

Enzymatic hydrolysis of water hyacinth biomass for the production of ethanol: Optimization of driving parameters

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An efficient conversion of lignocellulose into fermentable sugars is a key step in producing bioethanol in a cost effective and eco-friendly manner. Alternative source like water hyacinth biomass (WHB) (*Eichhornia crassipes*) may be used as a supplement for the routine feedstocks. The enzyme loading for optimum yield of total reducing sugar was investigated and the enzyme-substrate interaction optimised. The maximal reducing sugar and xylose yield was obtained using cellulase and xylanase loading of 46.12 and 289.98 U/g and 2.26% (w/v) substrate loading. The efficiencies of ethanol production from the WHB hydrolysate are very less and the maximal ethanol yield was 3.4969 g/L when *Pichia stipitis* was used, followed by 3.4496 and 3.1349 g/L for *Candida shehatae* and *Saccharomyces cerevisiae*.

Keywords: Enzymatic hydrolysis, Fermentation, Optimization, Response surface methodology, Water hyacinth

Steady increase in global warming is associated with the increment in the emission of CO₂ to the atmosphere by the continuous use of fossil fuels¹. Combined with a dependency on increasingly scarce fossil fuels, it has sparked a tremendous interest in alternative renewable fuel sources. Consequently, there has been an emphasis on plant biomass as a source of fermentable sugars. Often being touted as the world's most abundant polymers, cellulose and other plant carbohydrates are believed to be the renewable energy source that can provide liquid fuels and chemicals on a sustainable basis, in turn replacing fossil fuels and decreasing CO₂ emissions². Cellulosic bioethanol, also known as second-generation bioethanol, is seen as a more attractive alternative. It can be produced from all kinds of plant materials, ranging from rice straw to forest residues. Furthermore, lignocellulosic ethanol has the potential to produce large quantities of fuel with more significant reduction in greenhouse gas emissions³. Plant cell walls are highly recalcitrant to degradation, both microbial and mechanical, and hence it is one of the major challenges associated with the enzymatic conversion of cellulosic plant biomass into fermentable sugars^{4,5}. To address this challenge of

efficiently hydrolysing cell walls into fermentable sugars, also known as saccharification, two key aspects of cellulosic bioethanol production have been improved upon: pretreatment and enzyme optimisation. Pretreatment is a balancing act that involves unlocking the cell wall structure without forming inhibitors, which affect hydrolysis and fermentation⁶.

The main objective of the present work is to understand the enzyme-substrate interactions in the enzymatic hydrolysis of pretreated biomass. An efficient conversion of lignocellulose into fermentable sugars is a key step in producing bioethanol in a cost effective and ecofriendly manner. The most studied lignocellulosic substrates for the biological hydrolysis are wheat straw, rice straw and bagasse etc. However, due to stringent government directives, the non-edible lignocellulosic sources may provide a viable answer⁷. It has several advantages of being largely available in supply and no competition with food requirements. Therefore for avoiding this competition, alternative sources such water hyacinth biomass (WHB) (*Eichhornia crassipes*) can be used as a supplement. The enzyme loading for optimum yield of total reducing sugar was investigated and the enzyme-substrate interaction was optimised.

Bioconversion of cellulose and hemicellulose— Cellulose is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1-4)-glycosidic

