat straw than in woody material and this low ability of lignin degradation in woody substrate by the fungi could be attributed to the difference in the lignin properties of wood and straw (Taniguchi et al. 2005). Thus lignin structure could be seen as a detrimental factor for economic exploitation of plant residues.

Besides delignification, the availability of carbohydrates is also an important criterion for evaluating the biological pretreatment performance because the higher cellulose content in fermented substrates eventually provides higher accessibility of

<table>
<thead>
<tr>
<th>Components</th>
<th>Substrates</th>
<th>Time (days)</th>
<th>Control</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>Control</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td><em>L. camara</em></td>
<td>10.0</td>
<td>8.91 (10.87)</td>
<td>8.67 (13.27)</td>
<td>8.47 (15.33)</td>
<td>8.27 (16.27)</td>
<td>8.11 (18.87)</td>
<td>8.01 (19.87)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klason-Lignin (%)</td>
<td>33.18</td>
<td>31.64 (1.87)</td>
<td>30.91 (6.85)</td>
<td>30.41 (8.65)</td>
<td>30.28 (8.74)</td>
<td>30.12 (9.45)</td>
<td>29.94 (10.65)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holocellulose (%)</td>
<td>59.82</td>
<td>60.94 (1.34)</td>
<td>60.70 (4.87)</td>
<td>60.41 (6.85)</td>
<td>60.21 (8.65)</td>
<td>60.01 (9.45)</td>
<td>58.84 (10.65)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H/L</td>
<td>1.80</td>
<td>1.69 (0.99)</td>
<td>1.67 (3.14)</td>
<td>1.65 (5.31)</td>
<td>1.63 (7.48)</td>
<td>1.61 (9.56)</td>
<td>1.59 (11.65)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Percentage loss in fungal treated substrates with respect to control (untreated) substrates
*Percentage gain in fungal treated substrates with respect to control (untreated) substrates

Table 1 Analysis of compositional changes during the fungal treatment of *L. camara* and *P. juliflora* using *P. cinnabarinus* at different time intervals (5-25 days)
carbohydrates for acid or enzymatic saccharification (Shi et al. 2008). There are several reports where P. chrysosporium, the most widely studied white rot fungus for lignin degradation, resulted in higher amounts of lignin degradation but the fungus was also found to cause a significant loss in carbohydrate fraction (Taniguchi et al. 2005; Kuhar et al. 2008). However, our results showed an increase in holocellulose content in both the fungal fermented substrates by 3.32 and 4.87% compared to the control (unfermented) substrates after 15 days and thereafter it declined gradually (Table 1). The fungal delignification also increased the H/L ratio by 14.62 and 17.28% in L. camara and P. juliflora, respectively after 15 days (Table 1). The increase in H/L ratio may be due to the reason that the fungus produces a fairly good amount of laccase with very low activity of carbohydrate hydrolyzing enzymes (Alves et al. 2004), which might have resulted in higher degradation of lignin as compared to holocellulose in the P. cinnabarinus treated substrates. Similar results were also obtained during the biological pretreatment of wheat straw using Streptomyces cyaneus, which caused 16 and 8% degradation of lignin and holocellulose, respectively, after 15 days of SSF and eventually enhanced the H/L ratio of the fermented wheat straw (Berrocal et al. 2000).

Table 2: Compositional changes during the scale up of biological delignification of L. camara and P. juliflora using P. cinnabarinus at different levels (75.0, 200.0 and 500.0 g).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substrates (g)</th>
<th>L. camara</th>
<th>P. juliflora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>Weight (%)</td>
<td>61.94 (17.41)</td>
<td>167.04 (16.48)</td>
<td>424.05 (15.19)</td>
</tr>
<tr>
<td>Klason-Lignin (%)</td>
<td>30.75 (7.31)</td>
<td>30.89 (6.89)</td>
<td>30.85 (7.01)</td>
</tr>
<tr>
<td>Holocellulose (%)</td>
<td>62.58 (4.61)</td>
<td>62.36 (4.25)</td>
<td>62.45 (4.39)</td>
</tr>
<tr>
<td>H/L</td>
<td>2.03 (13.04)</td>
<td>2.02 (12.14)</td>
<td>2.03 (12.44)</td>
</tr>
</tbody>
</table>

| a | Percentage loss in fungal treated substrates with respect to control (untreated) substrates |
| b | Percentage gain in fungal treated substrates with respect to control (untreated) substrates |

The fungal delignification of lignocellulosic substrates was scaled up under similar conditions such as particle size, substrate to moisture ratio, pH, temperature, tray volume to substrate ratio. The study revealed that irrespective of the scale of treatment, the lignin degradation was found to be comparatively more or less same. The P. cinnabarinus when grown on L. camara and P. juliflora at larger scales (75.0, 200.0 and 500.0 g) caused 15.19–17.41 and 12.05–15.56% weight loss, respectively. Moreover, the fungal pretreatment of L. camara and P. juliflora removed lignin by 6.89–7.31 and 7.69–10.08% and also enhanced the holocellulose content by 4.25–4.61 and 2.9–3.97%, respectively, after 15 days of incubation. Furthermore, the H/L ratio of fermented L. camara and P. juliflora remained in the range of 2.02–2.03 and 2.31–2.39%, respectively (Table 2). The insignificant difference in H/L ratios and compositional changes in the fermented substrates at different scale experiments demonstrated the possibility to further scale up of the process.

Evaluation of biologically delignified substrates for acid saccharification

Acid hydrolysis of fungal fermented and the control (unfermented) substrates was evaluated. The acid hydrolysis of fungal fermented L. camara and P. juliflora resulted in 131.4 and 189.7 mg gds⁻¹ sugar yield as compared to sugar release of 108.2 and 133.2 mg sugar gds⁻¹ from control (unfermented) substrates, respectively (Fig. 1). The acid saccharification of fungal fermented materials has significantly improved in sugar release (21.4–42.4%) as compared to the unfermented substrates. The increased sugar release in fungal treated substrates might be because of the preferential lignin degradation ability in the biomass.
substrates by the fungus *P. cinnabarinus*, which in turn would have resulted in enrichment of carbohydrate content in the fermented substrates. Similar results have been reported in fungal treated wheat straw by Kuhar et al. (2008).

Acid hydrolysis not only release the reducing sugars but also release some lignin degradation compounds (phenolics), which are toxic to the fermenting microorganisms (Palmqvist et al. 1999; Mussatto and Roberto 2004; Chandel et al. 2007). The acid hydrolysates obtained from fungal treated *L. camara* and *P. juliflora* were found to have 18.5–19.9% less phenolics as compared to the hydrolysates of unfermented substrates (Fig. 1). This reduction in phenolics can be attributed to the fungal degradation of phenolic compounds (Kuhar et al. 2008).

An attempt has been made to evaluate the effect of fungal pretreatment on reduction in the usage of chemicals in detoxification of acid hydrolysates. Interestingly, the activated charcoal even when used at a lower concentration (1 and 1.5% w/v, 25–33% lower than control) removed more than 90% phenolics from acid hydrolysates of fungal treated substrates as compared to the acid hydrolysates of unfermented ones (Table 3). Our observations clearly indicated that fungal fermentation (delignification) of lignocellulosic material resulted in holocellulosic materials more vulnerable to acid hydrolysis, which in turn gives hydrolysates rich in pentose sugars and lesser phenolics.

### Evaluation of biologically delignified substrates for enzymatic saccharification

In comparison to the unfermented substrates, the fungal fermented *L. camara* and *P. juliflora* when hydrolysed with cellulases for 48 h, an increase of 25.1 and 21.1% (w/w) saccharification with release of reducing sugars (389.1 and 402.1 mg gds⁻¹), respectively, was achieved (Fig. 2). The enhanced enzymatic saccharification of fungal fermented substrates may be attributed to the partial degradation of lignin seal responsible for preventing the penetration of cellulase molecules (Taniguchi et al. 2005). Moreover, the improvement in enzymatic hydrolysis could be because the biological treatment increases the initial adsorption of cellulases to the cellulose, which in turn enhances the sugars released during the enzymatic hydrolysis (Yu et al. 2009a).

It has been observed that fungal fermentation with *P. cinnabarinus* has considerable potential in improving enzymatic saccharification of cellulosics into sugar rich syrup. Taniguchi et al. (2005) have reported 330 mg gds⁻¹ sugar released from rice straw fermented with *P. ostreatus* after 60 days. Zhang et al. (2007b) when enzymatically hydrolysed

**Table 3** Detoxification of phenolics from the acid hydrolysates obtained from different substrates (untreated and fungal treated *L. camara* and *P. juliflora*) using varied dosage of activated charcoal

<table>
<thead>
<tr>
<th>Amount of charcoal used (% w/v)</th>
<th>L. camara</th>
<th>P. juliflora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>SSF-treated</td>
</tr>
<tr>
<td>0.00</td>
<td>24.00 (0.00)</td>
<td>19.23 (0.00)</td>
</tr>
<tr>
<td>0.50</td>
<td>18.69 (22.13)</td>
<td>14.20 (26.15)</td>
</tr>
<tr>
<td>1.00</td>
<td>12.51 (47.88)</td>
<td>6.04 (68.60)</td>
</tr>
<tr>
<td>1.50</td>
<td>4.67 (80.54)</td>
<td>0.95 (95.04)</td>
</tr>
<tr>
<td>2.00</td>
<td>0.09 (99.62)</td>
<td>0.08 (99.56)</td>
</tr>
<tr>
<td>2.50</td>
<td>0.03 (99.87)</td>
<td>0.04 (99.78)</td>
</tr>
</tbody>
</table>

*The values in parenthesis is the percentage amount of phenolics removed*
Trametes versicolor and Echinodontium taxodii fermented bamboo culms for 120 days, the sugar released was approximately 360 and 200 mg gds⁻¹, respectively. Recently Yu et al. (2009a) have also reported 255.75 and 70.4 mg gds⁻¹ sugar yield from E. taxodii fermented chinese willow and china fir after 120 days, as calculated from their data. Thus the sugars released in our study from both the fungal fermented substrates after 15 days was higher (389.1 and 402.1 mg gds⁻¹) than the above cited studies. Although it’s difficult to make exact comparison in studies where lignocellulosic substrates are different and moreover fermentation period is variable. But comparatively it seems that the fungus used in this study holds considerably more potentials.

Conclusion

The conventional non-biological pretreatment methods require high energy, often generate toxic substances inhibitory to fermentation, which makes the processes uneconomical and environmentally inimical. The results of this fungal fermentation based study indicate that in order to increase the acid or enzymatic saccharification of hemicellulose and cellulose, it is prerequisite to degrade the lignin in the plant material to be used for producing value added compounds. The fungal fermentation have led to significant improvement in acid hydrolysis of hemicellulosic content and enzymatic hydrolysis of cellulose content in L. camara and P. juliflora. Moreover, fungal fermentation of plant material has resulted into material, which on acid hydrolysis produces hydrolysates with lesser amount of toxic compounds. Thus, the fungal delignification if optimized can be used as a potential alternative pretreatment method for improving the enzymatic saccharification of lignocellulosic residues and eventually for economizing the ethanol production as biofuel.

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TAPPI (1992) Technical Association of Pulp and Paper Industry, Atlanta, GA, USA


Bioethanol production from pentose sugars: Current status and future prospects

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ABSTRACT

The utilization of hemicellulose, the second most abundant polysaccharide, is must for the cost-efficient production of ethanol from second generation feedstocks. Xylan, the major hemicellulose in plant biomass yields mainly xylose as pentose sugars on hydrolysis. The progress in fermentation of pentose sugars has gone on slow pace as there are few microorganisms known, which are capable of pentose metabolism. The future perhaps lies in finding organisms that would ferment high density hydrolysates without purification. This obviously has to use the genetic and metabolic engineering routes. Either a direct or a sequential fermentation system needs to be worked out. This review provides an overview of the current pentose bioconversion processes and future prospects for bioethanol production.

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Keywords:
Hemicellulose
Pentose sugars
Bioethanol, Fermentation

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1. Introduction

Utilization of lignocellulosic biomass for ethanol production is one of the most promising alternatives for liquid fuel, which has near zero greenhouse gas emission with great socio-economic benefits [1–4]. Lignocellulose is the most abundant, renewable organic material on the biosphere, but by virtue of its structural properties they are resistant to bioconversion [5–7]. For cost-effective production of bioethanol from lignocellulosic biomass, the high efficiency utilization of both carbohydrate fractions, i.e., cellulose and hemicellulose is required.

Bioethanol production from lignocellulosic biomass involves several steps such as pretreatment, hydrolysis of complex carbohydrates, fermentation, and distillation for product recovery [8,9]. Various pretreatment methods have been explored to enhance the accessibility of lignocellulosic substrates. Among them, dilute acid pretreatment is the method of choice which has been studied widely [9–13]. However, there has been a drawback of non-selectivity and formation of fermentation inhibitory compounds such as furfural, 5-hydroxymethylfurural and phenolics [9,14,15]. In order to remove these toxic compounds from hydrolysates, strategies such as overliming, charcoal treatment, steam stripping, ion-exchange resin treatment, and biological methods such as enzymatic and microbial detoxification have been employed [1,16–18].

A number of cellulytic microorganisms are known to produce cellulases and hemicellulases, which can convert cellulose and hemicellulose respectively into soluble monomeric or oligomeric sugars. These sugars further can be fermented into ethanol by a number of bacterial, yeast and filamentous fungi. The fermentation process would be economically viable only if both hexose and pentose sugars present in the hydrolysates are converted to ethanol. The ability to ferment pentoses is not widespread among microorganisms. The most promising yeast species identified so far are Candida shehatae, Pichia stipitis and Pachysolen tannophilus [19–22]. But, the use of these yeasts for ethanol production from xyllose at commercial level is limited mainly due to their slow fermentation rates, carefully regulated oxygen requirement, sensitivity to inhibitors and low ethanol tolerance [22]. Although significant improvements have been made in the microorganisms for the efficient fermentation of pentose sugars into ethanol, however, the bioconversion of pentoses to ethanol is still one of the major bottlenecks for ethanol commercialization effort. In this paper, an overview of bioconversion of pentose sugars into bioethanol using fermentation and molecular strategies for improvement of pentose-fermenting strains utilizing hemicellulosic sugars are discussed.

2. Hemicellulose

Among the different structural units of lignocelluloses, hemicellulose comprises almost 15–30% of the total dry weight and hence represents an important energy and material resource for bioconversion [6,23–25]. The hemicelluloses comprised both linear and branched hetero-polymers of D-xylene, D-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid (Fig. 1A). The xylose-rich hemicelluloses in both soft and hard wood are usually termed as xylan. The hemicellulose from hardwoods and agricultural residues are typically rich in xylan, while, softwood contains more mannan and less xylan [6,7,24,26].

2.1. Softwood hemicellulose

Galacto-mannans are the principal hemicelluloses in softwoods. Their backbone is a linear chain built up by 1,4-linked β-D-glucopyranose and β-D-mannopyranose units (Fig. 1B). The mannose and glucose units in the backbone are partially substituted at C-2 and C-3 positions by acetyl groups, approximately 1 per 3–4 hexose units [6]. Arabinogalacturonoxylan is another major hemicellulosic sugar and is composed of 1,4-linked-β-D-xylpyranose units. This chain is substituted at C-2 by 4-O-methyl-α-D-glucuronic acid group with approximately two such units per ten xylose units. The xylose backbone is also substituted by α-L-arabino-pyranose units, on the average 1.3 residue per ten xylose units [6,25]. Arabinogalactan is a minor component in both softwoods and hardwoods. The backbone of this galactan is built up by 1,3-linked α-D-galacto-pyranose units, and almost every galactose unit is substituted at C-6 position.

2.2. Hardwood hemicellulose

The 4-O-acetyl-4-α-methyl-glucurono-β-D-xylan (commonly known as galacturonoxylan) is the major component of hardwood hemicelluloses [6,27] (Fig. 1B). The xylan content varies between 15 and 30% in different hardwood species. The backbone of xylan consists of β-D-xylpyanoose units linked by 1,4-bonds, while seven of ten xylose units are substituted by acetyl group at C-2 or C-3 position and in one of ten xylose units, the 4-O-methyl-α-D-glucuronic acid residue unit is linked at C-1, 2 positions to the hemicellulose backbone [6,25,28]. Gluco-mannan is another hemicellulose in hardwood (Fig. 1B), comprises 2–5% of the wood and is composed of β-D-glucopyranose and β-D-mannopyranose units by 1,4-bonds. Depending on the wood species, the glucose:manann ratio varies between 1:1 and 1:2 [6,24,25,29].

3. Pentose-fermenting microorganisms

During the last more than three decades, several laboratories around the world have examined the utilization of pentose sugars by different bacteria, fungi and yeasts for production of acids, alcohols and other fermentation products under cultivation conditions [30].

3.1. Bacteria

Most of the fungi cannot ferment pentoses anaerobically, while many bacteria can readily convert xylene to various products under anaerobic fermentation. The common pentose fermenting bacteria include Bacillus macerans, Bacillus polymyxa, Klebsiella pneumoniae, Clostridium acetobutylicum, Aeromonas hydrophila, Aerobacter sp., Erwinia sp., Escherichia sp., Leuconostoc sp., Lactobacillus sp., Thermoanaerobacterium saccharolyticum, and Zymomonas mobilis [22,31]. Among bacteria, thermophiles may be the best suited for the production of alcohol, polyols and ketones due to decreased cooling energy and low risk of contamination. Promising thermophilic pentose fermenting bacteria include T. saccharolyticum, Clostridium thermohydrosulfuricum, Clostridium thermosaccharolyticum, Clostridium thermosulfurogenes, Clostridium tetani and Thermoanaerobacter ethanolicus [32–34]. These thermophiles have many industrially important properties such as wide substrate range, less biomass, no specific oxygen requirement, less risk of contamination and continuous recovery of volatile products. But, the low product tolerance and byproduct formation during the fermentation make these bacteria commercially unviable; however, high temperature fermentation may save cooling energy as compared to low temperature fermentation. Moreover, the genetic modification of thermophilic organisms would solve the problem of product tolerance.
3.2. Filamentous fungi

The filamentous fungi have been known to ferment sugars for more than 80 years. Several fungal species belonging to genera Chalara [35], Fusarium [36], Rhizopus [37], Neurospora [38], Paecilomyces [39] and Trichoderma [40] have potential for fermenting xylose. Some other useful fungal strains have also been studied that can ferment more complex natural cellulosics substrates as well. Monilia sp., Neocallimastix sp., Trichoderma reesei and Fusarium oxysporum have shown the ability for direct conversion of cellulose/hemichellulose to ethanol/organic acid in single step fermentation [40,41]. Despite the pentose fermentation characteristics, these fungi have several physiological drawbacks such as, long fermentation period, low ethanol productivity, high viscosity fermentation broth, requirement of low critical oxygen levels and formation of byproducts in large amounts. However, a filamentous fungal system might be interesting because of their ability to grow on natural plant biomass, which yeast systems usually lack [22].

3.3. Yeast

The use of yeasts in conversion of carbohydrates to ethanol is known for generations. However, only a few strains are capable of converting pentoses [21]. The extensively studied yeast species for xylose fermentation are P. tannophilus, C. shehatae, P. stipitis and Kluyveromyces marxianus [19,20,22]. Many other yeast species are also reported for their xylose-fermenting capabilities, which include Brettanomyces, Clavispora, Schizosaccharomyces, several other species of Candida viz., C. tenus, C. tropicalis, C. utilis, C. blankii, C. friedrichii, C. solani and C. parapsilosis, and species from Debaryomyces viz., D. nepalensis and D. polymorpha [42,43]. Suh and coworkers have isolated a novel xylose-fermenting yeast ‘Enteroramus dimorbus’ from the microflora in the hindgut of beetles ‘Odontotaenius disjunctus’ [44]. Our group has also screened 20 yeast strains for pentose fermentation recently, where only few strains of Pichia and Pachysolen showed pentose fermenting capabilities [45]. The pentose fermenting yeasts were observed to be less tolerant to pH, ethanol and hydrolysate inhibitors when compared to Saccharomyces cerevisiae. Moreover their inability to produce ethanol as major end-product from xylose is a major drawback for ethanol production [21,22,25].

4. Xylose metabolizing pathways

The initial metabolic pathway of d-xylose in microorganisms involves its conversion to d-xylulose followed by xylulose kinase
reaction to d-xylulose-5-phosphate and then directed to pentose phosphate pathway (PPP). For d-xylene to d-xylulose conversion, bacteria generally use xylose isomerase (XI) enzyme, whereas yeasts and mycelial fungi employ a two step oxidation–reduction pathway [46]. In yeasts and fungi, the d-xylene is first reduced to xylitol by d-xylene reductase (XR) and subsequently oxidized to d-xylulose by xylitol dehydrogenase (XDH), which is further oxidized to form d-xylulose-5-phosphate. d-Xylulose is further metabolized via pentose phosphate pathway (PPP), in which the non-oxidative rearrangements of α-xylulose-5-phosphate by ribulosephosphate-3-epimerase, transaldolase (TAL) and transketolase (TK) results in the formation of glyceraldehyde-3-phosphate and fructose-6-phosphate, which can be converted to ethanol by fermentative reactions of the Embden–Meyerhoff–Parnas (EMP) pathway [47] (Fig. 2).

Fermentative yeasts generally possess both aerobic and anaerobic pathways along with adaptive regulatory mechanisms. Even if they can metabolize d-xylene anoxically, d-xylene-fermenting yeasts require functional mitochondria and oxygen for growth, regardless of the carbon source. During typical aerobic xylene fermentation, ethanol concentration peaks sharply and then declines as consumption exceeds production. However, ethanol uptake is not observed in anaerobic xylose fermentation, where the TCA cycle is not operational [22,48].

5. Fermentation of pentose sugars

The general requirements of an organism for ethanol production from pentose sugar hydrolysate should be high ethanol yield, high productivity, good tolerance against inhibitors as well as high ethanol concentrations and ability to ferment at relatively low pH. S. cerevisiae is one of the most commonly used yeasts for ethanol fermentation using glucose. However, it does not have the ability of fermenting pentose sugars. The most promising yeast species identified so far for the pentose fermentation are, C. shehatae, P. stipitis and P. tannophilus [19,20]. Various studies have been carried out for the fermentation of xylose rich hydrolysates from different lignocellulosic materials (Table 1). Moniruzzaman achieved 78% theoretical ethanol yield during the fermentation of enzymatic hydrolysate of steam exploded rice straw, however, a 2–3 h lag due to diauxic phenomenal metabolic shift from glucose to
Table 1

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Organism</th>
<th>Sugar (g/l)</th>
<th>Ethanol (g/l)</th>
<th>Ethanol yield (g/g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn stover</td>
<td>P. stipitis</td>
<td>40</td>
<td>15,92</td>
<td>0.4</td>
<td>[97]</td>
</tr>
<tr>
<td>Prosopis juliflora</td>
<td>P. stipitis</td>
<td>18</td>
<td>7.1</td>
<td>0.39</td>
<td>[9]</td>
</tr>
<tr>
<td>Rice straw</td>
<td>P. stipitis</td>
<td>33</td>
<td>14.9</td>
<td>0.45</td>
<td>[155]</td>
</tr>
<tr>
<td>Sunflower seed hull</td>
<td>P. stipitis</td>
<td>34</td>
<td>11</td>
<td>0.32</td>
<td>[52]</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>Pachysolen cannabuliflous</td>
<td>63.5</td>
<td>19</td>
<td>0.34</td>
<td>[156]</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>P. stipitis</td>
<td>35</td>
<td>12.4</td>
<td>0.35</td>
<td>[51]</td>
</tr>
<tr>
<td>Corn stover</td>
<td>P. stipitis</td>
<td>40</td>
<td>15</td>
<td>0.37</td>
<td>[50]</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>Candida shehatae</td>
<td>30</td>
<td>8.67</td>
<td>0.29</td>
<td>[1]</td>
</tr>
<tr>
<td>Corn stover</td>
<td>P. stipitis</td>
<td>60</td>
<td>25</td>
<td>0.42</td>
<td>[157]</td>
</tr>
<tr>
<td>Red oak wood chips</td>
<td>P. stipitis</td>
<td>36</td>
<td>14.5</td>
<td>0.4</td>
<td>[158]</td>
</tr>
<tr>
<td>Red oak spent sulphite liquor</td>
<td>P. stipitis</td>
<td>49</td>
<td>20.2</td>
<td>0.41</td>
<td>[159]</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>P. stipitis</td>
<td>52</td>
<td>22.3</td>
<td>0.43</td>
<td>[160]</td>
</tr>
<tr>
<td>Poplar</td>
<td>P. stipitis</td>
<td>39</td>
<td>12</td>
<td>0.31</td>
<td>[162]</td>
</tr>
<tr>
<td>Switch grass</td>
<td>P. stipitis</td>
<td>39</td>
<td>14</td>
<td>0.36</td>
<td>[162]</td>
</tr>
<tr>
<td>Rice straw</td>
<td>Candida shehatae</td>
<td>20</td>
<td>9</td>
<td>0.45</td>
<td>[19]</td>
</tr>
<tr>
<td>Rice straw</td>
<td>P. stipitis</td>
<td>15</td>
<td>6</td>
<td>0.4</td>
<td>[49]</td>
</tr>
<tr>
<td>L. camara</td>
<td>P. stipitis</td>
<td>16.8</td>
<td>5.16</td>
<td>0.33</td>
<td>[12]</td>
</tr>
</tbody>
</table>

xylose was also observed [49]. In another study using the acid and the auto-hydrolysate of rice straw, C. shehatae NCIM 3501 showed enhanced ethanol production in auto-hydrolysate (23.1 g/L) than in acid hydrolysate (20.0 g/L) because of lower inhibitor concentration [19]. The pentose fermentation process does not require intensive aerobic fermentation because of high cell mass synthesis, low ethanol yields and higher aeration energy consumption. However, aeration is required for the biomass production, which could be a major problem during the fermentation of non-detoxified hydrolysate. Interestingly, the fermentation of non-detoxified corn stover hydrolysate at higher aeration improved the ethanol production which was due to higher xylose consumption translating higher biomass concentration [50]. In a similar study the degree of aeration showed a prominent effect on xylose utilization, ethanol production and xylitol minimization during the fermentation of membrane treated sugar maple hydrolysate using P. stipitis NRRL Y-7124 [51]. Further, different detoxification strategies were used by various researchers to enhance the ethanol production [1,52].

The removal of toxic inhibitors from fermentation broth significantly improved the ethanol yield (2.4-fold) and productivity (5.7-fold), compared to neutralized hydrolysate. Similarly, the fermentation of sugarcane bagasse acid hydrolysate with C. shehatae NCIM 3501 showed maximum ethanol yield (0.48 g/g) from ion exchange treated hydrolysate, followed by treatment with activated charcoal (0.42 g/g), laccase (0.37 g/g), overliming (0.30 g/g) and neutralization (0.22 g/g) [1]. While in another study, the sequential application of overliming with sodium sulfite addition was observed to be the best detoxification method for the sunflower seed hull acid hydrolysate, for maximum ethanol yield (0.32 g/g) and ethanol productivity (0.065 g/L.h) [52]. Recently, P. stipitis NCIM 3498 was used to ferment the detoxified acid hydrolysates of two lignocellulosic feedstocks (Prosopis juliflora and Lantana camara), resulting in an ethanol yield ranging from 60 to 70% of the theoretical yields [9,12].

Although significant work has been carried out on pentose fermentation, but no economically feasible process has been developed so far. Therefore, in order to achieve improved ethanol production, the major focus of research on pentose fermentation is shifting toward the exploration of improved fermentation strategies and strain improvement.

5.1. Pentose fermentation using whole cell immobilization

The immobilization technique offers many advantages such as cell recycling, economic extraction of the product, easy maintenance of specific growth and dilution rate, maintenance of high cell density, high productivity, good mixing and mass transfer with low risk of contamination [53–56]. Though the immobilized microorganisms showed enhanced volumetric productivities as compared to free microbes, however, in most of the cases, the xylose and glucose are not utilized at the same time due to catabolite repression [47]. In an attempt to ferment glucose and xylose simultaneously, Grootjen and coworkers used a co-culture cultivation of alginate immobilized S. cerevisiae and P. stipitis in a conventional bioreactor, where P. stipitis cells inoculum were taken in comparably higher amount which allowed more xylose utilization under anaerobic conditions and the fermentation appears to be simultaneous [53]. Furthermore, the modified stirred tank reactor (STR) system equipped with two tetlon-made HPLC filters air diffusers with improved mixing and less shearing during culture strategy improved the ethanol yield up to 80% with calcium alginate immobilized P. stipitis and S. cerevisiae [54]. Interestingly, an agar sheet sandwiched between two chambered bioreactor has also been used for the co-immobilization of S. cerevisiae and C. shehatae during the mixed sugar (glucose and xylose) fermentation, however, the cell proliferation in the gel clogged the microporous membrane, which in turn limited the mass transfer [57]. In another study to overcome the problem of glucose catabolite repression, sieve plates adjusted STR with a movable device was used for the coculture of immobilized Z. mobilis and free P. stipitis to improve the fermentation efficiency [58]. Recently, a calcium alginate immobilized recombinant S. cerevisiae strain ZU-10 has been used for the fermentation of detoxified corn stover hemichellulosic hydrolysate, which showed the consumption of more than 92% xylose with an enhanced ethanol yield and productivities with higher tolerance to fermentation inhibitors [56].

Despite various improvements of these fermentations, the repeat culture with the same batch of immobilized microorganism under the same conditions resulted in decreased performance [58]. Alternatively yeast cells immobilized by self flocculation have shown many advantages such as no requirement of support matrix, maintained biomass and enhanced ethanol tolerance. Moreover flocculated yeast cells can be recovered by sedimentation from fermentation broth. However, CO₂ bubbles produced during ethanol fermentation can alter the settling zone and disturb the sedimentation of yeast floc, whereas a specially designed baffle can overcome this problem [59]. There are also some reports of yeast cells immobilization for biocatalysts development for simultaneous saccharification and fermentation (SSF). Fujita and coworkers constructed a yeast-based whole-cell biocatalyst displaying T. reesei xylanase II.
on the cell-surface and showed xylan degradation by recombinant cells [60]. Significant attempts have also been made for the development of yeast cells displaying cellulase activities on the cell surface so as to decrease the usage of exogenously added cellulase [61,62].

5.2. Pentose fermentation using recycling of cells

Increasing the cell density by cell recycling and cell retention has been a suitable way of increasing the volumetric productivity for slow growing microorganisms [63,64]. The cell recycling operation requires lesser amount of nutrients, achieves high dilution rate in continuous operations, decreases cell mass synthesis, and increases ethanol yields [57,65]. A high productivity system that involved a membrane bioreactor with cell recycling of Z. mobilis ZM4 capable of converting both glucose and xylose to ethanol had been developed [66]. Similar strategy was also applied during the continuous cultivation of a recombinant xylose fermenting S. cerevisiae TMB 3001 on a xylose-glucose mixture [64]. Interestingly, the recycled cells get adapted to the fermentation inhibitors present in the hydrolysate and showed better results in lignocellulose hydrolysate containing mixed sugars [63]. Pruwadi and coworkers found similar results, where continuous cultivation of high cell density flocculating yeast in toxic dilute acid hydrolysate of spruce residues in a single and serial bioreactor adapted to the medium and reduced the requirement of any detoxification [67]. Recently, a fuzzy optimization of continuous fermentation with cell recycling for ethanol production was carried out [68]. From the computational results, the overall productivity of the continuous fermentation process with cell recycling allowed a higher dilution rate with 7.3-fold higher productivity [68].

Though in most of the earlier reports, the cell enrichment was carried out by cell resetting, centrifugation, microfiltration and ultrafiltration, but recently hydroclone has been used to recover the cells of S. cerevisiae IROST 5209 from the fermentation broth [69]. The hydroclone system offers an advantage of negligible cell disruption [69]. Furthermore, implementation of cell recycling in industrial process is possible only if the effluent passes the membrane without fouling. Industrial effluents are not suitable for membrane filtration due to suspended particles, solid residues and high viscosity. However, decreasing the viscosity in industrial effluents by enzymatic treatment is one of the possible solutions of this problem [60,6].

5.3. Pentose fermentation using simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) approach has also been employed to decrease sugar inhibition to cellulase, achieve improved ethanol production, and to reduce the overall process cost since both the processes took place in single reactor. The SSF technique reduces the processing time, which in turn leads to increase the apparent volumetric productivity of ethanol [71]. The other advantages of this approach are shorter fermentation time and reduced risk of contamination with external microflora due to the high temperature of the process, the presence of ethanol in the reaction medium and the anaerobic conditions [30,71,72]. Interestingly, in a comparison between the separate hydrolysis and fermentation (SHF) and SSF of steam exploded corn stover, it was observed that SSF gave a 13% higher overall ethanol yield than SHF (72.4% versus 59.1% of the theoretical) [72]. However, the performance of the SSF process is limited by the competence between optimum temperatures for enzymatic saccharification and microbial fermentation [73]. Hence, the coexistence of two process conditions for low-temperature microbe and high-temperature fungal enzyme may hinder the efficacy of the SSF process.

To improve process efficiency, many microorganisms have been tested with cellulosic produced by T. reesei mutants [30]. Besides, there are some microorganisms such as Clostridium, Cellulomonas, Trichoderma, with high cellulolytic and hemicellulolytic activity and are highly capable of fermenting mono- or oligo-saccharides into ethanol. Application of thermotolerant yeast strains may be beneficial in high temperature SSF processes, where fungal cellulase can also exhibit higher activity [74]. Ballesteros and colleagues tested different treatments to improve the thermotolerance of some species belonging to the genera Saccharomyces, and Kluyveromyces and the best results were obtained with K. marxianus [74]. The strain was further mutagenised and the mutated K. marxianus strain CECT 10875 achieved a SSF yield of 50–72% in 72–82 h using various lignocellulosic feedstocks such as Populus sp. and Eucalyptus sp., Sorghum sp. bagasse, wheat straw and Brasicar carinata residue [75]. The efficacy of Kluyveromycetes was further confirmed by other researchers as well, where Kluyveromyces strain NCIM 3358 in SSF experiments of softwood resulted in more than 70% ethanol conversion [76]. Similarly, during the SSF of lignocellulose wastes with this thermotolerant yeast at 10% (w/v) initial substrate consistency, a final ethanol concentration of 2–2.5% was obtained after 72 h [77].

In another strain improvement report, a thermophilic anaerobic bacterium T. saccharolyticum, capable of utilizing xylan and biomass derived sugars, was engineered by removing the genes encoding acetate kinase, phosphate acetyl transferase and β-lactate dehydrogenase. The engineered thermophilic strain ALK2 thus obtained was able to reduce the requirement of externally added fungal cellulase by 2.5-fold [34]. Recently, a respiratory-deficient mutant of the thermotolerant yeast Candida glabrata (Cgrd1), was subjected to ethanol production by high-temperature SSF under aerobic conditions to achieve maximum ethanol (17.0 g/L) within 48 h at 66.6% of its theoretical yield and with 0.35 g/L/h productivity [78].

However, more efforts are required in the development of microorganisms for economically feasible industrial ethanol production from pentose sugars. Since enzymatic hydrolysis is the rate-limiting step in SSF, increasing the rate of hydrolysis will lower the cost of ethanol production via SSF [30].

6. Strain improvement for pentose fermentation

6.1. Strain improvement through mutagenesis

There are several reports where mutagenised recombinant strains showed enhanced ethanol production over their parent strains [47,81]. In an early report, a recombinant S. cerevisiae strain T1J mutagenised with ethyl methane sulfonate (EMS) was found to have lower XR activity but high XD and xylulokinase (XKS) activities than the parent strain, which in turn resulted in 1.6-fold increase in ethanol production [82]. Further, a mutant of S. cerevisiae TMB 3001 capable of utilizing xylose under anaerobic condition was developed by sequential EMS mutagenesis and adaptation of the mutant strain under microaerobic and anaerobic conditions [83]. Later on similar strategy was used to develop two EMS mutagenised S. cerevisiae strains 3399 and 3400 with improved growth on xylose [80]. Since the most efficient xylose utilizing microbes are not able to metabolize the pentose sugars anaerobically, the strategies of natural selection and random mutations were also tested [84]. Besides EMS, several other mutagens had also been used to obtain mutants derepressed for pentose metabolism. Sreenath and Jeffries used 2-deoxyglucose (2-DOG) mutated strain, showing considerable improvement in xylose utilization [63]. While in another report, a UV mutagenised P. stipitis NRRL Y-7124 strain was found to produce higher ethanol than the wild strain [85].
Site-directed mutagenesis is another strategy used to obtain the mutants for better xylose fermentation. Watanabe and coworkers used multiple site-directed mutagenesis of the NAD⁺-dependent XDH from *P. stitpis* and introduce a structural zinc atom for the complete reversal of the coenzyme specificity [81]. The selected mutants were found to exhibit significant thermostability and enhanced catalytic activity with NADP⁺. Similarly, several PsXDH mutants were generated with complete reversal of coenzyme specificity toward NADP⁺ by multiple site-directed mutagenesis within the coenzyme-binding domain and with increased thermostability by refining the structural zinc-binding loop without affecting their activities [86]. In addition one of the *S. cerevisiae* mutant (MA-85) under the control of a strong constitutive promoter showed particularly high ethanol production from xylose and low xyitol yield by fermentation of not only xylose as the sole carbon source, but also a mixture of glucose and xylose [87–89]. Additionally using this approach, an ethanologenic *Escherichia coli* mutant, devoid of foreign genes, has also been developed by combining the activities of pyruvate dehydrogenase and the fermentative alcohol dehydrogenase and the mutant was found able to ferment glucose or xylose to ethanol with 82% ethanol yield under anaerobic conditions [79].

6.2. Strain improvement through protoplast fusion

Protoplast fusion provides characteristic advantage such as promotion of high frequencies of genetic information between organisms for which poor or no genetic exchange has been demonstrated or which are genetically uncharacterized [90–92]. In the presence of a fusogenic agent such as polyethylene glycol (PEG), protoplasts are induced to fuse and form transient hybrids or diploids. Several reports on protoplast fusion between pentose and hexose utilizing yeasts showed efficient utilization of both sugars with higher biomass yield. Heluane and coworkers successfully transferred the genes of xylose utilization from *P. tantanophilus* to *S. cerevisiae* [90]. The hybrids reassembled the *S. cerevisiae* parent morphologically but displayed the ability to use the pentose sugars (xylose) similar to *P. tantanophilus*. The same has been supported by other workers, where a fusant of *Schizosaccharomyces pombe* and *Lentinula edodes* were found to utilize xylan as carbon source [91]. In another study, the protoplasts of thermotolerant *S. cerevisiae* and mesophilic xylose-utilizing *C. shehatae* were fused by electrofusion and the fusant yeast gave an ethanol yield of approximately 0.459 g/L with productivity of 0.67 g/L/h and fermentation efficiency of 90% and showed higher temperature tolerance up to 40 °C as well [92]. Moreover, using a combinatorial approach, a xylose fermenting fusant (F6) of *C. shehatae* and *S. cerevisiae* was developed, showing improved ethanol production (28%) than its parental strain [93]. In this strategy the *C. shehatae* was first adapted for ethanol tolerance and then mutagenised by UV irradiation and thus a respiration deficient mutant RD-5 was selected. Further, the protoplasts of RD-5 and *S. cerevisiae* were fused and the resultant fusant strain F6 showed 28% higher ethanol production than the parent *C. shehatae* strain, with the production level of 18.75 g/L from 50 g/L xylose. Recently a strategy of genome shuffling was also used, in which the genomes of 6 UV mutagenised *P. stitpis* strain (WT, PS302, GS301, GS302, GS401 and GS402) were shuffled and after the 3rd and 4th rounds of genome shuffling, putative improved mutant colonies were pooled, re-grown and spread on hardwood spent sulphite liquor (HWSLL) gradient plate again [94]. *P. stitpis* WT and PS 302 could not grow in any of the HWSLL concentrations, while 2 mutants (GS401 and GS402) from the 4th round could grow in 80% (v/v) HWSLL while another 2 mutants (GS301 and GS302) from the 3rd round could grow in 85% (v/v) HWSLL. Thus the study concluded that the mutated strains showed improved inhibitors tolerance against HWSLL [94].

6.3. Strain improvement through adaptation

Fermentation of wood-derived hydrolysates is sometimes problematic because of the toxic inhibitors released during thermochemical hydrolysis. However, the adaptation approach can be an alternative means to improve the microbial strains [95–97]. There are several reports on enhancement of ethanol yield and productivity using adapted strains of *P. stitpis* and *C. shehatae* for the fermentation of undetoxified or partially detoxified hydrolysates [97–99]. For instance, an ethanologenic yeast when adapted against inhibitors by repeated sub-culturing in a medium with furfural and HMF up to a concentration of 10–20 mM was found to grow more efficiently than its parent strain in the presence of inhibitors [98]. Another strategy of natural selection and breeding was used to develop non-recombinant strains of *S. cerevisiae* that could grow efficiently on xylose [99]. By breeding and natural selection over 23 mating cycles and 1463 selection days, a non-genetically modified *S. cerevisiae* (MBG-2303) was obtained, which grew aerobically on xylose and demonstrated 57-fold higher biomass production than the control strain [98]. Moreover, using a combinatorial approach of *P. stitpis* CBS 6054 in solid agar produce more ethanol (19.4 g/L) than liquid adapted (18.4 g/L) and unadapted strains (16.3 g/L) [95]. Recently, studies were carried out on adaptation of *P. stitpis* CBS 5776 strain which on fermentation of steam exploded prehydrolysate of corn stover showed improved ethanol yield of 15.92 g/L with 80.34% theoretical yield [97].

Moreover, the evolutionary adaptation approaches have also been applied to recombinant strains to improve their fermentation capability. Lawford and group improved the xylose-fermenting recombinant strain *Z. mobilis* 39767 to tolerate higher concentration of acetic acid by subculturing in a medium containing 10–50% of hydrolysate and the adapted isolates demonstrated a significant improvement in ethanol productivity compared to un-adapted strains [100]. Similarly, an engineered *E. coli* KO11 was developed to tolerate high ethanol concentration using a long term adaptation strategy of alternative serial selections for liquid and solid medium. The mutants thus developed, i.e., LY01, LY02 and LY03 demonstrated more than 50% survival rate in 10% ethanol (0.5 min exposure) and also reduced the fermentation time [96]. In almost all previous efforts of evolutionary adaptation the organism was first subjected to genetic engineering, which was followed by adaptive selection [83,101,102]. However, recently a new strategy consisting genetic engineering, mutation with EMS followed by two-step evolutionary adaptation (under sequential aerobic and oxygen limited conditions) has also been attempted [4]. The strain thus developed showed fourfold increase in its specific growth rate compared to the parental strain. Interestingly the activity of critical enzymes of xylose metabolism (XR, XDH and XK) remain unchanged suggesting that chemical mutagenesis and evolutionary adaptation might have created a new genetic traits making the mutants capable of xylose metabolism [4].

6.4. Strain improvement through genetic manipulation

Substantial progress in the genetic engineering of different microbes for the conversion of xylose or pentose sugars to ethanol has been achieved [4,88,103]. Although the genetically engineered host strains of bacteria and yeast showed tremendous improvement in final ethanol yields and efficient utilization of pentose sugars (Table 2), the information about the usage of genetically modified organisms for large scale pentose fermentation is scarcely available [25].