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bonds. Cellobiose is the smallest repetitive unit of cellulose and can be converted into glucose residues. The cellulose-hydrolysing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases, cellobiohydrolases (exoglucanases), and β -glucosidases. The endoglucanases catalyze random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasing cellobiose. β -glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose. Bioconversion of cellulose into fermentable sugars is a biorefining area that has attracted enormous research efforts, as it is a prerequisite for the subsequent production of bioenergy. Sugars and starch comprise the feedstock for 90% of the produced ethanol today, but the most prevalent forms of sugar in nature are cellulose and hemi-cellulose. Lignocellulosic biomass can be converted to ethanol by hydrolysis and downstream fermentation processing. This process is much more complicated than just fermentation of C_6 sugar and is still far from being cost effective as compared to the production of bioethanol from starch or sugar crops. In hydrolysis, the cellulosic part of the biomass is converted into sugars, and fermentation converts these sugars to ethanol. Lignocellulosic biomass consists of 10–25% lignin, which contains no sugar, and therefore impossible to convert into sugars. Lignin is therefore a residue in ethanol production, and it represents a big challenge to convert it into a value-added product⁸. Hemicellulose is the second most abundant renewable biomass and accounts for 25–35% of lignocellulosic biomass. Hemicelluloses are heterogeneous polymers built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. Hemicelluloses in hardwood contain mainly xylans, while in softwood glucomannans are most common. There are various enzymes responsible for the degradation of hemicellulose. In xylan degradation, for instance, endo-1, 4- β -xylanase, β -xylosidase, α -glucuronidase, α -L-arabinofuranosidase and acetylxylan esterase act on the different heteropolymers available in nature. In glucomannan degradation, β -mannanase, and β -mannosidase cleave the polymer backbone. Like cellulose, hemicellulose is also an important source of fermentable sugars for biorefining applications. Xylanases are being produced and used as additives in feed for poultry and as additives to wheat flour for

improving the quality of baked products, at the industrial scale⁸.

Cellulolytic enzymes— For microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell associated. Components of cellulase systems were first classified based on their mode of catalytic action and have more recently been classified based on structural properties. Three major types of enzymatic activities found are: The endoglucanases attack the region of low crystallinity in cellulose fibre, and thereby create free ends. The exoglucanases remove cellobiose from the free ends of the chain. And, the β -glucosidases convert cellobiose to glucose.

Due to their heterogeneity and complex nature, the complete breakdown of xylans requires the action of a large variety of hydrolytic enzymes. These enzymes can be classified into two main groups: xylanases, that hydrolyse internal linkages in xylan, and xylosidases that release xylose units from xylobiose and xylo- oligomers.

The complex structure of xylan needs different enzymes for its complete hydrolysis. Endo-1, 4- β -xylanases (1,4- β -D-xylanxylanohydrolase, E.C.3.2.1.8) depolymerise xylan by the random hydrolysis of xylan backbone and 1,4- β -D-xylosidases (1,4, β -D-xylan xylohydrolase) (E.C.3.2.1.37) split off small oligosaccharides. The side groups present in xylan are liberated by α -L-arabinofuranosidase, α -D-glucuronidase, and galactosidase and acetyl xylan esterase⁸.

Optimizing enzyme composition— Extensive research is being done on improving the performance of the enzymes. This involves screening for new enzyme-producing microorganisms, random mutagenesis of fungal strains and genetic engineering of individual enzymes. The development is on increasing specific activity, modifying carbohydrate binding molecules (CBMs) to alter interaction with cellulose, increasing tolerance towards end products, improving thermal stability to enable operation at higher temperatures and in some cases modifying pH optima⁹.

The present study investigates the effect of enzymatic hydrolysis using enzymes like cellulase, xylanase on alkali, acid and untreated water hyacinth and then optimizing the enzyme concentration with substrate loading and studying the interaction among the enzymes for a maximum total reducing sugar as

well as xylose yield. Optimization of enzymatic hydrolysis of alkali pretreated biomass was carried out since it gave better results during batch study.

Materials and Methods

Biomass preparation—Water hyacinth plants were obtained from an unpolluted pond within the CSIR-CMERI grounds. Only the shoots and leafy parts of the plants were used and the roots were discarded. The shoots and leaves were first reduced to a particle size of about 2-3 cm and then dried at 106 °C for 6 h. After drying, particle size was further reduced to 1-2 mm by grinding. This grounded water hyacinth biomass (WHB) was used for the experiments. It was stored in air tight containers at room temperature. The dried WHB was pre-treated with dilute H₂SO₄ (5%) with soaking time 1 h and treatment time 10 min at 130 °C and also with NaOH solution (5%) with soaking time 1 h and treatment time 10 min at 150 °C. Biomass loading was 10% (w/v) as that was the maximum possible loading. The acid treated and alkali treated WHB was then washed thoroughly under running water until pH was neutralized. The washings were then dried at room temperature and stored in air tight container till further use.