6.4.1. Genetic engineering of *E. coli*

Ingram’s group have done extensive work on the development of efficient recombinant *E. coli* strains for ethanol production.
They eliminated the dependence of host on alcohol dehydrogenase (ADH) activity by combining adh B and pdc (coding for pyruvate decarboxylase, PDC) genes of *Z. mobilis* to form pet operon [104,105]. Considering a number of factors influencing ethanol production such as substrate range and growth conditions, *E. coli* strain ATCC 11303 was chosen as the host for the pet plasmid [106]. Further, to improve the genetic stability, the pet operon was integrated into the chromosome of ATCC11303 [99]. Since the strain containing the integrated genes produced only low levels of ethanol, the spontaneous mutation strategy generated a hyper ethanol producing *K. oxytoca* strain. This strain was further modified by deletion of the succinate production gene (*frd*) to prevent the formation of succinate, a major byproduct of *E. coli* metabolism. The finally developed strain KO11 was able to convert glucose and xylose to ethanol at theoretical yields of 100% in rich media containing ample yeast extract [107]. To further improve pentose fermentation by KO11, a number of spontaneous mutants defective in glucose transport were selected and two such strains SL28 and SL40 when fermented using individual or mixture of xylose and glucose produced ethanol more efficiently (by 20%) than the parent strain KO11 [108]. The recombinant *E. coli* strain was further improved to achieve better xylose fermentation, the glycolytic flux and the growth rate of recombinant strain [109]. However, from an industrial point of view, these recombinant strains still had the drawback of requiring nutrient rich medium for ethanol production. In order to overcome, a lactate producing recombinant of KO11 was reengineered for

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**Table 2**

List of pentose utilizing recombinant yeasts and bacterial strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sugar/sugar mix used (g/l)</th>
<th>Ethanol production (g/l)</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol productivity (g/l/h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli KO11</td>
<td>80 X</td>
<td>41.6</td>
<td>102</td>
<td>0.87</td>
<td>[107]</td>
</tr>
<tr>
<td>E. coli KO11</td>
<td>90 X</td>
<td>41</td>
<td>89</td>
<td>0.85</td>
<td>[96]</td>
</tr>
<tr>
<td>E. coli KO11(140 X)</td>
<td>140</td>
<td>59.5</td>
<td>88</td>
<td>0.66</td>
<td>[96]</td>
</tr>
<tr>
<td>E. coli LY01</td>
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<td>63.2</td>
<td>90</td>
<td>0.59</td>
<td>[110]</td>
</tr>
<tr>
<td>E. coli FBR5</td>
<td>A-X G</td>
<td>44.0</td>
<td>90</td>
<td>0.92</td>
<td>[110]</td>
</tr>
<tr>
<td>E. coli SE2378</td>
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<td>27.8</td>
<td>82</td>
<td>NA</td>
<td>[79]</td>
</tr>
<tr>
<td>Z. saccharomyces</td>
<td>AKL-2</td>
<td>33.1</td>
<td>92</td>
<td>2.2</td>
<td>[41]</td>
</tr>
<tr>
<td>Z. mobilis CP4</td>
<td>25 X</td>
<td>11.0</td>
<td>86</td>
<td>0.57</td>
<td>[115]</td>
</tr>
<tr>
<td>Z. mobilis CP4</td>
<td>G-X</td>
<td>24.2</td>
<td>95</td>
<td>0.81</td>
<td>[115]</td>
</tr>
<tr>
<td>Z. mobilis ATCC 39767</td>
<td>G:X:A</td>
<td>33.5</td>
<td>82-84</td>
<td>0.82-0.65</td>
<td>[117]</td>
</tr>
<tr>
<td>Z. mobilis CP4</td>
<td>30:30:20</td>
<td>23</td>
<td>94</td>
<td>0.32</td>
<td>[163]</td>
</tr>
<tr>
<td>Z. mobilis ZM4</td>
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<td>62</td>
<td>90</td>
<td>1.29</td>
<td>[66]</td>
</tr>
<tr>
<td>Z. mobilis AX10</td>
<td>20:40:40</td>
<td>42</td>
<td>84</td>
<td>0.61</td>
<td>[119]</td>
</tr>
<tr>
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<td>1.6</td>
<td>14</td>
<td>0.07</td>
<td>[164]</td>
</tr>
<tr>
<td>S. cerevisiae TJ1</td>
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<td>106</td>
<td>0.02</td>
<td>[165]</td>
</tr>
<tr>
<td>S. cerevisiae 1400</td>
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<td>90</td>
<td>0.92</td>
<td>[166]</td>
</tr>
<tr>
<td>S. cerevisiae 1400</td>
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<td>27</td>
<td>66</td>
<td>1.2</td>
<td>[166]</td>
</tr>
<tr>
<td>S. cerevisiae 1400</td>
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<td>6</td>
<td>[121]</td>
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<tr>
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<td>21.6</td>
<td>54</td>
<td>NA</td>
<td>[123]</td>
</tr>
<tr>
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<td>19</td>
<td>76</td>
<td>0.27</td>
<td>[48]</td>
</tr>
<tr>
<td>S. cerevisiae TMB 3251</td>
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<td>17</td>
<td>68</td>
<td>0.24</td>
<td>[48]</td>
</tr>
<tr>
<td>S. cerevisiae TMB 3255</td>
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<td>20.5</td>
<td>82</td>
<td>0.29</td>
<td>[48]</td>
</tr>
<tr>
<td>S. cerevisiae H2084</td>
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<td>20.5</td>
<td>82</td>
<td>NA</td>
<td>[113]</td>
</tr>
<tr>
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<td>50 X</td>
<td>5</td>
<td>50</td>
<td>NA</td>
<td>[80]</td>
</tr>
<tr>
<td>S. cerevisiae TMB 3001</td>
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<td>2.4</td>
<td>48</td>
<td>NA</td>
<td>[83]</td>
</tr>
<tr>
<td>S. cerevisiae BH42</td>
<td>G:X</td>
<td>28</td>
<td>56</td>
<td>NA</td>
<td>[167]</td>
</tr>
<tr>
<td>S. cerevisiae TMB 3120</td>
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<td>4.6</td>
<td>92</td>
<td>0.064</td>
<td>[168]</td>
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<tr>
<td>S. cerevisiae TMB 3050</td>
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<td>14.5</td>
<td>58</td>
<td>NA</td>
<td>[103]</td>
</tr>
<tr>
<td>S. cerevisiae RW8 217</td>
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<td>8.6</td>
<td>86</td>
<td>NA</td>
<td>[101]</td>
</tr>
<tr>
<td>S. cerevisiae TMB 3270</td>
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<td>72</td>
<td>0.32</td>
<td>[135]</td>
</tr>
<tr>
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<td>[128]</td>
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<td>0.032</td>
<td>[130]</td>
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<tr>
<td>S. cerevisiae DR PHO13</td>
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<td>0.25</td>
<td>0.093</td>
<td>[130]</td>
</tr>
<tr>
<td>S. cerevisiae TMB 3066</td>
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<td>21.5</td>
<td>86</td>
<td>0.073</td>
<td>[128]</td>
</tr>
<tr>
<td>S. cerevisiae CMB JHV</td>
<td>20 X</td>
<td>6.4</td>
<td>0.32</td>
<td>NA</td>
<td>[169]</td>
</tr>
<tr>
<td>S. cerevisiae BP1000</td>
<td>20 X</td>
<td>6.8</td>
<td>0.34</td>
<td>NA</td>
<td>[139]</td>
</tr>
<tr>
<td>S. cerevisiae MA-N5</td>
<td>45 X</td>
<td>15.8</td>
<td>0.36</td>
<td>0.24</td>
<td>[88]</td>
</tr>
<tr>
<td>S. cerevisiae RBW</td>
<td>50 X</td>
<td>19.5</td>
<td>78</td>
<td>NA</td>
<td>[170]</td>
</tr>
<tr>
<td>202-APX</td>
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<td>NA</td>
<td>[170]</td>
</tr>
<tr>
<td>S. cerevisiae INVSC1</td>
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</tr>
<tr>
<td>S. cerevisiae ADP8</td>
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<td>[172]</td>
</tr>
<tr>
<td>S. cerevisiae MA-R4</td>
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<td>0.36</td>
<td>[174]</td>
</tr>
<tr>
<td>S. cerevisiae MA-R5</td>
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<td>16.65</td>
<td>0.37</td>
<td>0.50</td>
<td>[84]</td>
</tr>
<tr>
<td>S. cerevisiae ZJ-10</td>
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<td>30.2</td>
<td>75.6</td>
<td>0.50</td>
<td>[56]</td>
</tr>
<tr>
<td>S. cerevisiae LRE 513</td>
<td>50 X</td>
<td>8.13</td>
<td>32.5</td>
<td>0.113</td>
<td>[4]</td>
</tr>
</tbody>
</table>

A, arabinose; G, glucose; Gal, galactose; M, maltose; and X, xylose.
ethanol production by deleting genes encoding for fermentative routes for NADH and randomly inserting a promoter-less cassette containing the complete Z. mobilis ethanol pathway into K011 chromosome [96].

Using different approach, Dien and coworkers developed new ethanologenic strains of E. coli such as FBR3, FBR4 and FBR5 [110]. These strains were generated by transforming a xylose-utilizing isolate of strain E. coli FMJ39 with plasmid pLOI297 containing Z. mobilis pyruvate to ethanol converting enzymes. Alternatively a homo-ethanologenic strain of E. coli SE2738 from wild type E. coli K-12 W3110 was also developed, where the mutant strain exhibited 82% theoretical ethanol yield when grown on xylose under anaerobic conditions [79]. In a recent study, E. coli cells for efficient ethanol production from hexoses and pentoses were developed using elementary mode analysis to dissect the metabolic network into its basic building blocks [111]. During this strategy the functional space of the central metabolic network was reduced, with eight gene knockout mutations, from over 15,000 pathway possibilities to 6 pathway options that support cell function. Furthermore the engineered traditional genetic network of Z. mobilis photosynthetic cells selectively grows only on pentoses, even in the presence of glucose, with a high ethanol yield. Later on using the similar approach, a glycerol to ethanol converting E. coli strain was designed by reducing the functional space of central carbon metabolism to a total of 28 glycerol utilizing pathways [112]. More recently an attempt has been made to engineer E. coli for the production of ethanol from fatty acid feedstocks, resulting in ethanol yield higher than the theoretical maximum obtained from sugars [113].

6.4.2. Genetic engineering of Z. mobilis

The xylose utilization in Z. mobilis was developed by integrating XI, XKS, and tkt, which when grown on xylose as the sole carbon source resulted in 86% theoretical ethanol yield [115]. In another work, the constructed operons encoding xylose assimilation and pentose phosphate pathway enzymes were transformed into Z. mobilis to generate pZB5 strain for the effective fermentation of xylose to ethanol [36,115]. Further to construct improved strains with higher ethanol productivity and yields, pZB5 was transformed into Z. mobilis ethanol producing strain ZM4; ATCC 31821, which showed the capability of converting a mixture of 65 g/L of glucose and 65 g/L of xylose to 62 g/L ethanol in 48 h with an overall yield of 0.46 g/g [60]. Following similar approach, another group incorporated five genes of arabino sugar utilization from E. coli ara A (coding for l-arabinose isomerase), ara B (coding for l-ribulokinase), ara D (coding for l-ribulose-5-phosphate-4-epimerase), tal and tkt in Z. mobilis ATCC 39787 [116]. The resultant strain showed more than 90% ethanol yield from l-arabinose. However, 40% of the cells lost their ability to ferment arabinose when grown on complex medium. A number of other improvements have also been made in Z. mobilis strains and the new strain Z. mobilis AX101 fermented both arabinose, xylose and glucose and carried seven necessary recombinant genes as part of chromosomal DNA [117–119]. The co-fermentation process yield of Z. mobilis AX101 was about 84%, with the order in sugar utilization as glucose followed by xylose and arabinose [119]. However, these strains showed acetic acid sensitivity [119]. To address the problem of sensitivity to toxic fermentation inhibitors, a new strain of Z. mobilis ZM4/Acr (pZB5) was developed with increased acetate resistance that has enhanced performance in the presence of 12 g sodium acetate per litre at pH 5 [120]. Z. mobilis ZM4 produced ethanol with high specific productivity and was able to ferment both C-5 and C-6 sugars. The transformed Z. mobilis ZM4 performed best under anaerobic conditions, but also exhibited tolerance to aerobic conditions. However, the genetic and physiological basis of ZM4’s response to various stresses has only been understood poorly. Recently, transcriptomic and metabolomic profiles for ZM4 under aerobic and anaerobic fermentations have been elucidated using microarray, high-performance liquid chromatography and gas chromatography–mass spectrometry (GC–MS) analysis [120].

6.4.3. Genetic engineering of S. cerevisiae

S. cerevisiae produces ethanol from hexose sugars but cannot ferment xylose or arabinose. However, the yeast is able to metabolize a xylose isomer, xyulose and recombinant DNA technologists have taken advantage of this in creating xylose-fermenting strains. Ho and coworkers were the first to successfully create a recombinant S. cerevisiae strain capable of effective xylose fermentation and xylose and glucose co-fermentation [121]. The recombinant plasmids with XR and XDH genes from P. stiptis and XKS gene from S. cerevisiae were transformed into S. cerevisiae for the co-fermentation of glucose and xylose. In contrast report the overexpression of endogenous XKS from S. cerevisiae resulted in fermentative growth on xylose [122]. Similarly some other workers have also reported about lower consumption of xylose in such strains [123–125]. However, Toivari and coworkers reported successful xylose fermentation to ethanol through over-expression of the endogenous XKS 1 and PsXR and XDH genes [126]. Recently following similar strategies, the improved xylose utilization and high ethanol production have been reported by various groups [86,127–129]. Based on these reports it can be concluded that the controlled overexpression of XKS gene in S. cerevisiae improved the xylose consumption as well as ethanol production. Jefferson and coworkers identified some interesting spontaneous or chemically induced mutants of recombinant S. cerevisiae that can overcome the growth inhibition caused by overexpression of ScXKS and PsXKS gene [130]. In order to achieve reduction in xyitol formation during xylose fermentation, recombinant S. cerevisiae strains expressing PsXR and PsXDH and overexpression of ScXKS were constructed that lowered the oxidative PPP activity through the GND1 (6-phosphogluconate dehydrogenase) and ZWF1 genes (glucose-6-phosphate dehydrogenase) [48]. These mutants showed increase in ethanol yield and xylose consumption rate compared to the parent strain. Furthermore an attempt to overexpress Klyveromyces lactis GDP1 gene (NADP-dependent glyceraldehyde 3-phosphate dehydrogenase, GAPDH) in a xylose-fermenting S. cerevisiae strain was also found to enhance ethanol production [131]. In another approach to enhance ethanol yields, the metabolic flux toward ethanol formation appeared to be a significant strategy to improve the intracellular cofactor concentrations in S. cerevisiae [132]. The impact of over-expression of NADH kinase (encoded by the P055 gene) on glucose and xylose metabolism in recombinant xylose-fermenting S. cerevisiae has also been studied [133]. The expression of NADH kinase in cytosol instead of mitochondria redirected the carbon flow from CO2 to ethanol during aerobic growth on glucose, whereas under anaerobic growth the flux directed toward ethanol and acetate fermentation. The cytosolic NADH kinase appeared to revert these effects during anaerobic metabolism of xylose by channeling carbon flow from ethanol to xyitol [133]. The heterologous expression of a xylose isomerase (XI) may also be a good approach to enable yeast cells to metabolize xylose. In this aspect, Brat and coworkers screened nucleic acid databases for sequences encoding putative XIs and cloned them to express a highly active XI from the anaerobic bacterium Clostridium phytofermentans in S. cerevisiae, which resulted in an efficient metabolism of α-xylose as the sole carbon and energy source by recombinant yeast cell [134].

Alternative protein engineering approach has also been investigated to reduce xyitol formation and enhancing ethanol yield
using recombinant S. cerevisiae. Using this approach an improved ethanol production accompanied by decreased xyitol formation was achieved in recombinant S. cerevisiae expressing mutated PxXR (having reduced affinity for NADPH). PxDH and ScXKS [135]. In this aspect, several NADH-prefering XR mutants from Candida tenuis have been developed [136–139]. Recently one such S. cerevisiae strain harboring the K274R-N276D CxXR double mutant showed enhanced ethanol production with decreased xyitol formation [140]. The heterologous expression of xylose specific transporters in recombinant S. cerevisiae for improved ethanol production has also been tested. The SUT1 gene [141] coding a sugar transporter in P. stipitis, has been successfully expressed in S. cerevisiae [142]. Moreover, the glucose/xylose-facilitated diffusion transporter and glucose/xylose symporter from Candida intermedia, encoded by Gx1 and Gx2 genes [143] have been expressed in S. cerevisiae [144], where the recombinant xylose-fermenting S. cerevisiae strain harboring Gx1 showed faster xylose uptake and ethanol production [145]. Recently, a combinatorial approach of genetic engineering, chemical mutagenesis and evolutionary adaptation has been used to improve the xylose utilization. The S. cerevisiae strain W303-La was introduced with XI and XK5 gene from P. stipitis NRRL7124 to make S. cerevisiae LEK 122. Thereafter, the selected strain was chemically mutagenised with EMS followed by their evolutionary adaptation for xylose utilization and growth under oxygen-limited conditions [4].

7. Future prospects

Ethanol has always been considered a better alternate to gasoline, as it reduces the dependence on fossil fuel reserves and promises cleaner combustion leading to a healthier environment. Interestingly, the world’s focus is switching over from corn and sugarcane to second generation feedstocks as renewable raw material for production of bioethanol [146–149]. In recent years, significant developments in hemicellulose to ethanol conversion have been achieved. However the industrial activities for bioethanol production are limited mainly because of the cost of raw material (lignocellulosic biomass) processing to obtain high yield of fermentable sugars and unavailability of efficient fermentation strategies. The process of hemicellulose conversion to bioethanol requires adequate breakdown of lignocellulosic biomass with maximum pentose sugar yield and efficient utilization of pentose sugars during fermentation. Although various pretreatment methods and their effects on biomass composition and sugar yields have widely been studied and described in literature but efficient fermentation of high yield hemicellulosic hydrolysates is essential to maximize the ethanol productivity and subsequent cost effective ethanol recovery [25]. The generation of microbial inhibitors during the pretreatment process is a major concern, which affects the economics of bioethanol production from lignocellulosic materials. Different detoxification strategies have been applied to remove these inhibitors from hemicellulosic hydrolysates to improve their fermentability [9,12,21,150], this additional step increases the overall ethanol production cost. There is an imperative need for bioprospecting of new robust microbial strains capable of converting the pentose sugars efficiently from un- or partially detoxified hydrolysates [151].

Since the efficient utilization of pentose sugars is a prerequisite for cost effective and sustainable ethanol production, the major research has now focused on further improving ethanol productivities using genetic modification and chemical additives [152,153]. Although several research groups have taken lead in developing a number of improved xylose utilizing recombinant organisms, it appears that xylose utilization and fermentation capabilities are commercially unattractive. Therefore, the genetic engineering approaches should be more focused on developing new improved strains with higher substrate tolerance and improved production kinetics. Additionally, the better elucidation of pentose sugars transport at the molecular level and characterization of kinetic and regulatory properties, including quorum-sensing mechanisms, should be given high priority because it may provide the basis for developing improved strains that can simultaneously utilize pentose and hexose sugars released during biomass hydrolysis. High-throughput screening techniques and better expression systems for efficient production of membrane proteins, and enzyme complexes such as cellulosomes are in progress.

Recently, the chromosomal integration of genes encoding the xylose utilization pathway enzymes into industrially applied yeast strains are reported necessary for large scale fermentation of xylose from lignocellulosic biomass to ethanol [89]. Therefore, further studies at micro levels are also required to combine the functional genomics with metabolic engineering strategies, which may provide further clues for developing robust strains. Moreover, the approach of flux analysis to divert or increase the activity of certain crucial enzymes involved in the main metabolic pathways could be improved with efficient xylose utilization needs more attention.

To make the bioethanol production process successful at industrial scale with reduction in capital and operation cost, some integrated unit operations using robust microorganisms for better product yields should be adopted [154]. An ideal up-scaling strategy needs to be fully integrated to evaluate the complete system (e.g., enzymes, nutrients, product yields and titers, and fermenting yeasts) with sufficient flexibility to investigate alternative process configurations. From a process scale-up perspective, the challenges lie not only with finding the most efficient organism for hemicellulose conversion but also to make an intelligent use of the entire process integration, a biorefinery concept.

Acknowledgements

The authors are thankful to Prof. P. Tauro, Manglore Biotech, Manglore, India and Prof. P. Gunasekaran, Department of Microbial Genetics, Madurai Kamaraj University, India for their help in editing the manuscript. The authors also acknowledge the financial assistance from Department of Biotechnology (DBT), Council of Scientific and Industrial Research (CSIR) and University of Delhi, Delhi India.

References


Bioethanol production from Lantana camara (red sage): Pretreatment, saccharification and fermentation

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Abstract

Lantana camara contains 61.1% (w/w) holocellulose and can serve as a low-cost feedstock for bioethanol production. Acid hydrolysis (3.0%, v/v H2SO4, 120 °C for 45 min) of L. camara produced 187.14 mg/g total sugars along with fermentation inhibitors such as phenolics (8.2 mg/g), furfurals (5.1 mg/g) and hydroxy methyl furfurals (6.7 mg/g). Sequential application of overliming (pH 10.0) and activated charcoal (1.5%, w/v) adsorption was used to remove these toxic compounds from the acid hydrolysate. The acid-pre-treated biomass of L. camara was further delignified through combined pretreatment of sodium sulphite (5.0%, w/v) and sodium chloride (3.0% w/v), which resulted in about 87.2% lignin removal. The enzymatic hydrolysis of delignified cellulosic substrate showed 80.0% saccharification after 28 h incubation at 50 °C and pH 5.0. Fermentation of acid and enzymatic hydrolysates with Pichia stipitis and Saccharomyces cerevisiae gave rise to 5.16 and 17.7 g/L of ethanol with corresponding yields of 0.32 and 0.48 g/g after 24 and 16 h, respectively.

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1. Introduction

Lantana camara, commonly known as red sage, is one of the world’s top 100 worst invasive species. It has invaded millions of hectares of grazing land globally (Day et al., 2003). In Australia alone, since its introduction as an ornamental plant in the 1840’s it has spread to infest four million hectares (www.weeds.org.au). While in India, the weed has invaded most of the tropical and sub-tropical parts and is found in areas from the sea coast to 5000 ft in altitude (Sankaran, 2007). The approximate total biomass produced by L. camara per year ranges from 15 to 17 tonnes/ha (Bhatt et al., 1994), which projects the availability of Lantana biomass in huge quantity. Therefore, the abundance of L. camara biomass (lignocellulosic material) is likely to offer a potential feedstock for ethanol production.

Currently, the biomass to ethanol conversion technology relies mainly on chemical and enzymatic treatments. Chemical hydrolysis of biomass with dilute sulphuric acid has long been recognized as a critical step for removing the hemicellulosic fraction from the lignocellulosic substrate to economize the biological conversion of cellulosic biomass to ethanol. However, the pentose sugar-rich acid hydrolysate also contains toxic byproducts such as furfural, hydroxy methyl furfural (HMF) and phenolics, which significantly affect yeast cell metabolism during fermentation (Palmqvist and Hahn-Hagerdal, 2000; Chandel et al., 2007). Although various detoxification methods have been investigated for the removal of fermentation inhibitory compounds (Palmqvist and Hahn-Hagerdal, 2000; Chandel et al., 2007), of which overliming and activated charcoal adsorption methods are widely used (Miyafuji et al., 2003; Gupta et al., 2009).

An appropriate delignification strategy is essential for the efficient enzyme hydrolysis of cellulosic biomass as lignin hinders the saccharification process. The cellulose components such as β-glucosidase and endoglucanase showed higher binding affinity towards lignin compared to carbohydrates, which in turn lowered the saccharification efficiency (Kaya et al., 2000). Various delignification approaches have been exploited in the past such as alkali pretreatment (Carillo et al., 2005), hydrogen peroxide pretreatment (Saha and Cotta, 2007), sulphite pretreatment (Kuhad et al., 1999), ammonia fiber expansion pretreatment (Teymour et al., 2005) and sodium chloride pretreatment (Gupta et al., 2009).

The efficient conversion of biomass into ethanol requires optimum utilization of both pentose and hexose sugars. The widely studied yeast for hexose fermentation, Saccharomyces cerevisiae, is unable to utilize pentose sugars while, among the pentose fermenting yeasts only Pichia stipitis, Pachysolen tannophilus and Candida shehatae are proven to be highly efficient in the conversion of xyllose to ethanol (Abbi et al., 1996a,b). The fermentation of both types of sugars can be carried out either by co-cultivation or
through separate fermentation. Co-cultivation has several drawbacks such as the diauxic phenomenon (inefficient utilization of xylose in presence of glucose) (Grootjen et al., 1991), and competition between organisms for nutrients requirements. Recently some studies on separate fermentation of individual hydrolysates using sugar specific yeast strains have been reported (Gupta et al., 2005; Laser et al., 2009).

The aim of the present work was to extract maximum sugars with minimum inhibitory compounds from lignocellulosic substrate, *L. camara*, using less stringent acid hydrolysis conditions. We attempted to remove the toxic substances produced during acid hydrolysis employing sequential overliming and activated charcoal adsorption. The delignification of pretreated biomass was carried out by a combinatorial approach using sodium chlorite and sodium sulphite. The pentose and hexose sugar hydrolysates were subsequently fermented separately using *P. stipitis* and *S. cerevisiae*.

2. Methods

2.1. Biomass collection and preparation

*L. camara* was collected from Aravali Hills, University of Delhi South Campus, New Delhi, India. Dried substrate was comminuted by the combination of chopping and milling to attain a particle size of 1–2 mm using laboratory Knife mill (Metrax Scientific Instrumentation Pvt., New Delhi, India). The chopped plant material was washed thoroughly with tap water and dried overnight at 60 °C.

2.2. Biomass composition analysis

The chemical composition of *L. camara* was analysed for holocellulose, Klason lignin, pentosans, ash and moisture content. The raw material was consecutively extracted with alcohol–benzene (1:2, v/v) mixture. The extractive-free *L. camara* dust was processed for chemical analyses following the TAPPI (1992) protocols.