Fourier Transform Infrared (FTIR) experiment—The spectra were studied on WHB using a Shimadzu spectrometer (Japan). For this, 3 mg of the sample was dispersed in 300 mg of spectroscopic grade KBr and subsequently pressed into disks at 10 MPa for 3 min. The spectra were obtained with an average of 25 scans and a resolution of 4 cm⁻¹ in the range of 4000–400 cm⁻¹.

X-ray diffraction (XRD) experiment—The XRD analysis of WHB was performed using Rigaku diffractometer (Rigaku, Tokyo, Japan) using Cu K α radiation at 40 kV and 30 mA in the scanning angle of 5-50° at the scanning speed of 0.5°/min. Crystallinity of cellulose was calculated according to the empirical method proposed by Segal *et al.*¹⁰ for native cellulose:

$$CrI(\%) = \left[\frac{I_{002} \cos^{-1} 18.0^\circ}{I_{180}} \right] \times 100 \quad \dots (1)$$

where *CrI* is the crystalline index, *I*₀₀₂ is the maximum intensity at the (002) lattice diffraction and *I*₁₈₀ is the intensity diffraction at 18°, 2 θ degrees.

Biomass saccharification—Enzymatic biomass saccharification was carried out using crude enzyme extracts, cellulase from *Trichoderma reesei* and

xylanase from *Trametes versicolor* (Sigma Aldrich®), each used separately. The hydrolysis was performed in 50 mM citrate buffer (pH 4.8), at 50 °C, in stoppered 100 mL flasks with gentle agitation (125 rpm) and the volumes were adjusted to achieve required biomass loading of 4% for acid treated, alkali treated and untreated WHB. The experiment was carried out for 60 h. Samples were collected at regular intervals of time. The sugars released were monitored by by 2, 5 dinitrosalicylic acid method^{11,12} for reducing sugar yield and modified Tollen's test for xylose yield¹³. The sugar hydrolysate was obtained by centrifugation. The hydrolysate was first centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was collected and filter sterilized. The hydrolysate was collected in screw cap bottles and stored at 4 °C until further use.

Optimization of biomass saccharification using commercial enzymes—The combined effect of cellulase and xylanase in combination with the effect of substrate loading was assessed using response surface methodology. The loading of cellulase, xylanase, and the substrate loading was optimized for enhancing the reducing sugar and xylose yields using a response surface Box-Behnken experiment design (Box and Behnken, 1960). The design matrix with 17 experimental runs in two blocks with 5 replicates of the midpoint was used for the experiment. The variables selected for optimization, i.e. cellulase concentration, xylanase concentration, and the substrate loading were coded as X₁ and X₂ and X₃, respectively. Incubations were carried out as described above for 48 h and the substrate used was alkali treated WHB. The model, constructed as a response function of the variables on cellulase production was a second-order polynomial as follows:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^k \sum_{j=1+l}^k \beta_{ij} x_i x_j + \epsilon \dots (2)$$

where *Y* is response (dependent variable), β_0 is constant coefficient, β_i , β_{ii} , β_{ij} are coefficients for the linear, quadratic and interaction effect, *x*_{*i*}, *x*_{*j*} are factors (independent variables), the error is represented by ϵ . In this work a second order polynomial equation was obtained using the uncoded independent variables as mentioned in eq. 3:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_{11} + \beta_{22} x_{22} + \beta_{33} x_{33} + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad \dots (3)$$

Regression analysis and estimation of the coefficients were performed using Design Expert®7.0.0 trial version. The contributions of individual parameters and their quadratic and interaction effects on cellulase production were determined. The best combination of parameters for obtaining maximal reducing sugar yield was determined by using the numerical optimization function in Design Expert®7.0.0 trial version.