2.3. Optimization of dilute acid pretreatment

The optimization of dilute acid hydrolysis was carried out at different temperatures (100–140 °C), time periods (30 and 45 min) and H₂SO₄ concentration (1–5%, v/v) at 10.0% (w/v) solid content. The hydrolysates recovered were filtered through double-layered muslin cloth, and the remaining biomass was washed properly with tap water and dried overnight at 60 °C.

2.4. Detoxification of acid hydrolysate

The acid hydrolysate of *L. camara* was mixed with calcium hydroxide to raise the pH of the hydrolysate to 10.0. The whole slurry was stirred for 30 min by an overhead stirrer (Remi Motors Ltd., Mumbai, India). After bringing to room temperature, the hydrolysate was neutralized with concentrated H₂SO₄ and then centrifuged (10,000g, 15 min). The overlimed hydrolysate was further detoxified by mixing 1.5% (w/v) activated charcoal with continuous stirring for 30 min at room temperature and the resulting sugar was recovered using vacuum filtration.

2.5. Delignification

The acid-pretreated residue of *L. camara* was treated with different dosages of sodium sulphite (5.0–20.0% w/v) alone and in combination with sodium chloride (3.0% w/v) at different temperatures (100–140 °C) and time intervals (30 and 45 min). The delignified biomass was then filtered through double-layered muslin cloth and the cellulosic residue was washed thoroughly with tap water until a neutral pH was achieved and dried overnight at 60 °C.

2.6. Enzyme hydrolysis

Commercial cellulase (6.0 FPU/mg) from *Trichoderma reesei* (ATCC 26921) and β-glucosidase (250 U/mL) from *Aspergillus niger* (Novozyme 188) were purchased from Sigma (St. Louis, MO, USA).

The delignified cellulosic residue of *L. camara* was suspended in 0.05 M citrate phosphate buffer (pH 5.0) at 50 °C with a solid content of 5% (w/v) and soaked in a rotary incubator shaker (Innova-40, New Brunswick Scientific, NJ, USA) for 2 h. The soaked suspension was further supplemented with cellulase (3 FPU/mL) and β-glucosidase (Novozyme 188) (9 U/mL). Enzymatic hydrolysis was performed at 50 °C and 150 rpm for 36 h. A dose of 0.005% sodium azide was introduced to avoid any microbial contamination; and 1.0% (v/v) of Tween 80 was added to facilitate the enzymatic action. Samples were withdrawn every 4 h and subsequently analysed for glucose released in the reaction mixture.

2.7. Ethanol fermentation

2.7.1. Microorganisms

*P. stipitis* NCIM 3498 was obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India and was maintained in a medium having (g/L): xylose, 20; yeast extract, 3; peptone, 5; malt extract, 3; agar, 20 at pH 5.0 ± 0.2 and temperature 30 °C (Nigam, 2001). The *S. cerevisiae* strain from our laboratory was maintained in medium containing (g/L): glucose, 30; yeast extract, 3; peptone, 5; agar, 20 at pH 6.0 ± 0.2 and temperature 30 °C (Chen et al., 2007).

The *P. stipitis* inoculum was prepared as described by Nigam (2001) using (g/L): xylose, 50.0; yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; at pH 5.0 ± 0.2 and temperature 30 °C. *S. cerevisiae* inoculum was developed by growing the cells at 30 °C for 24 h in a culture medium containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; (NH₄)₂HPO₄, 0.25; pH 6.0 ± 0.2 (Chen et al., 2007). Cells were grown to an optical density (OD₆₀₀) of 0.6.

2.7.2. Fermentation

The fermentation of acid and enzymatic hydrolysates was carried out separately in an *in situ* sterilizable 12 L fermentor (B-Lite Sartorius India Ltd., Bengaluru, India) with a working volume of 10 L. The acid hydrolysate (16.83 g/L sugars) supplemented with (g/L): NH₄Cl, 0.5; KH₂PO₄, 2.0; MgSO₄·7H₂O, 0.5; yeast extract, 1.5; CaCl₂·2H₂O, 0.1; FeCl₃·2H₂O, 0.1; ZnSO₄·7H₂O, 0.001 (pH 5.5 ± 0.2), was inoculated with 10% (v/v) inoculum of *P. stipitis* (OD₆₀₀ 0.6) and incubated at 30 °C for 36 h under shaking conditions (150 rpm). The cellulose hydrolysate (34.75 g/L sugars, pH 6.0 ± 0.2) aided with 3 g/L yeast extract and 0.25 g/L (NH₄)₂HPO₄ was inoculated with *S. cerevisiae* (10.0% v/v). The pH of the medium was adjusted with 2 N HCl and 2 N NaOH. The silicone-based anti-foaming agent (10.0% v/v) was used to control the foaming, whenever required. The dissolved oxygen concentration was monitored continuously throughout the process using a dissolved oxygen probe and a air flow at 0.4 L/min (lpm) was maintained through out the study. Samples were withdrawn at regular intervals of 4 h and centrifuged at 10,000g for 15 min at 4 °C. The cell free supernatant was used to determine the ethanol and residual sugar concentration.
2.8. Fourier Transform Infrared Spectroscopy (FTIR) characterization of biomass samples

The untreated, acid-pretreated and delignified L. camara materials were characterized according to Singh et al. (2005). A spectrum FTIR (FTIR-1, Perkin-Elmer, MA, USA) was used to characterize the KBr-discs of different samples, which were prepared by grinding 1.0 mg sample/100 mg pre-dried KBr. The spectra were recorded in the range of 400–4000 cm⁻¹.

2.9. Analytical methods

The hydrolysates were analysed using high performance liquid chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan) for estimating the presence of carbohydrates. An Aminex (Bio-Rad, Hercules, CA, USA) column (300.0 × 7.8 mm) was used, with 0.04 M H₂SO₄ as an eluent with the flow rate of 0.5 mL/min keeping the oven temperature at 60 °C with a RID detector. Furans, furfurals and lignin derivatives were estimated with Luna 5U C18 column (250.0 × 7.8 mm) with an ethylene-wax (cross bond-polyethylene glycol) column (30.0 mm × 0.25 mm) at an oven temperature of 85 °C and flame ionization detector (FID) at 200 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Darmstadt, Germany). Nitrogen with a flow rate of 0.5 mL/min was used as the carrier gas.

Total reducing sugars were estimated by the DNS method of Miller and the enzymatic saccharification efficiency was calculated as:

\[
\text{Saccharification (\%)} = \frac{\text{Amount of glucose released}}{\text{Total sugar concentration in the pretreated substrate}} \times 100
\]

Moreover, the total phenolics released were determined by the Folin–Ciocalteu reagent method (Singleton et al., 1999) using vanillin as a standard. The OD₄₉₀ of culture filtrate was measured with a double beam spectrophotometer (Specord 200, Analytical Jena, Germany) and the dry biomass of yeast cells was measured after drying the pellets at 70 °C until constant weight.

2.10. Statistical analysis

All the experiments were performed in triplicate and the results are presented as mean ± standard deviation.

3. Results and discussion

3.1. Compositional analysis of L. camara

The comminuted and oven dried wood pieces (40–60 mesh size) of L. camara were found to contain holocellulose (61.1 ± 2.53%) comprising cellulose (44.1 ± 1.72%) and pentosans (17.0 ± 0.81%); lignin (32.25 ± 1.57%), ash (2.30 ± 0.11%) and moisture (4.35 ± 0.13%). The presence of 61.1% of total carbohydrates (holocellulose) makes it a potential and renewable material for bioethanol production.

3.2. Dilute acid hydrolysis

Among the different temperatures, the maximum sugar yield (191.43 ± 1.91 mg/g) was obtained when L. camara was hydrolysed with 5% (v/v) H₂SO₄ at 140 °C for 45 min. No significant difference in sugars were observed when the substrate was treated with 3.0% (v/v) H₂SO₄ at 120 °C for 45 min (Table 1). Increasing the pretreatment period beyond 45 min, resulted in a decrease in the final sugar concentration (data not shown). This may be due to the degradation of sugars at severe conditions. The acid hydrolysate obtained at optimum condition of hydrolysis when analysed was found to be majorly consisted of (g/L): xylose (14.29 ± 0.82), glucose (2.21 ± 0.09), arabinose (1.49 ± 0.08), HMF (0.67 ± 0.004), furfural (0.51 ± 0.03) and phenolics (0.82 ± 0.05). The furfural and HMF are degradation products of pentose and hexose sugars, respectively, while, phenolics are lignin degradation byproducts. Furfural and HMF are known to decrease fermentability by reducing the activities of several yeast enzymes e.g., alcohol dehydrogenase, alcohol dehydrogenase and pyruvate dehydrogenase (Modig et al., 2002). The furfurals and HMF are degradation products ofpentose and hexose sugars, which has been observed that the accumulation ofacetaldehyde also inhibits the microbial growth (Taherzadeh et al., 2000).

Among different detoxification treatments, overliming (Chandel et al., 2007) and activated charcoal (Miyafuji et al., 2003; Chandel

<table>
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<tr>
<th>Temperature (°C)</th>
<th>Acid concentration (% v/v)</th>
<th>Time (min)</th>
<th>Sugar concentration (g/L)</th>
<th>Sugar yield (mg/g dry substrate)</th>
<th>Phenolics concentration (g/L)</th>
<th>Phenolics yield (mg/g dry substrate)</th>
<th>Sugar concentration (g/L)</th>
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et al., 2007) are commonly used. However, no single method is efficient at removing all the inhibitors present in acid hydrolysates. Thus, we attempted a sequential treatment of overliming and activated charcoal adsorption for the detoxification of acid hydrolysates from *Prosopis juliflora* with our earlier work on the detoxification of acid hydrolysates (Kuhad et al., 1999).

### 3.3. Delignification of *L. camara*

Since a significant negative correlation exists between the percentage of lignin in plant material and its enzymatic digestibility (Kaya et al., 2000), in order to improve the enzymatic hydrolysis of *L. camara*, the lignin removal is essential. The chemical delignification of acid treated *L. camara* displayed a continuous increase in phenolics with the increase in sodium sulphite from 5.0% to 20.0% (w/v) (Table 2). The acid treated *L. camara* when treated with 20.0% sodium sulphite at 140 °C for 45 min resulted in 77.0% delignification with phenolics yield (248.33 ± 7.59 mg/g). However, the use of 3.0% sodium chlorite along with sodium sulphite (5.0% w/v) reduced the sodium sulphite requirement from 20.0% to 5.0% and delignified the *L. camara* by 87.16% with phenolics yield (281.1 ± 9.37 mg/g). Sodium chlorite is an elemental chlorine free compound, which on heating produces chlorine dioxide that acts as a delignifying agent and depolymerizes the lignin (Hamzeh et al., 2006; Gupta et al., 2009). Earlier we had used sodium sulphite for the pretreatment of sugarcane bagasse (Kuhad et al., 1999) and sodium chlorite for *P. juliflora* (Gupta et al., 2009) in our laboratory. The chemical delignification pretreatments not only remove the lignin but also act as a swelling agent, which in turn enhances the surface area of the substrate made accessible for enzymatic action (Kuhad et al., 1999).

### 3.4. FTIR characterization

The FTIR spectroscopy is extensively used for lignocellulose characterization since it presents a relatively easy method of obtaining direct information on chemical changes that occurs during various chemical pretreatments (Ristolainen et al., 2002). The FTIR spectra of untreated, acid treated and delignified *L. camara* depicted that as compared to hemicellulose and cellulose, a large difference was found in the fingerprint region (1830–730 cm⁻¹) for lignin’s IR spectrum (Electronic Annexure I). All spectra had a sharp peak at 898 cm⁻¹, which is characteristic of β-glycosidic linkages between the sugar units, as it has been earlier reported by Gupta et al. (1987). A prominent band of hemicellulose was observed at 1370 cm⁻¹, which was subsequently removed in acid treated substrate. The acid treated substrate showed a reduction in the band at 1238 and 1738 cm⁻¹, which are indicative of hemicellulose–lignin linkage and C=O stretching due to carbohydrate linked with lignin, respectively (Lin and Dence, 1992). Results also showed a band at 655 cm⁻¹ that is a characteristic feature of lignosulphonates (Nada et al., 1998) occurring after the action of sulphuric acid over lignin. The absence of this band in delignified substrate shows removal of lignin (Singh et al., 2005). The aromatic C=C stretch from aromatic ring of lignin gave two peaks at 1508 and 1458 cm⁻¹ in untreated and at 1509 and 1457 cm⁻¹ in acid treated substrates. The FTIR spectra of delignified substrate after chlorite treatment of acid-pretreated substrate revealed noticeable changes at the bands relating to aromatic ring vibration at 1508–

### Table 2

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Delignifying agents (% w/v)</th>
<th>Phenolics yield (mg/g dry substrate)</th>
<th>Delignification (%)</th>
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</tr>
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<td>5 A + 3 B</td>
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<td>87.16</td>
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<td>87.18</td>
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</tr>
</tbody>
</table>

![Fig. 1.](image-url) Enzymatic saccharification profile of delignified *L. camara*. Where, dark square is saccharification rate (mg/g/h), white square is saccharification yield (mg/g) and the triangle is sugar concentration (g/L).

### References

Kaya et al., 2000, in order to improve the enzymatic hydrolysis of *L. camara*, the lignin removal is essential. The chemical delignification of acid treated *L. camara* displayed a continuous increase in phenolics with the increase in sodium sulphite from 5.0% to 20.0% (w/v) (Table 2). The acid treated *L. camara* when treated with 20.0% sodium sulphite at 140 °C for 45 min resulted in 77.0% delignification with phenolics yield (248.33 ± 7.59 mg/g). However, the use of 3.0% sodium chlorite along with sodium sulphite (5.0% w/v), reduced the sodium sulphite requirement from 20.0% to 5.0% and delignified the *L. camara* by 87.16% with phenolics yield (281.1 ± 9.37 mg/g). Sodium chlorite is an elemental chlorine free compound, which on heating produces chlorine dioxide that acts as a delignifying agent and depolymerizes the lignin (Hamzeh et al., 2006; Gupta et al., 2009). Earlier we had used sodium sulphite for the pretreatment of sugarcane bagasse (Kuhad et al., 1999) and sodium chlorite for *P. juliflora* (Gupta et al., 2009) in our laboratory. The chemical delignification pretreatments not only remove the lignin but also act as a swelling agent, which in turn enhances the surface area of the substrate made accessible for enzymatic action (Kuhad et al., 1999).

#### 3.4. FTIR characterization

The FTIR spectroscopy is extensively used for lignocellulose characterization since it presents a relatively easy method of obtaining direct information on chemical changes that occurs during various chemical pretreatments (Ristolainen et al., 2002). The FTIR spectra of untreated, acid treated and delignified *L. camara* depicted that as compared to hemicellulose and cellulose, a large difference was found in the fingerprint region (1830–730 cm⁻¹) for lignin’s IR spectrum (Electronic Annexure I). All spectra had a sharp peak at 898 cm⁻¹, which is characteristic of β-glycosidic linkages between the sugar units, as it has been earlier reported by Gupta et al. (1987). A prominent band of hemicellulose was observed at 1370 cm⁻¹, which was subsequently removed in acid treated substrate. The acid treated substrate showed a reduction in the band at 1238 and 1738 cm⁻¹, which are indicative of hemicellulose–lignin linkage and C=O stretching due to carbohydrate linked with lignin, respectively (Lin and Dence, 1992). Results also showed a band at 655 cm⁻¹ that is a characteristic feature of lignosulphonates (Nada et al., 1998) occurring after the action of sulphuric acid over lignin. The absence of this band in delignified substrate shows removal of lignin (Singh et al., 2005). The aromatic C=C stretch from aromatic ring of lignin gave two peaks at 1508 and 1458 cm⁻¹ in untreated and at 1509 and 1457 cm⁻¹ in acid treated substrates. The FTIR spectra of delignified substrate after chlorite treatment of acid-pretreated substrate revealed noticeable changes at the bands relating to aromatic ring vibration at 1508–
and 1457–1458 cm\(^{-1}\). The disappearance of these bands showed that lignin was largely removed in comparison to polysaccharides during the chlorite pretreatment (Sun et al., 2005). The absorbances at 1428, 1331, 1281, 1111, 1056 and 899 cm\(^{-1}\) were associated with the typical value of cellulose (Sun et al., 2005).

3.5. Enzymatic saccharification of delignified \textit{L. camara}

During the course of enzymatic saccharification, a perpetual increase in sugar concentration was observed till 28 h, which on prolonged incubation remained almost constant (Fig. 1). However, the optimal saccharification yield (777.13 mg/g) was achieved after 28 h with a saccharification rate of 24.29 g/L/h (Fig. 1). The maximum rate of saccharification was achieved after 4 h of incubation, which thereafter it started declining as reported earlier (Gupta et al., 2009). The decline in hydrolysis rate could be due to the increasing resistance of the substrate during the course of hydrolysis or other factors. End product inhibition of the enzymes by glucose and cellobiose may also have a major impact on cellulose hydrolysis (Kuhad et al., 1999). Moreover, the enzymatic saccharification

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Fermentation profile of detoxified acid hydrolysate of \textit{L. camara} by \textit{P. stipitis}.</th>
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</thead>
<tbody>
<tr>
<td>Time (h)</td>
<td>Ethanol (g/L)</td>
</tr>
<tr>
<td>0</td>
<td>0.05 ± 0.003</td>
</tr>
<tr>
<td>4</td>
<td>0.27 ± 0.009</td>
</tr>
<tr>
<td>12</td>
<td>1.49 ± 0.060</td>
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<tr>
<td>16</td>
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<tr>
<td>20</td>
<td>4.11 ± 0.234</td>
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<tr>
<td>24</td>
<td>5.16 ± 0.371</td>
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<tr>
<td>28</td>
<td>4.49 ± 0.174</td>
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<td>32</td>
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<table>
<thead>
<tr>
<th>Table 4</th>
<th>Fermentation profile of enzymatic hydrolysate of \textit{L. camara} by \textit{S. cerevisiae}.</th>
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<td>Time (h)</td>
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<tr>
<td>8</td>
<td>15.7 ± 0.760</td>
</tr>
<tr>
<td>12</td>
<td>16.5 ± 0.462</td>
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<tr>
<td>16</td>
<td>17.7 ± 0.955</td>
</tr>
<tr>
<td>20</td>
<td>15.9 ± 0.845</td>
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<tr>
<td>24</td>
<td>15.0 ± 0.540</td>
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</tbody>
</table>

**Fig. 2.** Mass balancing of the complete bioconversion process for ethanol production from \textit{L. camara}.
fication efficiency (77.7%, w/w) obtained was in agreement with other earlier reports about saccharification of pretreated substrates with a saccharification efficiency of 67.6%, 70.0% and 80.0% (w/w) from chlorite pretreated sugarcane bagasse (Adsul et al., 2005), alkali treated rice straw (Jeya et al., 2009) and chlorite pretreated P. juliflora (Gupta et al., 2009), respectively.

3.6. Fermentation

Both acid and enzymatic hydrolysates were fermented with P. stipitis and S. cerevisiae, respectively. The detoxified xylose rich hydrolysate obtained after dilute acid hydrolysis (16.83 ± 0.04 g/L sugars), when fermented with P. stipitis, resulted in ethanol production of 5.16 ± 0.37 g/L with yield 0.33 g/g and productivity of 0.23 g/L/h after 24 h. The biomass yield and productivity were found to be 0.37 g/g and 0.26 g/L/h, respectively (Table 3). However, fermentation of cellulose hydrolysate (34.75 ± 1.54 g/L sugars) using S. cerevisiae resulted in a maximum ethanol content of 17.7 ± 0.96 g/L with yield of 0.48 g/g and productivity of 1.11 g/L/h with a biomass yield (0.22 g/g) and productivity (0.47 g/L/h) after 16 h incubation (Table 4). Our results were in agreement with previous reports on fermentation with P. stipitis (Kapoor et al., 2008; Gupta et al., 2009). The ethanol yield obtained from cellulose hydrolysate using S. cerevisiae in our case (0.48 g/g) was higher or comparable to some of the previous reports of ethanol yield from corn cob (0.48 g/g) (Chen et al., 2007) and from P. juliflora (0.49 g/g) (Gupta et al., 2009). The S. cerevisiae produced maximum ethanol after 16 h and it declined thereafter (Table 4). The decline in ethanol production after 16 h incubation can be due to the consumption of accumulated ethanol by the organism. According to Ramon-Portugal et al., 2004, when the ethanol accumulated in the medium, the microbial population was adapted to consume simultaneously sugar and ethanol. The increased yeast biomass could be because of the utilization of the yeast extract present in fermentation medium. The increase biomass in pentose and hexose fermentation is in accordance with the observations of Wang et al. (2004) and Lau and Dale (2009).

Moreover, a mass balance study of the ethanol production from L. camara has also been elucidated in Fig. 2.

4. Conclusion

Bioethanol from lignocellulosics is a globally accepted alternative fuel. The production of ethanol from L. camara would have the dual advantage of producing energy and serving as an effective method of weed management. The present study demonstrates that the pretreatment (acid hydrolysis and delignification) of L. camara can provide cellulose material, which eventually is hydrolyzed to hexose sugars. Thus pentose and hexose sugars can be separately fermented to ethanol. Developing a fermentation system with yeast strains capable of fermenting both hexose and pentose sugars simultaneously would economize ethanol production from lignocellulosic biomass.

Acknowledgements

The authors are thankful to Dr. A.J. Varma, Polymer Science Division, National Chemical Laboratory, Pune for FTIR analysis of our samples. The authors also thank Ms. Urvashi Kuhad, Department of English, University of Delhi, Delhi, for editing the manuscript. The financial support from Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India is highly acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.06.043.

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Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*

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**Abstract**

The newspaper is comprised of (w w\(^{-1}\)) holocellulose (70.0%) with substantial amount of lignin (16.0%). Bioconversion of the carbohydrate component of newspaper to sugars by enzymatic saccharification, and its fermentation to ethanol was investigated. Of various enzymatic treatments using cellulase, xylanase and laccase, cellulase enzyme system was found to deink the newspaper most efficiently. The saccharification of deinked paper pulp using enzyme cocktail containing exoglucanase (20 U g\(^{-1}\)), β-glucosidase (60 U g\(^{-1}\)) and xylanase (80 U g\(^{-1}\)) resulted in 59.8% saccharification. Among additives, 1% (v v\(^{-1}\)) Tween 80 and 10 mol m\(^{-3}\) CoCl\(_2\) improved the enzymatic hydrolysis of newspaper maximally, releasing 14.64 g L\(^{-1}\) sugars. The fed batch enzymatic saccharification of the newspaper increased the sugar concentration in hydrolysate from 14.64 g L\(^{-1}\) to 38.21 g L\(^{-1}\). Moreover, the batch and fed batch enzymatic hydrolysates when fermented with *Saccharomyces cerevisiae* produced 5.64 g L\(^{-1}\) and 14.77 g L\(^{-1}\) ethanol, respectively.

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**1. Introduction**

Presently most of the bioethanol is produced using corn kernel or sugarcane molasses but lignocellulosics, the second generation biofuel substrate and the least explored renewable resource is yet to be tapped. Lignocellulosics are the most abundant biomass available on earth, comprising mainly of cellulose and hemicellulose [1,2]. Among various lignocellulosics, the recycled books, magazines and newspaper could have value addition via deinking and reuse of fiber either in manufacturing of new paper or in ethanol production.

An efficient conversion of newspaper to ethanol depends mainly on the extent of carbohydrate saccharification, however, the enzymatic conversion of newspaper is majorly hindered by toner’s ink, which forms a physical barrier, restricting the hydrolysable sites as well as release fermentation inhibitory heavy metal ions [3,4]. Among various enzymes tested to deink the newspaper pulp [5], only cellulases were found to be the most effective [6]. The deinked paper pulp can be saccharified using either acid [7,8] or enzymes [9–11], however, enzymes are preferred over acid because enzymatic hydrolysates are free from any fermentation inhibitory products. The enzymes such as cellulases have been found to be the most effective for this purpose [12]. It is well known that combined action of cellulase and hemicellulase results in a higher sugar production as compared to
cellulase alone [13]. Despite the high extent of saccharification efficiency, the lower substrate consistency in the enzymatic suspension resulted into lower sugar concentration during the enzymatic hydrolysis. Raising the substrate concentration in batch hydrolysis helps to obtain higher sugar concentration, but also causes mixing and heat transfer problem due to rheological properties of dense fibrous suspension [14]. While in fed batch hydrolysis, such problem could be avoided by adding the substrate gradually to maintain the low level of viscosity [15]. In the present study, the optimization of enzymatic saccharification using both cellulase and xylanase was carried out to improve the hydrolysis of the newspaper. In order to increase the sugar concentration in enzymatic hydrolysate, the fed batch enzymatic hydrolysis of deinked newspaper was carried out. Thereafter, the enzymatic hydrolysates comprising both the hexose and pentose sugars were fermented to ethanol using Saccharomyces cerevisiae.

2. Materials and methods

2.1. Raw material and chemicals

The newspaper procured locally was shredded mechanically into small pieces and the processed substrate was dried in an oven at 80 °C until constant weight was obtained.

Commercial cellulase from Trichoderma reesei (ATCC 26921), β-glucosidase from Aspergillus niger (Novozyme 188), xylanase from Thermomyces lanuginosus, 1-hydroxybenzotriazole (HOBT) and 3.5-di nitro salicylic acid (DNS) were purchased from Sigma (St. Louis, Missouri, U.S.A.). Ethanol was purchased from Merck (Darmstadt, Germany). Laccase was produced in our laboratory as described earlier [16]. Rest of the chemicals and media components of highest purity grade were purchased locally.

2.2. Microorganism and culture conditions

S. cerevisiae RCK-1 was procured from culture collection of Department of Microbiology, University of Delhi South Campus, New Delhi, India and was maintained on agar slants containing (g L−1): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; agar, 20.0 at pH 6.0 ± 0.2 and temperature 30 °C. Inoculum of S. cerevisiae was prepared in medium containing (g L−1) glucose, 5.0; yeast extract, 3.0; peptone, 5.0; (NH₄)₂HPO₄, 0.25 media constituents at pH 5.5 ± 0.2 [15].

2.3. Compositional analysis of newspaper

The chemical composition of newspaper was analysed for holocellulose, α-cellulose, pentosans, moisture and ash content following the Technical Association of Pulp and Paper Institutes (TAPPI) methods [17].

2.4. Deinking of newspaper

The deinking of newspaper was carried out using flotation method at 2.0% (w v−1) consistency having various enzyme systems such as cellulase, xylanase, laccase with HOBT (LH) and laccase without HOBT (LB). The waste paper slurry was incubated at 50 °C for cellulase [18], 60 °C for xylanase [19] and 30 °C for laccase [16].

2.5. Enzymatic hydrolysis

2.5.1. Batch enzymatic hydrolysis

Enzymatic hydrolysis of deinked newspaper was carried out at 2% (w v−1) consistency in a 50 mol m−3 citrate phosphate buffer (pH 5.0) containing 0.005% (w v−1) sodium azide. Before enzyme loading, slurry was preincubated at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, Germany) at 2.5 Hz for 2 h. Thereafter, to obtain the optimized enzyme doses for the saccharification of deinked newspaper pulp, varied doses of exoglucanase (5–30 U g−1), β-glucosidase (15–90 U g−1), and xylanase (20–100 U g−1) were added to the preincubated slurry. Different types of additives such as nonionic surfactants (Tween, Polyethylene glycol and Triton X-100) and metal ions were also examined to study their effect on the extent of saccharification efficiency. Samples were withdrawn at regular intervals of 4 h, centrifuged at 2.5 Hz for 15 min in a centrifuge (Sigma, Germany) and the supernatant was analysed for total reducing sugars released.

2.5.2. Fed batch enzymatic hydrolysis

Fed batch enzymatic hydrolysis was carried out at optimized conditions of saccharification with an initial substrate consistency of 2% (w v−1) in the suspension. The substrate was added twice at 20th and 40th hour to get a final substrate concentration of 60 g L−1. The samples were withdrawn at regular intervals of 4 h and analysed for the release of total reducing sugars.

2.6. Fermentation of enzymatic hydrolysate

The fermentation studies of enzymatic hydrolysate were carried out in 2.0 L Erlenmeyer flasks. The batch and fed batch enzymatic hydrolysates containing 14.64 g L−1 and 38.21 g L−1 sugars, respectively, supplemented with 3.0 g L−1 yeast extract and 0.25 g L−1 (NH₄)₂HPO₄ were inoculated with 10% (v v−1) S. cerevisiae. The flasks were incubated at 30 °C, 3.33 Hz and pH 5.5 ± 0.2. The pH was adjusted with 2 N HCl and 2 N NaOH. Samples were withdrawn at regular intervals of 4 h and centrifuged at 2.5 Hz for 15 min at 4 °C. The cell free supernatant was used for the determination of ethanol produced and sugar consumed.

2.7. Enzyme assays

All the cellulase assays were carried out in 50 mol m−3 citrate phosphate buffer (pH 5.0) unless otherwise stated. Endoglucanase and exoglucanase activities were determined following International Union of Pure and Applied Chemistry (IUPAC) methods [20]. For the estimation of endo and exoglucanase, 2.0% carboxymethyl cellulose (CMC) and 50.0 mg Whatman No. 1 filter paper, respectively, were used as the substrates. The release of sugars was determined by the DNS method [21]. One unit of endo and exoglucanase activity is defined as the amount of enzyme required to produce 1.0 mmol m−3 of glucose equivalents per min under assay conditions. β-glucosidase activity was determined by taking
0.1% p-nitrophenol β-D-glucopyranoside (pNPc) as substrate and the activity was calculated as the enzyme required to liberate 1.0 mmol m⁻³ of p-nitrophenol (pNP) per minute under the assay conditions. Xylanase activity was quantified by measuring the release of reducing sugar groups by DNS at 60 °C using 1% (w v⁻¹) birch wood xylan in citrate phosphate buffer (200 mol m⁻³), pH 6.0 [19]. One unit of xylanase was defined as amount of enzyme required to release 1.0 mmol m⁻³ of xylose in 1 min under the assay conditions. The activity of laccase was determined as described previously [22]. The reaction was set by adding 0.2 mL of culture supernatant in 1.8 mL of 10 mol m⁻³ guaiacol in 100 mol m⁻³ citrate phosphate buffer (pH 5.4). The reaction mixture was incubated at 26 °C for 10 min and color development was assayed at 470 nm on a UV–VIS spectrophotometer (Analytical Jena, Specord 205, Germany). Heat denatured enzyme served as control. One unit of laccase was defined as the change in absorbance of 0.01 mL⁻¹ min⁻¹ at 470 nm.

### 2.8. Analytical methods

Ethanol was estimated by gas chromatography (GC) (Perkin Elmer, Clarus 500) with an elite-wax (cross bond-polyethylene glycol) column (30.0 m × 0.25 mm), at oven temperature 85 °C and flame ionization detector (FID) at 200 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Germany). Nitrogen with a flow rate of 0.5 mL min⁻¹ was used as carrier gas. The extent of deinking (Chromophore (Merck, Germany). Nitrogen with a flow rate of 0.5 mL min⁻¹ was used as carrier gas. The extent of deinking (Chromophore) released) of newspaper was measured with a UV–visible spectrophotometer at 528 nm, the wavelength at which treated filtrate gave maximum absorbance peak. Total reducing sugars were estimated by the DNS method [21] and the saccharification efficiency was calculated as follows:

\[
\text{Saccharification efficiency(%) = } \frac{\text{Reducing sugar concentration obtained} \times 100}{\text{Total carbohydrate content in the substrate}}
\]

### 3. Results and discussion

#### 3.1. Compositional analysis of newspaper

Newspaper was found to contain (w w⁻¹), β-cellulose (51.0%), pentosans (19.0%), lignin (16.0%), moisture (8.0%) and ash (2.0%). The high carbohydrate content (holocellulose, 70.0% w w⁻¹) in the newspaper made it a substrate of choice for bioethanol production. The results obtained here were similar to earlier studies, where lignin, holocellulose and moisture content in newspaper were found to be 16–22%, 60–75% and 7–10%, respectively [7,8].

#### 3.2. Deinking of newspaper celluloses

The dyes fixed on the repeating units of cellulose backbone block the sequential hydrolysis of paper by the enzymes and inhibited the hydrolysis kinetics significantly [4,23]. Therefore, many enzyme systems were used to study their effect on the deinking of newspaper. The comparative efficiency of different enzyme systems to deink the newspaper has been shown in Fig. 1. The maximum deinking was obtained with cellulase system, while the minimum deinking occurred when treated with laccase with out HOBT (LB), however the deinking efficiency of newspaper using different enzyme systems were in an order of cellulase > xylanase > laccase with HOBT (LH) > laccase with out HOBT (LB) (Fig. 1). The possible mechanism may be that the enzymatic action of cellulase had released the cellulose micro-fibril matrix in which the toners are embedded. In earlier reports, a combined action of cellulase and xylanase enzymes brought about 62.0% deinking [5]. The results showed that when compared, LB enzyme system caused a significant increase in deinking efficiency than LH, this may be due to the fact that laccase itself cannot enter into pulp fibers due to size limitations, however the presence of mediator could help in transmitting electron between laccase and lignin. In this case, firstly the mediator was oxidized by laccase in presence of O₂, then the oxidized mediator diffused into pulp fiber and cause a series of oxidative degradation reactions [24]. With the removal of lignin, the bonding between fiber and ink particles is loosened, which aid to the deinking process [24]. Although there are several reports of using alkaline conditions for deinking [25–27], however, in the present work, we have demonstrated the deinking process at low pH, because, as the toners are not only associated with cellulose fibrils but also with the white pigment fillers and coating such as calcium carbonate, therefore, the slight acidic conditions could lead to the improvement of the deinking process by the removal of CaCO₃ and fillers [5,28].

#### 3.3. Enzymatic hydrolysis of deinked newspaper

#### 3.3.1. Batch enzymatic hydrolysis

3.3.1.1. Effect of enzyme dosage on the hydrolysis. Different dosages of exoglucanase (5–30 U g⁻¹), were used to enzymatically hydrolyze the deinked newspaper (Fig. 2). The sugars release increased with increase in enzyme dosage up to 20 U g⁻¹, which resulted in 23.94% saccharification after 24 h of incubation. Where as, an increase in the enzyme dosage beyond 20 U g⁻¹ did not cause any further improvement in the saccharification (Fig. 2). In earlier reports, enzymatic hydrolysis of paper material at loading of 45 FPU g⁻¹ [29] and 120 FPU g⁻¹ [30] have resulted in 46.5 and 90% saccharification, respectively, after 6 days. The lower saccharification efficiency may be possibly due to feed back inhibition by the cellulobiose moieties, which are released by the action of exoglucanase [31]. To overcome the problem of feedback inhibition, varied doses of β-glucosidase (15–90 U g⁻¹) were tested in the enzyme system containing exoglucanase (20 U g⁻¹) that
could hydrolyze the cellobiose moieties and reduce the cellobiose accumulation, which might have resulted in enhanced saccharification. Among different dosages of β-glucosidase used, addition of 60 U g⁻¹ of β-glucosidase caused 13.8% increase in sugar yield (264.13 mg g⁻¹), while further increase in dosage of β-glucosidase did not enhance the saccharification (Fig. 3). This is also in accordance with earlier reports where the addition of β-glucosidase increased the saccharification of recycled paper sludge from 45 to 100% was observed [30].

In order to enhance the enzymatic saccharification of deinked newspaper, different doses of xylanase (20–100 U g⁻¹) were added to the optimized enzymatic suspension (20 U g⁻¹ exoglucanase and 60 U g⁻¹ β-glucosidase). Among different dosages of xylanase used, the addition of 80 U g⁻¹ xylanase to the enzymatic cocktail resulted in optimum sugar yield (419.54 mg g⁻¹) (Fig. 4). The effect of combined action of cellulases and hemicellulases demonstrated that compared to cellulase alone, the addition of xylanase with cellulase enhanced the saccharification efficiency from 37.7 to 59.8%. This is because the enzymatic saccharification with xylanase cleaves the xylan backbone and releases the lignin from carbohydrate—lignin complex, which helps in improvement of saccharification of lignocellulose [19].

3.3.1.2. Effect of additives on the hydrolysis. Out of various surfactants used, Tween 80 and Triton X-100 improved the sugar release by 10–30%, while Tween 20, 40, 60 and PEG 400,
4000 and 6000 at any loading were not statistically distinguishable and reduced the sugar release by 30–40% from the control. The increase in newspaper saccharification with Tween 80 could be because of (i) the presentation of unproductive enzyme adsorption to lignin [32] and (ii) the protection of the enzymes from thermal deactivation [33]. Moreover, on increasing the concentration of Tween 80 more than 1% declines the rate of hydrolysis, which might be because of the detergent property of the surfactant, which denatures the enzymes [34].

Different metal ions were also tested to study their effect in enzymatic saccharification and it was observed that the presence of cobalt (10 mol m\(^{-3}\)) increased the saccharification efficiency by 17.1%, which is in agreement with the earlier reports [35,36]. While, other metal ions (Cu, Hg, Fe) at 10 mol m\(^{-3}\) concentration were found to inhibit the enzymatic hydrolysate of newspaper, respectively. However, the yeast did not utilize pentose sugars from the hydrolysates and non-utilization of pentose sugars limits the economization of ethanol production from cellulosics. Therefore, in order to improve the ethanol fermentation from lignocellulosic waste, the reuse of newspaper for the production of bioethanol seems feasible approach for energy conservation and waste minimization. The fed batch enzymatic hydrolysis of newspaper cellulosics improved the release of sugars (pentose and hexose) and subsequently the ethanol yield. However, the yeast did not utilize pentose sugars from the hydrolysates and non-utilization of pentose sugars limits the economization of ethanol production from cellulosics. Therefore, in order to improve the ethanol fermentation from lignocellulosic waste,
it is necessary to develop recombinant yeast strain capable of fermenting both sugars simultaneously.

Acknowledgement

The authors are thankful to Dr. Y. P. Khasa for his help during the preparation of this manuscript. The financial support from University of Delhi, Delhi, India is highly acknowledged.

References


Separate hydrolysis and fermentation (SHF) of Prosopis juliflora, a woody substrate, for the production of cellulosic ethanol by Saccharomyces cerevisiae and Pichia stipitis-NCIM 3498

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Fermentation

A B S T R A C T

Prosopis juliflora (Mesquite) is a raw material for long-term sustainable production of cellulosics ethanol. In this study, we used acid pretreatment, delignification and enzymatic hydrolysis to evaluate the pretreatment to produce more sugar, to be fermented to ethanol. Dilute H$_2$SO$_4$ (3.0%, v/v) treatment resulted in hydrolysis of hemicelluloses from lignocellulosic complex to pentose sugars along with other byproducts such as furfural, hydroxymethyl furfural (HMF), phenolics and acetic acid. The acid pretreated substrate was delignified to the extent of 93.2% by the combined action of sodium sulphite (5.0%, w/v) and sodium chlorite (3.0%, w/v). The remaining cellulosic residue was enzymatically hydrolyzed in 0.05 M citrate phosphate buffer (pH 5.0) using 3.0 U of filter paper cellulase (FPase) and 9.0 U of β-glucosidase per mL of citrate phosphate buffer. The maximum enzymatic saccharification of cellulosic material (82.8%) was achieved after 28 h incubation at 50 °C. The fermentation of both acid and enzymatic hydrolysates, containing 18.24 g/L and 37.47 g/L sugars, with Pichia stipitis and Saccharomyces cerevisiae produced 7.13 g/L and 18.52 g/L of ethanol with corresponding yield of 0.39 g/g and 0.49 g/g, respectively.

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1. Introduction

Worldwide high demand for energy, uncertainty of petroleum resources and concern about global climatic changes has led to the resurgence in the development of alternative liquid fuels. Ethanol has always been considered a better choice as it reduces the dependence on reserves of crude oil and promises cleaner combustion leading to a healthier environment. Developing ethanol as fuel beyond its current role of fuel oxygenate, would require lignocellulosics as a feedstock because of its renewable nature, abundance and low cost (Saha et al., 2005).

Lignocelluloses are mainly comprised of cellulose, a polymer of six-carbon sugar, glucose; hemicellulose, a branched polymer comprised of xylose and other five-carbon sugars and lignin consisting of phenyl propane units. The presence of lignin limits the fullest usage of cellulose and hemicellulose. To convert these energy rich molecules into simpler forms, it is necessary to remove the lignin from lignocellulosic materials. A number of pretreatments such as concentrated acid hydrolysis (Liao et al., 2006), dilute acid hydrolysis (Cara et al., 2008), alkali treatment (Carrillo et al., 2005), sodium sulphite treatment (Kuhad et al., 1999; Kapoor et al., 2008), sodium chlorite treatment (Sun et al., 2004), steam explosion (Oghren et al., 2005), ammonia fiber explosion (Teymoouri et al., 2005) lime treatment (Kim and Holtzapple, 2005), and organosolv treatment (Xu et al., 2006) have been used frequently to remove lignin and improve the saccharification of the cell wall carbohydrates.

Of these methods, dilute acid treatment and enzymatic hydrolysis have been the most popular ones. Dilute acid hydrolysis is a fast and convenient method to perform but it leads to the accumulation of fermentation inhibitory compounds such as furfurals, hydroxymethyl furfurals (HMF) and phenolics. These compounds, depending on their concentration in the fermentation media, can inhibit microbial cell and affect the specific growth rate and cell-mass yield. Several treatments e.g., ion exchange (Canilha et al., 2004; Chandel et al., 2007), overliming (Martinez et al., 2001; Chandel et al., 2007), activated charcoal adsorption (Mussatto et al., 2004; Canilha et al., 2004; Chandel et al., 2007), and laccase oxidation treatment (Chandel et al., 2007) have been reported for the detoxification of hydrolysate to improve the fermentability of acid hydrolysates into ethanol. However, the combination of pH adjustment by overliming followed by activated charcoal adsorption has been shown to improve the detoxification of hemicellulosic hydrolysate (Converti et al., 1999).

The acid hydrolysis pretreatment removes the hemicellulosic portion and some fraction of lignin but rest of the lignin remains intact to the cellulosic substrate. Kaya et al. (2000) had reported that during enzymatic hydrolysis of lignocellulosic biomass,
cellulase components, β-glucosidase and endoglucanase have more binding affinity towards lignin than to the carbohydrates, resulting in lower efficiency of saccharification. Hence, to achieve maximum hydrolysis of cellulosics, which is a prerequisite for ethanol fermentation, an appropriate delignification treatment of biomass is required. In the present work, the combination of sodium sulphite and sodium chloride for the delignification of cellulosic biomass has been attempted.

The cellulosic and hemicellulosic sugars obtained through acid and enzymatic hydrolysis can efficiently be used for ethanol fermentation either by separate fermentation of individual hydrolysate or fermentation of mixed hydrolysate using co-culture. However, in co-culture cultivation, optimum growth conditions of the yeasts would be different and might result in lower efficiency and lower product yield. Hence, for better efficiency of ethanol production, the approach of separate hydrolysis and fermentation (SHF) was preferred (Olsson and Hahn-Hagerdal, 1993).

In the present study, _Prosopis juliflora_ (Mesquite), a perennial deciduous thorny shrub, the common vegetation of semi-arid region of Indian subcontinent, was used as a raw material for the production of cellulosic ethanol. The mesquite has recently been suggested to be used as raw material for long-term sustainable production of cellulosic ethanol (Hopkins, 2007). Its nature to tolerate drought, grazing, heavy soil, sand as well as saline dry flats and no competence with animal feed demand made it a potential low value substrate for ethanol production. Here, an attempt was made to saccharify _P. juliflora_ into reducing sugars and eventually to ethanol fermentation.

### 2. Methods

#### 2.1. Raw material and chemicals

_Prosopis juliflora_ wood, collected from University of Delhi South Campus, New Delhi, India, was comminuted by a combination of chipping and milling to attain a particle size of 1–2 mm using a laboratory knife mill (Metrex Scientific Instrumentation, Delhi, India). The processed wood was washed thoroughly and dried overnight at 60 °C.

Commercial cellulase from _Trichoderma reesei_ (ATCC 26921) (6.5 FPU/mg), β-glucosidase (Novozyme 188) (250 U/g) from _Aspergilus niger_ and 3.5-dinitrosalicylic acid (DNS) were purchased from Sigma, St. Louis, Missouri, USA. Ethanol was purchased from Merck (Darmstadt, Germany) at 150 rpm for 2 h. Thereafter, a mixture of 3.0 U of filter paper cellulase (FPhase) and 9.0 U of β-glucosidase per mL of citrate phosphate buffer (pH 5.0) containing 0.005% sodium azide. Before enzyme loading, slurry was acclimatized by incubating at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, India) for 15 min to remove the precipitate formed during neutralization. The overlimed hydrolysate was further detoxified by treating with activated charcoal (1.5%, w/v) with constant stirring at room temperature for 30 min and the sugar syrup was recovered through vacuum filtration.

#### 2.6. Chemical delignification of acid hydrolysate

The acid hydrolysate was overlimed at room temperature by adding dried lime (Ca(OH)₂) till the pH reached 10.0, with constant stirring for 30 min by an overhead stirrer (Remi Motors Ltd, Mumbai, India). After overliming, the hydrolysate was neutralized with concentrated H₂SO₄ and centrifuged at 10,000 rpm for 15 min to remove the precipitate formed during neutralization. The overlimed hydrolysate was further detoxified by treating with activated charcoal (1.5%, w/v) with constant stirring at room temperature for 30 min and the sugar syrup was recovered through vacuum filtration.

#### 2.7. Enzymatic hydrolysis of delignified cellulosic substrate

Enzymatic hydrolysis of cellulose (delignified acid treated plant material) was carried out at a 5.0% (w/v) consistency in 0.05 M citrate phosphate buffer (pH 5.0) containing 0.005% sodium azide. Before enzyme loading, slurry was acclimatized by incubating at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, India) at 150 rpm for 2 h. Thereafter, a mixture of 3.0 U of filter paper cellulase (FPhase) and 9.0 U of β-glucosidase per mL of citrate phosphate buffer was added to preincubated cellulose slurry and reaction continued for 36 h. Samples were withdrawn at regular interval of 4 h, centrifuged at 10,000g for 15 min and the supernatant was analysed for total reducing sugars released. The extent of hydrolysis was calculated as follows:
Reducing sugar concentration obtained = \frac{\text{Potential sugar concentration in the pretreated substrate}}{100}

Different surfactants (1.0%, v/v or w/v) including nonionic surfactants (Tween 20, Tween 40, Tween 60, Tween 80 and Triton X-100), polyethylene glycols (PEG 4,000, PEG 6,000 and PEG 10,000) and ionic surfactants (sodium dodecyl sulphate (SDS) and cetyl trimethyl ammonium bromide (CTAB)) were used for studying their effect in improving enzymatic saccharification.

2.8. Ethanol fermentation

The acid and enzymatic hydrolysates were fermented in an in situ sterilizable fermenter (B-Lite Sartorius India Ltd., Bangalore, India) having geometric volume of 13.5 L and working volume of 10.0 L. The acid hydrolysate (9.0 L) containing 18.24 g/L sugars was supplemented with nutrients (g/L) NH4Cl, 0.5; KH2PO4, 2.0; MgSO4 7H2O, 0.5; Yeast extract, 1.5; CaCl2 2H2O, 0.1 and ZnSO4 7H2O, 0.001 (pH 5.5 ± 0.2) and inoculated with 10.0% (v/v) culture of P. stipitis (OD 0.6). While cellulosic hydrolysate having 37.47 g/L sugars supplemented with yeast extract, 3.0 g/L and (NH4)2HPO4, 0.25 g/L was fermented with S. cerevisiae (OD 0.6). The dissolved oxygen was monitored continuously. An attempt was also made to test the efficacy of the yeast to increase ethanol production by growing it in medium having initial sugar load of 100 g/L.

2.9. Analytical methods

The hydrolysates were analysed using high performance liquid chromatography (HPLC) (Shimadzu Kyoto, Japan) for the presence of carbohydrates. Aminex (Bio-Rad, Hercules CA USA) column (300.0 × 7.8 mm) was used with 0.04 M H2SO4 as an eluent with flow rate of 0.5 mL/min keeping oven temperature at 60 °C with RID detector. Furans, furfurals and lignin derivatives were estimated with Luna 5 U C18 column (250.0 × 4.6 mm). The analysis was done by using acetonitrile: 10% phosphoric acid (22:78) as mobile phase with flow rate 0.5 mL/min and oven temperature 35 °C with UV-detector. Ethanol was estimated by Gas chromatography (GC) (Perkin Elmer, Clarus 500) with an elite-wax (cross bond-polyethylene glycol) column (30.0 m × 0.25 mm) at oven temperature of 85 °C and flame ionization detector (FID) at 200 °C. The ethanol standards were prepared using commercial grade ethanol (Merck, Darmstadt, Germany). Nitrogen with a flow rate of 0.5 g L-1 min-1 was used as carrier gas. Total reducing sugars were estimated by the DNS method and the total phenolics released were determined by the Folin–Ciocalteu reagent method (Singleton et al., 1999) using vanillin as standard. The optical density (\(A_{600\ nm}\)) of culture filtrate was measured using a double beam spectrophotometer (Specord 200). Dry biomass of yeast cells was measured after drying the yeast pellets at 70 °C till constant weight.

Table 1

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*Values in parenthesis are sugar and phenolic yield with respect to total carbohydrate and total lignin content of the substrate.
3. Results

3.1. Proximate chemical composition of P. juliflora

Prosopis juliflora wood contained 66.20% holocellulose (47.50% cellulose and 18.70% pentosans), 29.10% Klason lignin, 2.68% moisture and 2.02% ash content.

3.2. Dilute acid hydrolysis of P. juliflora

The woody material when hydrolyzed with different concentration of dilute H\textsubscript{2}SO\textsubscript{4}, at 100 °C and 120 °C, the release in sugar increased with increase in acid concentration up to 3.0% (v/v) H\textsubscript{2}SO\textsubscript{4} and it declined thereafter. While at 140 °C, the acid concentration beyond 2.0% (v/v) resulted in continuous decrease in release of sugar. The maximum sugars (204.84 mg/g) were released, when the woody material was treated with 3.0% H\textsubscript{2}SO\textsubscript{4} at 120 °C for 60 min. However, no significant difference in sugar released was observed when hydrolysis was carried either for 45 or 60 min. (Table 1). The acid hydrolysis also resulted in release of phenolics ranging from 2.0–4.5% (w/w) of phenolics present in substrate. Under optimized hydrolysis conditions (3.0% H\textsubscript{2}SO\textsubscript{4}, 120 °C, 60 min), the hydrolysate was found to contain furfural (0.34 mg/mL), HMF (0.58 mg/mL) and caffeic acid (0.13 mg/mL).

3.3. Detoxification of acid hydrolysate

The sequential detoxification of acid hydrolysate using overliming and activated charcoal resulted in a drastic decrease in the concentrations of inhibitors. Overliming resulted in reduction of HMF (0.34 mg/mL), HMF (0.58 mg/mL) and caffeic acid (0.13 mg/mL).

Table 2

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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(43.9)</td>
<td>(49.25)</td>
<td>(65.29)</td>
<td>(64.52)</td>
<td></td>
</tr>
<tr>
<td>S 10</td>
<td></td>
<td>189.98</td>
<td>198.87</td>
<td>231.1</td>
<td>234.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(65.29)</td>
<td>(68.34)</td>
<td>(79.41)</td>
<td>(80.56)</td>
<td></td>
</tr>
<tr>
<td>S 15</td>
<td></td>
<td>207.76</td>
<td>238.87</td>
<td>241.1</td>
<td>241.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(71.4)</td>
<td>(82.09)</td>
<td>(82.85)</td>
<td>(82.85)</td>
<td></td>
</tr>
<tr>
<td>S 20</td>
<td></td>
<td>215.54</td>
<td>244.43</td>
<td>244.43</td>
<td>246.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(74.07)</td>
<td>(84)</td>
<td>(84.76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S + C 5 + 3</td>
<td></td>
<td>260.15</td>
<td>270.44</td>
<td>270.37</td>
<td>270.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(80.04)</td>
<td>(92.81)</td>
<td>(92.6)</td>
<td>(92.51)</td>
<td></td>
</tr>
</tbody>
</table>

*Values in parenthesis are yield of phenolics with respect to total lignin content present in substrate.

* Delignifying chemicals used; S, sodium sulphite and C, sodium chlorite.

3.4. Delignification of pretreated wood

A regular increase in delignification of woody material was observed with the increase in concentration of sodium sulphite from 5.0% to 20.0% (w/v) (Table 2). The treatment of woody biomass with 20.0% (w/v) sodium sulphite at 140 °C and 60 min resulted in release of 246.65 mg/g of phenolics. However, when sodium chloride (3.0%, w/v) was used along with sodium sulphite (5.0%, w/v), it reduced the concentration of sodium sulphite from 20.0% (w/v) to 5.0% (w/v), and released maximum phenolics at 120 °C for 30 min. Moreover, at optimized conditions, the release of phenolics (271.10 mg/g) was found to be 8.4% (w/w) higher than the phenolics released by the sodium sulphite alone (20.0%, w/v, 140 °C and 60 min).

3.5. Enzymatic saccharification of delignified cellulosic material

During the time course of enzymatic saccharification of delignified cellulosic substrate, a regular increase in release of sugars was observed till 28 h of incubation, which remained almost constant thereafter, however, after attaining the maximum rate of saccharification (49.58 mg/g/h, 4 h), the saccharification rate decreased regularly showing the reciprocal relationship with saccharification yield (Fig. 1). Moreover, the maximum yield of saccharification, 586.16 mg/g, was achieved after 28 h incubation, with saccharification rate of 20.60 mg/g/h (Fig. 1).

Different ionic and nonionic surfactants were evaluated for their ability to improve the enzymatic hydrolysis of delignified P. juliflora. The amount of sugar released in the presence of Tween 80 increased maximally by 47.51% compared to control and followed by PEG 4000 (43.81%) and Tween 40 (39.17%). However, the quantity of sugar released in presence of ionic surfactants e.g., CTAB and SDS, decreased by 60.12% and 48.24%, respectively (Fig. 2).

3.6. Fermentations of hemicellulosic and cellulosic hydrolysates

The hemicellulosic hydrolysate containing 18.24 g/L sugars when fermented with P. stipitis produced 7.13 g/L ethanol with a yield of 0.39 g/g and productivity of 0.30 g/L/h after 24 h. After 24 h of fermentation, P. stipitis produced biomass (5.96 g/L) with yield and productivity, 0.33 g/g and 0.25 g/L/h, respectively (Table 3).
3. Fermentation of cellulosic hydrolysate (37.47 g/L) using *S. cerevisiae*, gave maximum ethanol (18.52 g/L) with yield (0.49 g/g) and productivity (1.16 g/L/h) after 16 h, whereas, the biomass was found to be 8.02 g/L having yield (0.21 g/g) and productivity (0.50 g/L/h) (Table 4). However, *S. cerevisiae*, when grown in medium containing 100 g/L glucose produced 42.5 g/L ethanol with a yield of 0.43 g/g and productivity 2.66 g/L/h (Fig. 3).

4. Discussion

Dilute acid pretreatment method is commonly used to saccharification of any lignocellulosic biomass. It has the dual advantage of solubilizing hemicellulose and further converting it to fermentable sugars. In the present work, dilute sulphuric acid pretreatment of milled wood of *P. juliflora* was optimized to achieve maximum sugar yield at minimum severity conditions. The optimum pretreatment conditions i.e., 3.0% (v/v) acid at 120°C for 45 min, gave

---

**Table 3**
Fermentation profile of detoxified acid hydrolysate using *P. stipitis*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ethanol (g/L)</th>
<th>Sugar (g/L)</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol productivity (g/L/h)</th>
<th>Biomass (g/L)</th>
<th>Biomass yield (g/g)</th>
<th>Biomass productivity (g/L/h)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.17</td>
<td>18.24</td>
<td>0.01</td>
<td>0.00</td>
<td>0.15</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>0.19</td>
<td>17.43</td>
<td>0.01</td>
<td>0.05</td>
<td>1.47</td>
<td>0.08</td>
<td>0.37</td>
</tr>
<tr>
<td>8</td>
<td>3.08</td>
<td>15.09</td>
<td>0.14</td>
<td>2.91</td>
<td>2.91</td>
<td>0.16</td>
<td>0.36</td>
</tr>
<tr>
<td>12</td>
<td>3.33</td>
<td>11.17</td>
<td>0.28</td>
<td>3.59</td>
<td>3.59</td>
<td>0.20</td>
<td>0.30</td>
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<tr>
<td>16</td>
<td>4.41</td>
<td>9.02</td>
<td>0.3</td>
<td>4.55</td>
<td>4.55</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
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<td>0.26</td>
</tr>
<tr>
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<td>3.51</td>
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<td>5.96</td>
<td>0.33</td>
<td>0.25</td>
</tr>
<tr>
<td>28</td>
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<td>2.87</td>
<td>0.38</td>
<td>6.12</td>
<td>6.12</td>
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<tr>
<td>32</td>
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<td>6.23</td>
<td>6.23</td>
<td>0.34</td>
<td>0.19</td>
</tr>
</tbody>
</table>

**Table 4**
Fermentation profile of enzymatic hydrolysate using *S. cerevisiae*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ethanol (g/L)</th>
<th>Sugar (g/L)</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol productivity (g/L/h)</th>
<th>Biomass (g/L)</th>
<th>Biomass yield (g/g)</th>
<th>Biomass productivity (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.49</td>
<td>37.47</td>
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<tr>
<td>4</td>
<td>11.61</td>
<td>15.29</td>
<td>0.28</td>
<td>2.44</td>
<td>1.91</td>
<td>0.05</td>
<td>0.48</td>
</tr>
<tr>
<td>8</td>
<td>16.13</td>
<td>5.51</td>
<td>0.43</td>
<td>2.02</td>
<td>3.90</td>
<td>0.10</td>
<td>0.49</td>
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<tr>
<td>12</td>
<td>17.47</td>
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<td>1.46</td>
<td>6.05</td>
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<td>0.50</td>
</tr>
<tr>
<td>16</td>
<td>18.52</td>
<td>1.86</td>
<td>0.49</td>
<td>1.16</td>
<td>8.02</td>
<td>0.21</td>
<td>0.50</td>
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<tr>
<td>20</td>
<td>18.81</td>
<td>1.67</td>
<td>0.45</td>
<td>0.84</td>
<td>7.99</td>
<td>0.21</td>
<td>0.40</td>
</tr>
<tr>
<td>24</td>
<td>14.90</td>
<td>1.32</td>
<td>0.40</td>
<td>0.62</td>
<td>7.95</td>
<td>0.21</td>
<td>0.33</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of different surfactants on the enzymatic hydrolysis of delignified *P. juliflora* after 36 h. (T, Tween (1%, v/v); TX, Triton (1%, v/v); CTAB, cetyl trimethyl ammonium bromide (1%, w/v); SDS, sodium dodecyl sulphate (1%, w/v); PEG, polyethylene glycol (1%, w/v)).

**Fig. 3.** Time course for the production of ethanol from glucose (100 g/L).
a saccharification yield of 200.50 mg/g. While any further increase in pretreatment stringency caused the increase in release of toxic compounds without much effect on sugar yield. Increase in toxic compounds with corresponding increase in acid concentration, suggested to use the minimal acid concentration with reduced inhibitory compound and achieving maximum sugar hydrolysis.

The mechanistic rationale for the inhibitory action of HMF and HMF on yeast cultures could be the result of decrease in the activities of aldehyde dehydrogenase (AIDH), pyruvate dehydrogenase (PDH) and alcohol dehydrogenase (ADH) (Modig et al., 2002). Detoxification of acid hydrolysate using Ca(OH)₂ effectively reduced the level of inhibitors. This may be the result of either polymerization or chemical transformations of these inhibitors at higher pH (Martinez et al., 2001). Previous overliming reports showed the similar trends of decrease in inhibitors present in acid hydrolysates (Martinez et al., 2001; Chandel et al., 2007). While activated charcoal being hydrophobic in nature removes the hydrophobic inhibitory compound i.e., furan and phenolics more effectively (Saha, 2004; Chandel et al., 2007). Our results showed that detoxification of acid hydrolysate using Ca(OH)₂ followed by activated charcoal adsorption had resulted in almost complete removal of majority of fermentation inhibitory compounds except acetic acid, which was removed partially (58%). However, the left over concentration of acetic acid was lower than the minimum inhibitory concentration i.e., 5.0 g/L as reported by Taherzadeh et al. (1997). Moreover, the yeast can grow on a media containing acetic acid up to 20 g/L at pH (5.5) (Taherzadeh et al., 2000). Converti et al. (1999) had also shown the detoxification of hydrolysates through sequential steps of overliming and activated charcoal adsorption. However, removal of phenolics using laccase had also been reported (Chandel et al., 2007).

The chemical delignification of lignocellulosic material has previously been reported to achieve better enzymatic saccharification as compared to untreated sample (Saha, 2004; Kapoor et al., 2008). Earlier, Kuhad and coworkers had reported sodium sulphite (13.7%, w/v) for efficient delignification of biomass (Kuhad et al., 1999; Kapoor et al., 2006). In the present study, the maximum delignification was observed in P. juliflora when pretreated with combination of sodium sulphite (5.0%, w/v) and sodium chlorite (3.0%, w/v). It is interesting to note that the overall concentration of sodium sulphite (20.0%, w/v) was drastically reduced to 5.0% (w/v) with addition of merely 3.0% (w/v) sodium chlorite as delignification agent. Sodium chlorite is an elemental chlorine free compound, which at high temperature produces chlorous (HClO₂) and hypochlorous acids (HOC). The subsequent oxidation of chlorous by hypochlorous acid regenerates chlorine dioxide (ClO₂) that further react with lignin (Hamzeh et al., 2006). Chlorine dioxide has had tremendous success as a replacement for chlorine because it is easily substituted in a conventional chlorination stage without any special modification, produces fewer potentially toxic byproducts and causes less damage to the wood fibers (Svenson et al., 2005).

During the enzymatic saccharification of delignified substrate, a regular decrease in the rate of hydrolysis was observed after 4 h of saccharification, which may be due to the end product inhibition of the enzymes (Kuhad et al., 1999). However, to weaken the feedback inhibition caused by the cellobiose accumulation, β-glucosidase at a 3-fold concentration to FPase was used. In the present experiment, 5.0% (w/v) substrate consistency was found to obtain maximum hydrolysis, however, further increase in substrate consistency led to the reduced extent of hydrolysis (data not shown). This decrease in hydrolysis efficiency may be because of mixing and heat transfer problem due to the rheological properties of a dense fibrous suspension, which ultimately cause insufficient adsorption of the cellulase to the cellulose (Chen et al., 2007). As the enzymatic saccharification of cellulose involve transport of enzyme molecules and soluble sugars between the solid substrate and bulk reaction solution, a modification of the surface and interfacial properties of the reaction system may improve saccharification (Hemmantinejad et al., 2002). For further improvement in the saccharification efficiency of delignified P. juliflora, various ionic as well as nonionic surfactants were investigated. Among different surfactants tested, Tween 80, a nonionic surfactant has supported the enzymatic saccharification of delignified P. juliflora maximally (Fig. 2). Kaar and Holtzappe (1998) had also proposed that Tween protects the enzymes from thermal deactivation during the enzymatic hydrolysis. This may be the result of reduced contact of enzyme with the air-liquid interface due to surface activity of the surfactant. The reduction in surface tension of the solution inhibits the non-productive attachment of the exoglucanase to the lignin surface and allows the saccharifying exoglucanase greater access to cellulose, which results in increase of sugar release (Hemmatinejad et al., 2002).

The acid and enzymatic hydrolysates of P. juliflora were fermented by P. stipitis and S. cerevisiae, respectively. Several evidences suggested that separate fermentation by substrate specific organisms work better instead of using mixed hydrolysate with either single culture or co-culture method (Delgenes et al., 1996). Co-culture fermentation associates both hexose and pentose fermenting yeasts that trade-off for oxygen requirement between the two microorganisms. Whereas, ethanol production from mixed hydrolysates using single culture, circumvent the fact that utilization of xyllose becomes a subject of glucose catabolite repression (Olsson and Hahn-Hagerdal, 1993). The acid hydrolysate of lignocellulosics comprises mainly pentose sugars and very few microorganisms e.g., Candida shehatae, P. stipitis and Pachysolen tannophilus, which can utilize pentose sugars efficiently have been identified so far (Abbi et al., 1996). Among different pentose utilizing yeasts, P. stipitis has shown great potential by having broad substrate specificity and no absolute vitamin requirement for pentose utilization (du Preez et al., 1986). In our experiment, the ethanol yield (0.39 g/g) from hemcellulosic hydrolysate using P. stipitis was very much in agreement with previous reports having yield, 0.37 g/g from aspen wood (Delgenes et al., 1996), 0.39 g/g from α-xyllose (du Preez et al., 1986) and 0.36 g/g from P. juliflora (Kapoor et al., 2008). However, the ethanol yield obtained from cel lullosic hydrolysate using S. cerevisiae (0.49 g/g) was even higher than the previous results of ethanol yield 0.40 g/g from sweet sorghum (Mamma et al., 1995) and 0.48 g/g from corn (Chen et al., 2007). A low aeration rate of 0.4 lpm was maintained throughout the process, as low aeration conditions are attributed to obligate hypoxic induction of essential fermentative enzymes (Mc Millan and Boynton, 1994) and also in slightly aerobic conditions the production of ethanol enhanced by lowering down the production of glycerol as byproduct (Alfenore et al., 2004). However, S. cerevisiae when grown in medium having 100 g/L sugar produced 42.5 g/L ethanol, suggesting thereby to increase the initial sugar loading either by concentration of the hydrolysates or by supplementation with sugar to improve cellulosic ethanol production.

5. Conclusion

Mesquite (P. juliflora) wood could serve as novel material for the production of ethanol. The separate hydrolysis and fermentation could prove better in improving the ethanol production from cellulotic hydrolysates by minimizing the problem of catabolite repression. The ethanol production may be enhanced by increasing the initial sugar load in cellulotic hydrolysates. There is still a need to develop: (i) a more efficient and economic pretreatment process (ii) a hyper-cellulase producing strain for improved saccharification and (iii) an improved recombinant yeast strain capable of utilizing both pentose and hexose sugars, which in turn would increase ethanol production.
Acknowledgements

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References


Xylanase production from an alkalophilic actinomycete isolate \textit{Streptomyces} sp. RCK–2010, its characterization and application in saccharification of second generation biomass

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\textbf{A B S T R A C T}

Xylanase production by a newly isolated \textit{Streptomyces} sp. RCK–2010 was optimized for varying culture conditions following one factor at a time (OFAT) and response surface methodology (RSM) approaches. An initial medium pH 8.0, agitation 200 rpm, incubation temperature 40 °C and inoculum size 1.0% (v/v) were found to be optimal for xylanase production (264.77 IU/ml), after 48 h of incubation. Among various carbon sources tested, the actinomycete secreted higher level of xylanase on wheat bran. The production medium when supplemented separately with various nitrogen sources, the enhanced xylanase production was observed with beef extract followed by peptone. RSM employing central composite design (CCD) was used to optimize the xylanase production using wheat bran, beef extract and peptone as model factors. The RSM showed that the optimum level of wheat bran (2.5% w/v), peptone (0.2% N\textsubscript{2} equivalent) and beef extract (1.2% N\textsubscript{2} equivalent) resulted in almost 3.0 fold improvement in xylanase production (2310.18 IU/ml). To the best of our knowledge this is the best xylanase volumetric productivity (1155 IU/ml/day) by any \textit{Streptomyces} spp. reported in the literature. The enzyme was most active at 60 °C and pH 6.0 and almost 40% stable after 4 h at optimum temperature. Saccharification of steam exploded rice straw with xylanase (60 IU/g dry substrate) supplemented with cellulase (24 FPU/g dry substrate) and \textbeta-glucosidase (60 IU/g dry substrate) resulted in 88% (w/w) saccharification of the cellulosic substrate.

1. Introduction

Economical feasibility of biofuel production from lignocellulosic materials, the second-generation biomass, is a major technological challenge. Currently the major attraction for bioethanol production is cellulose fraction, while, hemicellulose, the second most abundant natural polymer is yet to be tapped. Xylan, the major hemicellulose, consists of 1, 4-\beta-linked \textalpha-xylene units substituted with different side groups such as \textalpha-arabinose, \textalpha-galactose, acetyl, feruloyl, \textalpha-coumaroyl and glucuronic acid residue [1,2]. Depending on its state, xylan can be used for various purposes such as, polymeric xylan as adhesives and emulsifiers [3], arabino-xylooligocaccharides as prebiotics [4,5], whereas monomeric xylene can be fermented to ethanol [6,7].

Among various methods used to breakdown the xylan backbone, its hydrolysis using xylanases is one of the most environmental benign alternative, as xylanases are highly specific in their nature and application [2,8,9]. Xylanases (EC. 3.2.1.8) are also attracting extensive interest due to their wide applications in pulp bleaching, bioethanol production and oligosaccharides production [7,10,11]. Furthermore, tolerance to high pH and temperature are desirable properties of xylanases for their effective use in various industries. The alkalitolerant xylanases reduce the chlorine requirements for pulp bleaching, while the higher temperature will enhance their rate of reaction. Though a variety of microorganisms have been reported to produce xylanolytic enzymes [2,8,10], there are few reports on the production of alkali and thermostable xylanase from actinomycetes. The present study deals with the production of thermostable and alkalitolerant xylanase from an alkalitolerant actinomycete isolate \textit{Streptomyces} sp. RCK–2010.

Since, the production of xylanases is strongly influenced by their culture conditions and medium constituents, to maintain a balance among process conditions and to minimize the amount of un-utilized components, optimization of medium composition is essential [8]. The optimization process is generally carried out using one factor at a time (OFAT) approach, but it does not consider interaction among variables [12]. While, the optimization using statistical approaches such as response surface methodology (RSM) offers quick screening of large experimental domain and modeling of interactive effects of process variables, which enables each
reaction parameters to be optimized incoherence with others for achieving maximum enzyme production [12,13]. Moreover, multivariate experiments in RSM also reduce the number of necessary optimization and give more precise results than those obtained by univariate strategies [13] and results in significant improvement of enzyme production.

In the present study, the optimization of xylanase production from an actinomycete isolate Streptomyces sp. RCK-2010 following OFAT and RSM approach was carried out. Moreover, an attempt has been made to evaluate the efficiency of xylanases in improving the hydrolysis of steam exploded rice straw, the abundantly available second generation feedstock, into sugars.

2. Experimental

2.1. Raw material and chemicals

The steam exploded rice straw having 77% (w/w) holocellulose (18% hemi-cellulose, 9% cellulose) was procured from Dr. A. J. Varna, Polytechnic. The Chemistry Division, National Chemical Laboratory, Pune, India. The substrate was washed thoroughly and dried at 60 °C in a hot air oven [14]. Xylan, cellulose, glucose, carboxymethyl cellulose, p-xylene and 3, 5-di-nitrosoacrylic acid were purchased from Sigma (St. Louis, USA). All other media components and chemicals were purchased locally.

2.2. Isolation, screening of xylanase producing actinomycetes and identification of the potent isolate

For isolation of alkalo-tolerant actinomycetes, the soil samples collected from University of Delhi South Campus, New Delhi, India were initially treated with alkali by suspending 1.0 g of each sample in sterile distilled water (pH 9.0) [10]. The samples were then serially diluted and spread on actinomycete isolation agar containing (g/L) sodium caseinate 2.0, t-asparagine 0.1, sodium propionate 4.0, K2HPO4 0.5, MgSO4 0.5, FeSO4 0.001 and agar 15.0 (pH 8.0) and incubated at 37 °C for 96 h. The actinomycetes colonies developed were purified by repeated transfer of cultures and the isolates were screened for xylanase production using Congo-red plate assay method and selected on the basis of hydrolysis zone [15].

For identification of the potent xylanase producing actinomycete isolate (R-10), a 500 bp region of 16S rRNA gene was amplified in a thermocycler (G–storm, USA) using the universal primers (CCAGCAACGGCGGTAAATCGC) and (ATCGGCTACTGGTACGACTTC). The PCR products were purified and sequenced as described earlier [13] and the nucleotide sequence has been deposited in the GenBank database (accession no. HQ658475). The sequence data was analyzed for the homology with the similar existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using BLAST search.

2.3. Microorganism and maintenance

Streptomyces sp. RCK-2010 was maintained on xylan agar plates containing (g/L) xylan 5.0, peptone 5.0, yeast extract 5.0, KH2PO4 1.0, MgSO4 0.1 and agar 20.0 at 37 °C. Subcultured every fortnight and stored at 4 °C. The modified Horikoshi medium having (g/L) KH2PO4 1.0, MgSO4 0.1, yeast extract 5.0, peptone 5.0, and glucose 5.0, was used for the xylanase production.

2.4. Optimization of xylanase production

In order to screen the effective parameters for the optimization of xylanase production from Streptomyces sp. RCK-2010, various process variables such as cultivation time (up to 60 h), temperature (30–42 °C), initial pH (5.0–9.0) of the medium, agitation (100–300 rpm), inoculum size (0.4–1.2% v/v of 18 h old culture), carbon sources and nitrogen sources were studied under submerged fermentation conditions using OFAT approach. The time course of xylanase production was carried out at 37 °C, pH 8.0 and 200 rpm with an inoculum size of 0.4% (v/v). Each factor examined for optimization was incorporated further in the subsequent experiments. All other experiment conditions were kept constant unless otherwise stated.

Further to study the interaction among the three effective parameters selected from OFAT method, i.e., wheat bran (A), beef extract (B) and peptone (C) on xylanase production from Streptomyces sp. RCK-2010, experiments were conducted using RSM approach. The maxima (+1) and minima (−1) values for wheat bran were 0.5 and 2.5% (w/v), while for both beef extract and peptone were 0.2 and 1.2% (N2 equivalent). The statistical software Package Design-Expert 6.0 Stat-Ease, USA was used to analyze the experimental design. The design was used for the determination of the optimum culture conditions for xylanase production. The analysis was used to identify the influence of variables on each other on the xylanase production and to determine the optimum fermentation conditions.

2.5. Characterization of xylanase from Streptomyces sp. RCK-2010

The xylanase produced under optimized conditions was purified partially with ammonium sulphate precipitation. The optimal pH for partially purified xylanase activity was determined using different buffers (0.2 M) (citrate–phosphate, pH 3.0–6.5; phosphate buffer, pH 6.0–7.5; Tris–HCl, pH 7.5–9.2; glycine–NaOH, pH 8.5–10.5 and carbonate–bicarbonate, pH 9.0–10.0) and the optimum temperature for xylanase activity was determined between 30 and 85 °C. The thermostability of the partially purified enzyme was studied by incubating the enzyme at 55 and 60 °C up to 4 h at pH 6.0, and the residual activities were estimated periodically.

2.6. Application of xylanase in saccharification of steam exploded rice straw

Enzymatic hydrolysis of steam exploded rice straw (10.0 g) was carried out at 5% (w/v) substrate concentration in 50 mM citrate phosphate buffer (pH 5.0). The substrate suspension was pre-incubated at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, Germany) at 150 rpm for 2 h. Thereafter, the slurry was supplemented with enzyme cocktail; cellulase (24 FPU/g), ß-glucosidase (60 IU/g) and crude enzyme extract containing varied dosages of xylanase (20–80 IU/ml). The enzymatic reaction without xylanase served as control. Tween 80 (1% v/v) was also added in the reaction mixture to facilitate the enzyme action. The enzymatic hydrolysis was performed at 50 °C and 150 rpm for 36 h. Samples of enzymatic hydrolysate were withdrawn regularly and analyzed for amount of glucose released.

2.7. Analytical methods

All the analyses have been carried in triplicates and the data presented here is the mean of the triplicates along with the standard deviation.

The xylanase activities were determined by measuring the amount of reducing sugars released from xylan (1% w/v) in citrate phosphate buffer; pH 6.0) at 60 °C for 10 min, as described earlier [4]. One international unit (IU) of xylanase activity was defined as amount of enzyme required to release 1 μM of reducing sugars as xylose from xylan per min under reaction conditions. The protein concentrations were measured by the Lowry’s method with BSA (Bovine serum albumin) as standard [16]. The reducing sugars
were estimated following DNS method of Miller [17] and the saccharification efficiency was calculated as follows:

\[
\text{Saccharification (\%)} = \frac{\text{Amount of sugar released in enzymatic saccharification}}{\text{Amount of carbohydrate present in the substrate}} \times 100
\]

3. Results and discussion

3.1. Selection and molecular identification of potent xylanase-producing actinomycete

Out of the 57 alkalo-tolerant actinomycetes isolated from soil samples, 13 isolates showed the ability to produce xylanase. Among these isolates, R-10 was found to exhibit the largest zone of hydrolysis on xylan agar plate flooded with congo-red [15]. Moreover, the identification of the actinomycete isolate R-10 was done by 16S rRNA gene sequences. The nucleotide BLAST similarity search analysis based on 16S rRNA gene sequence revealed that the isolate R-10 was closely related to the genus Streptomyces (Fig. 1) and the organism was termed as Streptomyces sp. RCK-2010.

3.2. Effect of pH, temperature, agitation and inoculum size on xylanase production

Time course of xylanase production by Streptomyces sp. RCK-2010 showed the maximum xylanase production (158.21 IU/ml) after 48 h of incubation and thereafter it gradually declined (Fig. 2). The reduction in xylanase yield could be due to nutrients depletion or due to proteolysis [18]. Among various incubation temperatures studied, the maximum xylanase production (170.72 IU/ml) with specific xylanase activity of 73.59 IU/mg protein was achieved at 40°C. The enzyme production was observed to be decreased by 12.3% on increasing the incubation to 42°C (Table 1). The optimum temperature of 40°C for xylanase production is also in correlation with our previous report on Streptomyces cyaneus [19].

The actinomycete isolate when grown under variable shaking conditions, the optimum xylanase of 170.79 IU/ml with specific activity 73.51 IU/mg protein was observed at 200 rpm after 48 h of incubation (Table 1). Increase or decrease in the agitation rates beyond 200 rpm resulted in low xylanase yields. The lower enzyme level under low agitation conditions may be attributed to the dissolved oxygen (DO) limitation, improper mixing of media components and cell clumping [20]. However, the decrease in enzyme activity on increasing the agitation might be as a result of the cells shearing [13].

Among wide range of initial pH of the production medium tested, pH 8.0 was found to be more effective in production of maximum xylanase (178.67 IU/ml; specific activity 82.17 IU/mg protein), which drastically decreased beyond pH 8.0 (Table 1). Earlier reports on xylanase production by several fungi and bacteria have also been shown to be markedly dependent on the initial pH of the medium [21,22]. Interestingly, the preference of higher pH (8.0) by Streptomyces sp. RCK-2010 qualified it as a alkalophilic actinomycete.

An inoculum concentration ranging from 0.4 to 1.20% (v/v) revealed 1.0% (v/v) as optimal inoculum level for maximum xylanase yield (264.77 IU/ml) and specific xylanase activity (115.11 IU/mg protein) (Table 1). However, lower enzyme yield (226.68 IU/ml) at higher inoculum level (1.2% v/v) could be the result of faster nutrient consumption. Hence, an optimal inoculum level is necessary for maintaining balance between the proliferating biomass and available nutrients to produce maximum enzyme level.

3.3. Effect of carbon and nitrogen sources on xylanase production

Among various carbon sources used Streptomyces sp. RCK-2010 exhibited clear preference for lignocellulosic agro-residues as compared to pure sugars (Table 2) and wheat bran was observed to be the optimum substrate for maximum xylanase production (761.37 IU/ml). This may be because that wheat bran acts as a complete nutritious feed containing various soluble sugars, which are helpful for the initiation of growth and replication of microorganisms and remains loose even under moist conditions providing a large surface area [8]. In addition, higher xylanase production on wheat bran may be due to lignin and silica content [11]. Wheat bran has been reported as an ideally suitable substrate for xylanase production for other microorganisms as well [23,24]. Interestingly, when monosaccharides and disaccharides were used as carbon sources, xylanase production ranged from 82 to 260 IU/ml. This implies that the enzyme is not specific to xylan-rich substrates and can be produced constitutively up to a certain level. But the use of pure sugars as substrate is uneconomical for large-scale production of xylanases, while agricultural residues are cost-effective substrates for xylanase production [25]. Further different concentrations of wheat bran ranging from 1.0 to 3.0% (w/v) were tested for xylanase production and the maximum xylanase production (805.12 IU/ml) was obtained when the actinomycete was grown on 2.5% (w/v) wheat bran (Fig. 3).

To optimize the effect of nitrogen sources for xylanase production, different organic and inorganic nitrogen sources were added by replacing the yeast extract and peptone from the production medium. The maximum activity of xylanase (617.58 IU/ml) with specific activity of 263.92 IU/mg protein was obtained with beef extract and followed by peptone (340.65 IU/ml) (Table 3). The enhanced production of xylanase in presence of beef extract as well as peptone may be attributed to organic nitrogen source mediated regulation of microbial growth and metabolism, as has
**Table 1**
Physiological parameters optimization for xylanase production from Streptomyces sp. RCK-2010 under submerged cultivation conditions following OFAT method.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (IU/ml)</td>
<td>30</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>90.11 ± 1.80</td>
</tr>
<tr>
<td>Specific activity (IU/mg protein)</td>
<td>1.92 ± 0.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Agitation (RPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (IU/ml)</td>
<td>100</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>49.25 ± 0.52</td>
</tr>
<tr>
<td>Specific activity (IU/mg protein)</td>
<td>2.02 ± 0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (IU/ml)</td>
<td>5</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>110.42 ± 5.41</td>
</tr>
<tr>
<td>Specific activity (IU/mg protein)</td>
<td>2.07 ± 0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Inoculum size (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (IU/ml)</td>
<td>0.4</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>169.6 ± 2.63</td>
</tr>
<tr>
<td>Specific activity (IU/mg protein)</td>
<td>2.07 ± 0.08</td>
</tr>
</tbody>
</table>

**Table 2**
Effect of carbon sources on xylanase production from Streptomyces sp. RCK-2010 at 40 °C with shaking (200 rpm) after 48 h of incubation following OFAT method.

<table>
<thead>
<tr>
<th>Carbon source (0.5% w/v)</th>
<th>Xylanase yield (IU/ml)</th>
<th>Protein mg/ml (mg/ml)</th>
<th>Specific activity (IU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose</td>
<td>181.01 ± 12.36</td>
<td>2.59 ± 0.14</td>
<td>69.78 ± 2.44</td>
</tr>
<tr>
<td>Glucose</td>
<td>260.03 ± 17.22</td>
<td>2.02 ± 0.07</td>
<td>128.71 ± 8.41</td>
</tr>
<tr>
<td>Pectin</td>
<td>115.31 ± 8.96</td>
<td>2.28 ± 0.06</td>
<td>91.84 ± 5.62</td>
</tr>
<tr>
<td>Mannose</td>
<td>203.61 ± 15.63</td>
<td>2.22 ± 0.02</td>
<td>97.81 ± 5.67</td>
</tr>
<tr>
<td>Lactose</td>
<td>151.63 ± 9.87</td>
<td>1.55 ± 0.07</td>
<td>103.0 ± 8.35</td>
</tr>
<tr>
<td>Fructose</td>
<td>158.42 ± 11.84</td>
<td>1.54 ± 0.05</td>
<td>74.42 ± 6.21</td>
</tr>
<tr>
<td>Sucrose</td>
<td>119.27 ± 9.14</td>
<td>1.60 ± 0.08</td>
<td>90.51 ± 4.58</td>
</tr>
<tr>
<td>Maltose</td>
<td>152.34 ± 9.21</td>
<td>1.68 ± 0.10</td>
<td>47.45 ± 2.10</td>
</tr>
<tr>
<td>Arabinose</td>
<td>82.81 ± 3.52</td>
<td>1.94 ± 0.08</td>
<td>42.57 ± 1.57</td>
</tr>
<tr>
<td>Xylose</td>
<td>225.33 ± 13.25</td>
<td>1.80 ± 0.11</td>
<td>125.46 ± 10.25</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>761.37 ± 2.24</td>
<td>2.51 ± 0.14</td>
<td>303.33 ± 8.54</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>505.11 ± 36.33</td>
<td>2.31 ± 0.12</td>
<td>216.93 ± 12.73</td>
</tr>
<tr>
<td>Prosopis juliflora</td>
<td>381.05 ± 19.22</td>
<td>1.91 ± 0.14</td>
<td>199.47 ± 14.37</td>
</tr>
<tr>
<td>Lantana camara</td>
<td>373.35 ± 27.13</td>
<td>1.76 ± 0.08</td>
<td>212.13 ± 15.49</td>
</tr>
<tr>
<td>Corn cob</td>
<td>325.19 ± 24.66</td>
<td>1.55 ± 0.11</td>
<td>184.85 ± 15.62</td>
</tr>
<tr>
<td>Corn stover</td>
<td>289.86 ± 22.63</td>
<td>1.89 ± 0.14</td>
<td>153.36 ± 7.58</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td>423.15 ± 36.51</td>
<td>1.63 ± 0.07</td>
<td>259.60 ± 5.36</td>
</tr>
<tr>
<td>Birchwood xylan</td>
<td>215.27 ± 13.11</td>
<td>1.53 ± 0.08</td>
<td>140.69 ± 10.24</td>
</tr>
</tbody>
</table>

**Table 3**
Effect of nitrogen sources on xylanase production from Streptomyces sp. RCK-2010 at 40 °C with shaking (200 rpm) after 48 h of incubation following OFAT method.

<table>
<thead>
<tr>
<th>Nitrogen source (0.55% N&lt;sub&gt;e&lt;/sub&gt; equivalent)</th>
<th>Xylanase yield (IU/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (IU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptone</td>
<td>340.69 ± 19.74</td>
<td>2.21 ± 0.12</td>
<td>154.15 ± 5.47</td>
</tr>
<tr>
<td>Beef extract</td>
<td>617.58 ± 36.98</td>
<td>2.34 ± 0.15</td>
<td>263.92 ± 3.65</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>281.69 ± 22.49</td>
<td>2.22 ± 0.08</td>
<td>126.89 ± 7.46</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>258.34 ± 23.30</td>
<td>2.15 ± 0.10</td>
<td>120.16 ± 7.25</td>
</tr>
<tr>
<td><strong>Inorganic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>88.35 ± 5.26 (2.43 26.35)</td>
<td>2.43 ± 0.21</td>
<td>36.35 ± 2.54</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>122.38 ± 6.93 (2.13)</td>
<td>2.13 ± 0.15</td>
<td>57.45 ± 4.29</td>
</tr>
<tr>
<td>NaNO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>205.08 ± 18.73</td>
<td>2.95 ± 0.09</td>
<td>69.51 ± 1.58</td>
</tr>
<tr>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;P</td>
<td>210.13 ± 18.73</td>
<td>2.57 ± 0.11</td>
<td>88.66 ± 3.65</td>
</tr>
<tr>
<td>Control&lt;sup&gt;+&lt;/sup&gt;</td>
<td>78.26 ± 5.42</td>
<td>1.87 ± 0.07</td>
<td>41.85 ± 2.17</td>
</tr>
</tbody>
</table>

<sup>+</sup> The control medium used was devoid of nitrogen source.
been reported that the complex nitrogen is an essential requirement for growth and enzyme production [26]. Moreover, it has also been observed that nitrogen can significantly affect the pH of the medium during the course of fermentation [27], thereby influences the microbial metabolism. Our results are very much in agreement with the earlier reports, where actinomycetes were found to produce higher xylanase on organic nitrogen sources [24,28,29]. In order to estimate the optimum dosage of nitrogen sources, the xylanase production was carried out at different dosage (0.5–0.9% N₂ equivalent) of both peptone and beef extract were carried out. Irrespective of the nitrogen sources tested, the maximum xylanase production was observed at 0.7% N₂ equivalent. The xylanase production with optimal level of beef extract and peptone as nitrogen sources was observed to be 889.21 and 503.24 IU/ml, respectively (Fig. 3).

3.4. Statistical optimization of xylanase production using RSM

The result of RSM experiment for studying the effect of three independent variables; wheat bran (A), beef extract (B) and peptone (C), are presented along with the mean predicted and observed responses in Table 4. The table showed the maximum and minimum levels of variables resulted in the RSM and also depicted that total four center points were set up at runs of 7, 12, 13 and 19 and almost similar xylanase activities (~1100 IU/ml) were observed. However, the maximum xylanase production (2310.18 IU/ml) was achieved at run no. 18 containing (% w/v): wheat bran 2.5, beef extract 1.2 and peptone 0.20. While, the minimum xylanase (439.9 IU/ml) was observed in run no. 16 containing minimum amount of wheat bran (0.50% w/v), beef extract (0.20% w/v) and peptone (0.20% w/v). From multiple regression analysis, it was observed that the second-order polynomial equation can explain xylanase production regardless of the significance of the coefficients:

\[ Y = 65.69 + 235.79 A + 801.24 B + 406.87 C + 240.83 AB - 794.90 BC \]

where \( Y \) is the response value. In current experiment, \( Y \) value is the level of xylanase production (IU/ml), A, B and C represent the coded levels of wheat bran, beef extract and peptone, respectively. The statistical significance of the regression model was checked by F-test. The model was highly significant, as manifested by the \( F \)-value and the probability value [(\( F_{\text{cal}} \) model > \( F_{\text{tab}} \)) > 0.0001] was achieved. The goodness of fit was manifested by the determination coefficient (\( R^2 \)). In this case the \( R^2 \) value of 0.9775 indicated that the response model can explain 97.75% of the total variations. The value of the adjusted determination coefficient (Adj\( R^2 \)) was also high enough (0.9695) to indicate the significance of the model. The parameter coefficient and the corresponding P-value suggested that among the independent variables, A (wheat bran) and B (beef extract) have a significant effect on xylanase production.

The 3D response surfaces plots were employed to determine the interaction of the medium components and the optimum levels that have the most significant effect on xylanase production. Fig. 4a describes the effects of wheat bran and beef extract on xylanase production, when peptone was fixed at its middle level (0.70 N₂ equivalents). The xylanase production yield increased with increase in both the components and the increase in xylanase activity as a function of increasing levels of wheat bran seems very similar to the increase as a function of increasing beef extract concentration (Fig. 4a). High levels of xylanase production with increase in wheat bran concentration could be due to the fact that wheat bran is nutrient reservoir for xylanolytic microorganisms and acts as carbon and nitrogen source. While, the interaction between beef extract and peptone (wheat bran at middle level)

---

**Table 4**

<table>
<thead>
<tr>
<th>Run</th>
<th>Factor-A wheat bran</th>
<th>Factor-B beef extract</th>
<th>Factor-C peptone</th>
<th>Response xylanase activity (IU/ml)</th>
<th>Predicted value</th>
<th>Actual value</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>1.2</td>
<td>0.2</td>
<td>1134.85</td>
<td>1066.9</td>
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</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>1.2</td>
<td>0.7</td>
<td>1956.23</td>
<td>2015.21</td>
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</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>0.7</td>
<td>0.7</td>
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<td>1192.65</td>
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<tr>
<td>4</td>
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<tr>
<td>6</td>
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<td>7</td>
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<td>0.7</td>
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<td>1190.55</td>
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<tr>
<td>8</td>
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<td>0.2</td>
<td>1.2</td>
<td>710.71</td>
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<tr>
<td>9</td>
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<td>0.7</td>
<td>825.40</td>
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<td>10</td>
<td>1.5</td>
<td>0.7</td>
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<td>1053.64</td>
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<tr>
<td>11</td>
<td>0.5</td>
<td>1.2</td>
<td>1.2</td>
<td>678.49</td>
<td>753.11</td>
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<tr>
<td>12</td>
<td>1.5</td>
<td>0.7</td>
<td>1.2</td>
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<tr>
<td>13</td>
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<td>0.7</td>
<td>0.7</td>
<td>1128.42</td>
<td>1101.52</td>
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<tr>
<td>14</td>
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<td>1553.59</td>
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<tr>
<td>15</td>
<td>2.5</td>
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<td>0.7</td>
<td>1532.79</td>
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</tr>
<tr>
<td>16</td>
<td>0.5</td>
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<td>0.2</td>
<td>372.17</td>
<td>439.90</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.5</td>
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<td></td>
</tr>
<tr>
<td>18</td>
<td>2.5</td>
<td>1.2</td>
<td>0.2</td>
<td>2275.06</td>
<td>2210.18</td>
<td></td>
</tr>
<tr>
<td>19</td>
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<td>1128.42</td>
<td>1093.25</td>
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</tr>
<tr>
<td>20</td>
<td>1.5</td>
<td>1.2</td>
<td>0.7</td>
<td>1431.45</td>
<td>1347.64</td>
<td></td>
</tr>
</tbody>
</table>
demonstrated that the xylanase production increased with increase in beef extract concentration, but the enhancement in peptone concentration did not significantly increased the xylanase production (Fig. 4b).

The xylanase production capability of Streptomyces sp. RCK-2010 has been compared with the other Streptomyces strains grown under optimized conditions (Table 5). To the best of our knowledge Streptomyces sp. RCK-2010 has been found to produce fairly good amount of xylanase (2310 IU/ml) compared to the majority of the Streptomyces strains reported in the literature (8–2360 IU/ml) and comparatively exhibited maximum volumetric productivity (1155 IU/ml/day) under submerged fermentation conditions (Table 5).

3.5. Effect of pH and temperature on the activity and stability of xylanase

Among various pH values (3–10) tested, the partially purified xylanase was active over a wide range of pH 5–9 with more than 80% of residual enzyme activities. The optimal pH for Streptomyces sp. RCK-2010 xylanase was 6.0 (Fig. 5). Although, most xylanases known today are active at either acidic or neutral pH [30–32], recently several alkalo tolerant xylanases have also been reported in efficient bleaching of paper pulp [4,33]. Moreover the alkaline xylanases, which are operationally stable at higher temperature, are more beneficial because of savings in cooling cost and time [4]. The xylanase from Streptomyces sp. RCK-2010 exhibited its temperature optima of 60 °C at pH 6.0 (Fig. 6). Similar temperature optima of 60–65 °C have been reported earlier for other xylanases [4,31,33]. While the profiles obtained for thermostability (at 55 and 60 °C) of partially purified xylanase from Streptomyces sp. RCK-2010 revealed that the enzyme was thermostable with approximately 50 and 40% residual activity at 55 and 60 °C, respectively, after 4 h of incubation (Fig. 7).

Table 5
Comparison of xylanase production by different species of Streptomyces.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Production (IU/ml)</th>
<th>Time (days)</th>
<th>Volumetric productivity (IU/ml/day)</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomyces sp. QC-11-3</td>
<td>84.26</td>
<td>2</td>
<td>42.1</td>
<td>Xylan</td>
<td>[36]</td>
</tr>
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</table>
3.6. Application of Streptomyces sp. RCK-2010 in saccharification of steam exploded rice straw

The time course of enzymatic saccharification of steam exploded rice straw with a combination of cellulase and xylanase revealed that the sugar yield increased continuously till 48 h (Fig. 8). Among various dosage of xylanase used along with cellulases, the enzyme dosage of 60 IU/g dry substrate resulted in maximum increase in saccharification (208 mg/g dry substrate) and thereafter it did not result in any significant improvement (Fig. 8). Though the xylan fraction in steam exploded rice straw was only 18%, the higher release of sugar might be due to the loosening of xylan backbone in the steam exploded substrate. The higher sugar yield may also be attributed to the release of glucan monomers bound tightly to the xylan backbone. The synergistic action of cellulases and xylanases led to a total saccharification efficiency of 88%. Our results are consistent with the earlier works [7,34,35] and indicate higher saccharification of lignocellulosic substrates with combined action of cellulases and xylanases.

4. Conclusion

The xylanase from Streptomyces sp. RCK-2010 shows potential in saccharification of second generation feedstocks into sugars for their eventual fermentation to ethanol.

Acknowledgements

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References

Application of lignocellulolytic enzymes produced under solid state cultivation conditions

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A B S T R A C T

In this paper, cellulose from brown-rot fungus Fomitopsis sp. RCK2010, thermostable and alkalostable xylanase from Bacillus pumilus MK001 and laccase from Ganoedera sp. rckk-02 were evaluated for (i) saccharification of alkali pretreated rice straw and wheat straw, (ii) upgrading of chick feed and (iii) decolorization of dyes, respectively. The cellulose from brown-rot fungus resulted in a sugar release of 151.48 and 214.11 mg/g, respectively, from rice straw and wheat straw, which was comparatively higher than the earlier reports. While xylan, one of the main anti-nutritional factors (AFS) present in the chick feed was removed to an extent of 11.6 mg/g xylose sugars at 50 °C using the thermostable xylanase. Besides, the treatment with thermostable xylanase also brought about a release of 0.85 (mg/g) of soluble phosphorous. Moreover, the laccase when used for the decolorization of Remazol Brilliant Blue R (RBBR) and xylidine ponceau cause almost complete decolorization in 2 and 4 h, respectively, depicting high rate of decolorization.

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1. Introduction

Enzymes have always played a substantial role in the production of variety of commercial products from many industries viz. food processing, beverage production, animal feed, leather, textile and detergent (Gavrilescu and Chisti, 2005; Kuhad and Singh, 2007). These enzymes not only make the process environmentally benign but also play an important role in improving the productivity and eventually the cost of product formation. Since last two decades, among various industrial enzymes, celluloses, xylanases and laccases (lignocellulolytic enzymes) are gaining enormous attention for their potential applications in bioconversion of cellulosic materials into value added commodities, biostoning and biopolishing of jeans, improving efficacy of detergents, maceration and color extraction from juices, enzymatic deinking, pulping, wastewater treatment, to improve the nutritional properties of animal feed, retting of flax, production of oligosaccharides, clarification of juices, biotransformation, treatment of dyes and other organic pollutants and development of biosensors, etc. (Xu, 2005; Kuhad and Singh, 2007; Kuhad et al., 2011).

Specially for more than a decade, hydrolysis of crop-byproducts such as rice and wheat straw, etc. by cellulases has picked up momentum for their conversion into sugars for ethanol production (Kuhad et al., 2010a; Deswal et al., 2011). The information on hydrolysis of crop byproducts (lignocellulosics) by cellulases from soft-rot and white-rot fungi is available in plenty, while the reports on hydrolysis of cellulosics with celluloses from brown-rot fungal celluloses are scanty (Baldrian and Valaskova, 2008). The brown rot-fungi differ substantially from soft-rot and white-rot fungi with respect to the cellulolytic enzymes produced and the pattern of cellulose degradation. These fungi are generally reported to lack the exoglucanases that can hydrolyze crystalline cellulose, yet they cause the most destructive type of wood decay and are important contributors to biomass recycling (Kuhad et al., 1997). However, recently we have observed that Fomitopsis sp. RCK2010, a brown-rot fungus, produces exoglucanases (Deswal et al., 2011).

In addition to prominent usage of xylanase in pulp and paper industry (Beg et al., 2001), they have been reported to improve feed value and performance of monogastric animals (Sinha et al., 2011). Xylanases have potential in improving chick feed by hydrolyzing the anti-nutritional factors present in the feed grains i.e. non-starch polysaccharides such as arabinoxylans. Other advantages of using xylanases as feed supplements are dehulling of cereal grains, better emulsification and flexibility of feed materials (Galante et al., 1998).

Textile effluents containing synthetic dyes constitute one of the most problematic wastewaters to be treated not only for their high chemical and biological oxygen demands, suspended solids and toxic compounds but also for their color (Diwaniany et al., 2010). Biological decolorization by means of ligninolytic enzymes is considered to be a suitable and eco-friendly method to dye degradation and color removal. Among ligninolytic enzymes, laccases have received much attention due to their ability to oxidize both...
phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants including synthetic dyes (Kuhad et al., 2004; Couto and Herrera, 2006). This ability makes them efficient for waste-water treatment of effluents containing chemically diverse and toxic dyes from textile industries. Moreover, the decolorization of dyes with laccases also reduces the operational cost and energy required in conventional chemical methods (Couto and Herrera, 2006).

In the present study, we have specifically studied the applications of lignocellulolytic enzymes produced under solid state cultivation conditions in the hydrolysis of crop-byproducts, improvement of chick feed and decolorization of industrial dyes. The cellulose from a brown rot fungal isolate Fomitopsis sp. RCK2010 (Accession number GU991381) has been evaluated for the hydrolysis of pretreated crop-byproducts (wheat straw and rice straw). The highly thermostable and alkalostable xylanase from Bacillus pumilus MK001 (Accession number AY389345) has been used for the improvement of chick-feed, while the laccase from Ganoderma sp. rckk-02 (Accession number AJ749970) has been tested for its ability to decolorize azo (xylidine ponceau) and anthraquinone dyes (Remazol Brilliant Blue R; RBBR).

2. Methods

2.1. Raw materials and biomass preparation

Lignocellulosic substrates (wheat bran, wheat straw and rice straw) obtained locally were dried and cut mechanically into small pieces by a chopper, as described earlier (Kuhad et al., 2010b; Gupta et al., 2011). Xylidine ponceau and RBBR dyes were purchased from Hi Media Laboratories Pvt. Ltd. (Mumbai, India). Phosphate buffer (pH 5.5) at 30 °C and anthraquinone dyes (Remazol Brilliant Blue R; RBBR). The cellulose from a brown rot fungal isolate Fomitopsis sp. RCK2010 (Accession number GU991381) has been evaluated for the hydrolysis of pretreated crop-byproducts (wheat straw and rice straw). The highly thermostable and alkalostable xylanase from Bacillus pumilus MK001 (Accession number AY389345) has been used for the improvement of chick-feed, while the laccase from Ganoderma sp. rckk-02 (Accession number AJ749970) has been tested for its ability to decolorize azo (xylidine ponceau) and anthraquinone dyes (Remazol Brilliant Blue R; RBBR).

2.2. Microorganisms and culture conditions

B. pumilus MK001, Fomitopsis sp. RCK2010 and Ganoderma sp. rckk-02 were procured from the culture collection of Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, New Delhi, India. The fungal cultures (Fomitopsis sp. RCK2010 and Ganoderma sp. rckk-02) were grown on malt extract agar (MEA) composed of (g/l): malt extract, 20.0; Ca(NO3)2.4H2O, 0.5; MgSO4.7H2O, 0.5; KH2PO4, 0.5; agar, 20.0 (pH 5.5) at 30 °C (Vasdev et al., 2005). While, B. pumilus strain MK001 was maintained on modified Horikoshi xylan–agar medium containing (g/l): xylan 5.0, peptone 5.0, yeast extract 5.0, KH2PO4 1.0, MgSO4.7H2O 0.1 and agar, 20.0 (pH 9.0) at 37 °C (Kapoor et al., 2007). The cultures were maintained by periodical static cultivation conditions for 7 days. The mycelial mat thus obtained was homogenized with pestle and mortar under sterile conditions and used as inoculum for the production of enzymes.

Prior to the enzymatic hydrolysis, the RCK2010 (Accession number GU991381) has been evaluated for the hydrolysis of pretreated crop-byproducts (wheat straw and rice straw). The highly thermostable and alkalostable xylanase from Bacillus pumilus MK001 (Accession number AY389345) has been used for the improvement of chick-feed, while the laccase from Ganoderma sp. rckk-02 (Accession number AJ749970) has been tested for its ability to decolorize azo (xylidine ponceau) and anthraquinone dyes (Remazol Brilliant Blue R; RBBR).

2.3. Lignocellulolytic enzyme production under solid state fermentation

Among lignocellulolytic enzymes, cellulose and laccase were produced from Fomitopsis sp. RCK2010 and Ganoderma sp. rckk-02, respectively, while the xylanase was produced from B. pumilus MK001. All the enzymes were produced under solid-state cultivation conditions. For the production of fungal enzymes (cellulose and laccase), 0.5% (w v−1) of both the fungal cultures having 0.25 ± 0.013 g of fungal dry mass were inoculated separately in 250 ml Erlenmeyer flasks, each having 5.0 g of dry wheat bran moistened with mineral salt solution (g/l): (NH4)2SO4, 0.5; KH2PO4, 0.5; MgSO4.0.5 and pH 5.5) to attain the final substrate-to-moisture ratio of 1:3.5 and incubated at 30 °C for 11 and 6 days, respectively. After incubation, the fungal fermented wheat bran was removed aseptically and the enzymes were extracted in 50 ml citrate buffer (100 mM, pH 5.5) (Sharma et al., 2005; Deswal et al., 2011).

For the production of xylanase, each 250 ml Erlenmeyer flasks containing 5 g of dry wheat bran moistened with mineral salt solution, containing (g/l): KH2PO4, 1.0; NaCl, 2.5; MgSO4.7H2O, 0.1; (NH4)2SO4, 1.0; CaCl2 0.1 and pH 9.0, to attain final substrate-to-moisture ratio of 1:4 were inoculated with 10% (v v−1) of seed culture of B. pumilus strain MK001 containing 106 CFU ml−1 and incubated at 37 °C for 5 days under static conditions. The enzyme was extracted in 50 ml citrate phosphate buffer (pH 6.0; 100 mM) (Kapoor et al., 2007).

2.4. Applications

2.4.1. Enzymatic hydrolysis of lignocellulosic biomass

2.4.1.1. Pretreatment of the substrates. Wheat straw and rice straw, the lignocellulosic, were pretreated with different dosage of sodium hydroxide (0.5–2.5% w v−1) and sulphuric acid (0.5–2.5% v v−1) to enhance their enzymatic susceptibility. The pretreated substrates were washed until neutral pH and dried in an oven at 60 °C till constant weight was achieved.

2.4.1.2. Enzymatic hydrolysis. Prior to the enzymatic hydrolysis, the pretreated substrate at 2.0% substrate consistency in 50 mM citrate phosphate buffer (pH 5.0) containing 0.005% (w v−1) sodium azide was acclimatized at 50 °C and 150 rpm for 2 h. Further the enzyme dosage of 25 U FPase/g dry substrate was added to the preincubated substrate suspension and the reaction mixture was incubated at 50 °C, 150 rpm for 24 h. Samples were withdrawn at regular intervals of 4 h, centrifuged at 10,000 rpm for 10 min and the filtrate was used for the analysis of reducing sugars following DNS method of Miller (1959).

2.4.2. Improvement of chick feed

For the pretreatment of chick feed with xylanase, the crude xylanase dosage was optimized by incubating 10.0 g of chick feed (moistened with 20 ml water) with variable amounts of crude xylanase (10–50 U/g chick feed) for 2 h at 50 °C. After incubation the suspension was centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for the release of sugars and soluble phosphorus.

2.4.3. Dye decolorization

To evaluate the potential of laccase for decolorization efficiency, 50.0 ml of azo (xylidine ponceau) and anthraquinone (RBBR) dyes (100 mg/l; pH 5.5) were mixed separately with the enzyme (10 U/ml of dye solution; unless otherwise specified) (with and without HBT), was incubated at 30 °C and 150 rpm in a rotary incubator shaker. Dye content was monitored at the maximum visible absorbance of each dye (respectively, 542 and 595 nm). A control
in which laccase was replaced by citrate phosphate buffer (100 mM, pH 5.5) was conducted in parallel. The decolorization percentage was defined as follows:

Decolorization percentage (\%) = \left( \frac{A_0 - A}{A_0} \right) \times 100

where, \(A_0\) is the dye absorbance of the control, \(A\) is the dye absorbance of the reaction sample.

In this study, decolorization of both the dyes was studied with respect to three operational parameters: effect of HBT and its concentration, reaction time and enzyme dose. To study the effect of HBT concentration on dye decolorization, varied doses of the mediator (0.05–2.0% w/v) were added to the reaction mixture. The optimum dose of the mediator was subsequently used for dye decolorization with varied laccase doses (5–30 U/ml) to obtain the optimal enzyme dosage for improved decolorization in minimum time. Decolorization was monitored at an interval of 2 h till complete decolorization was achieved for each enzyme dose.

2.5. Analytical methods

The compositional analysis of oven dried crop byproducts for lignin and holocellulose was carried out using the protocols of Technical Association of Pulp and Paper Industries (TAPPI, 1992). The cellulose activity was determined in accordance with the International Union of Pure and Applied Chemistry procedures (Ghose, 1987), while the laccase and xylanase were determined as described earlier (Sharma et al., 2005). Reducing sugars were assayed by dinitrosalicyclic acid (DNSA) method of Miller (1959), while the soluble phosphorous content was analyzed following the method of Fiske and Subbarow (1925).

All the experiments were done in triplicates and the values presented here are the mean values ± standard deviations within the range of 10%.

3. Results and discussion

3.1. Enzymatic saccharification of pretreated substrates

Lignocellulosic biomass cannot be hydrolyzed efficiently by enzymes without its suitable pretreatment, as the lignin in the cell wall hinders the enzymatic catalysis. Therefore in order to improve the enzymatic hydrolysis of rice straw and wheat straw the lignin removal is essential (Gupta et al., 2011). In the present study we evaluated the effect of both acid and alkali pretreatments on the enzymatic saccharification. During the enzymatic saccharification of alkali pretreated substrates, the sugar release increased with increasing in NaOH concentration up to 2.0% and thereafter remained almost constant. The enzymatic hydrolysis of rice straw and wheat straw pretreated with 2% (w/v) alkali resulted in a sugar release of 151.48 and 214.11 mg/g dry substrate, respectively (Fig. 1a and b). The maximum acidic saccharification from wheat straw (68.071 mg/g dry substrate) and rice straw (76.05 mg/g dry substrate) was achieved for cellulose from brown rot fungus *Fomitopsis* sp. RCK2010.
substrate) was observed, when both the substrates were pre-
treated with 0.5% (v/v) sulphuric acid (Fig. 1c and d). As com-
pared to untreated substrates (control), the higher residual lignin
and lower residual holocellulose in pretreated substrates may be
due to the removal of acid soluble carbohydrate fraction, resulting
in a decreased saccharification (Gupta et al., 2011). Also, during
acid pretreatment solubilized lignin can quickly condensate and
precipitate in acidic environments (Gupta et al., 2011). Our results
are well in accordance with the previous reports where Chen et al.
(2009) reported an increase in lignin content in acid hydrolyzed
corn stover. The results obtained in the present study revealed that
the enzyme system from Fomitopsis sp. RCK2010 has shown com-
paratively higher saccharification efficiency than the enzymes
from brown-rots reported earlier (Table 1).

3.2. Improvement of chick feed

Xylanases for the digestion of antinutritional factors (ANFs)
such as non-starch polysaccharides (NSPs) are scarce or not present
in monogastric animals. Consequently, the dietary NSPs remain
undigested and therefore negatively affect animal performance.
The adverse effect is associated with the viscous nature of NSPs,
their physiological and morphological effects on digestive tract,
interaction with epithelium, mucus and micro-flora of gut. How-
ever, the insoluble NSPs, like pentosans (arabinoxylans and
xylans), beneficially decrease transit time, enhance water holding
capacity and assist in faecal bulking in non-ruminant animals (Sin-
ha et al., 2011). Pretreatment of chick feed with xylanase has the
potential to improve feed efficiency, growth rate of the monoga-
tric animals and mitigate environmental pollution (Jozefiak et al.,
2007). Moreover, xylanases have been reported to reduce viscosity
and increase absorption by breaking down the non-starch polysac-
charides (NSPs) in the feeds of monogastric animals (Jozefiak et al.,
2007; Kuhad et al., 2011). In the present study an attempt was
made to study the effect of xylanase on the breakdown of NSP
present in chick feed and release of reducing sugars in the solution.
Among the different enzyme dosage (10–50 U/g dry chick feed)
used, xylanase when used at 30 U/g dry chick feed resulted in max-
imum release of reducing sugars (11.6 mg/g dry chick feed) and
soluble phosphorous (0.85 mg/g dry chick feed) at 50°C after 2 h
of incubation. The enzyme treatment of chick feed at higher
enzyme doses did not increase release of reducing sugars and sol-
uble phosphorous (Fig. 2). Xylanase from B. pumilus strain MK001
has the potential to improve chick feed, as evident from the release
of reducing sugars and increase in phosphate availability after
enzyme treatment of poultry feeds.

3.3. Dye decolorization by laccase from Ganoderma sp. rckk-02

In the present study, irrespective of the dyes, 0.1% of HBT, a re-
dox mediator, when used in conjunction with 10 U of laccase/ml of
the dye solution resulted in almost complete decolorization in 12 h
of incubation at 30°C (Table 2). This may be attributed to the fact
that redox mediators, the compounds which speed up the rate of
reaction by shuttling electrons from the biological oxidation of pri-
mary electron donors or from bulk electron donors to the electron-
accepting organic compounds. Our results are well in accordance
with the other reports which showed enhanced laccase activity

<table>
<thead>
<tr>
<th>Source</th>
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<td>70.9</td>
<td>Lee et al. (2008)</td>
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<td>Pinus densiflora</td>
<td>3.5</td>
<td>Lee et al. (2008)</td>
</tr>
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<td>Fomitopsis palustris</td>
<td>Avicel</td>
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<td>Yoon et al. (2007)</td>
</tr>
<tr>
<td>Gloephyllum trabeum</td>
<td>Spruce</td>
<td>25.51</td>
<td>Schilling et al. (2009)</td>
</tr>
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<td>Gloephyllum trabeum</td>
<td>Southern yellow</td>
<td>11.07</td>
<td>Schilling et al. (2009)</td>
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</tr>
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<td>Southern yellow</td>
<td>46.35</td>
<td>Tewalt and Schilling (2010)</td>
</tr>
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<td>Fomitopsis sp. RCK2010</td>
<td>Wheat straw</td>
<td>214.11</td>
<td>Present work</td>
</tr>
<tr>
<td>Fomitopsis sp. RCK2010</td>
<td>Rice straw</td>
<td>151.48</td>
<td>Present work</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of different dosage of xylanase for the removal of ANFs from the chick feed at 50°C, pH 9.0 after 2 h. Reducing sugars (●) and soluble phosphorous (▲).
Moreover in conjunction with 0.1% w/v \(C_0\) of HBT, the effect of different dosages of laccase (5–40 U/ml) on the decolorization of both the dyes (100 mg/l) was also evaluated. The rate of decolorization of both the dyes increased with increase in the enzyme dosage till 30 U/ml, and thereafter it did not increase significantly (Fig. 3a and b). The decolorization pattern of xylidine ponceau and RBBR dyes depicted that when only 5 U/ml laccase dye solution was used, the complete decolorization was observed after 24 and 18 h, respectively (Fig. 3a and b). Interestingly with increased dosage of 30 U/ml laccase of dye solution, the decolorization period for xylidine ponceau and RBBR dyes reduced to 4 and 2 h, respectively (Fig. 3a and b). The rate of decolorization of RBBR was higher than the xylidine ponceau, which may be attributed to the structural differences in the dyes (Diwaniyan et al., 2010). Comparatively, the laccase from \(Ganoderma\) sp. rckk-02 has shown better decolorization rates than the enzymes from other white-rot fungi reported earlier (Table 3).

### 4. Conclusion

In nature there are a wide variety of lignocellulolytic microorganisms, which due to their specific properties could be of more use in various industrial applications. In the present work, the unique filter paper cellulose producing quality of brown-rot fungus \(Fomitopsis\) sp. RCK2010, the thermostability of xylanase from \(B.\) \(pumilus\) MK001 and the high rate of decolorization by laccase from \(Ganoderma\) sp. rckk-02 was used for their specific applications. The
exploitation of such enzymes in specific industrial applications can improve the productivity and cost effectiveness of any product.

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Dadalea quercina fungus