Fermentation—*Pichia stipitis* (NCIM 3500) and *Candida shehatae* (NCIM 3497) were used for pentose and hexose fermentation simultaneously. They were obtained by the courtesy of National Collection of Industrial Microorganisms (NCIM), Pune. The medium used for inoculum preparation contained the following chemicals: D-xylose 50 g/L; glucose 5 g/L; yeast extract 3 g/L; malt extract 3 g/L; peptone 5 g/L; pH 5.0. Medium was sterilized by autoclaving at 120 °C for 15 min. *Saccharomyces cerevisiae* (MTCC 181) was obtained by the courtesy of CSIR-Institute of Microbial Technology (IMTECH), Chandigarh. The medium used for inoculum preparation contained: yeast extracts 10 g/L, peptone 20 g/L and D-glucose 10 g/L. 10% inoculum was used for ethanol production. To prepare the inoculum, a 250 mL Erlenmeyer flask containing 50 mL medium was inoculated from a fresh culture plate, and incubated at 30 °C on rotary shaker at 125 rpm. The culture was grown again for 18 h for *S. cerevisiae* and 20 h for *P. stipitis* and *C. shehatae* under conditions similar to those described above and the broth was centrifuged at 10,000 rpm for 10 min. The cell pellet was washed and suspended in sterile distilled water. The hydrolysate supplemented with NH₄Cl 0.5 g/L, KH₂PO₄ 2.0 g/L, MgSO₄·7H₂O 0.5 g/L, yeast extract 1.5 g/L, CaCl₂·2H₂O 0.1 g/L, FeCl₃·2H₂O 0.1 g/L, ZnSO₄·7H₂O 0.001 g/L was inoculated with 10% (v/v) inoculums of *P. stipitis*, *C. shehatae* and *S. cerevisiae* at pH 5.0 and incubated at 30°C with 150 rpm of agitation. The pH of the medium was adjusted with 2 N HCl and 2 N NaOH. Samples were withdrawn at regular intervals of time and centrifuged for 15 min. The supernatant was used to determine the ethanol and residual sugar concentration. Ethanol estimation was done spectrophotometrically by potassium dichomate method.

Analytical methods—The reducing sugars released were monitored by DNS method as per Miller¹¹ and Onsoni *et al.*¹², for reducing sugar yield.

Pentose sugar (xylose) content was determined by the modified Tollen's test for xylose yield using phloroglucinol (0.5 g/100 mL acetic acid)¹³.

Results and Discussion

Studies on removal of lignin from WHB—The initial pretreatment of WHB was done using dilute acid and dilute alkali, separately. Dilute acid pretreatment was carried out with 5% H₂SO₄, 1h soaking time and 10 min treatment at 130 °C. Alkali pretreatment was carried out with 5% NaOH; soaking time of 1h, temperature of 150 °C and treatment time of 10 min was used. The pretreatment was carried out to remove lignin from the plant biomass so as to increase its digestibility for maximum release of sugars for bioethanol production. The hemicelluloses and lignin were estimated by the method suggested by Goering and Van Soest¹⁴. The cellulose content was measured by the anthone method¹⁵. The WHB used for this work had a composition of 35 % cellulose, 33.8% hemi-cellulose, 15.5 % lignin as determined in the laboratory. It has been reported that the biomass of water hyacinth has about 48% hemicellulose, 13% cellulose 3.5% lignin¹⁶.

FTIR analysis—FTIR spectra of the untreated and treated samples indicated structural changes in the biomass upon pretreatment and saccharification. Bands at 1000 to 1200 cm⁻¹ were related to structural features of cellulose and hemicelluloses¹⁷. The change in the peaks in the given region (Fig. 1), suggests that there was increase in absorbance in these regions. The peak at 1735 cm⁻¹ was observed due to either the acetyl and uronic ester linkage of carboxylic group of the ferulic and p-coumeric acids of lignin and/or hemicelluloses¹⁸. A sharp band at 896 cm⁻¹, corresponding to the C1 group frequency or ring frequency, was attributed to the β-glycosidic linkages (1→4) between xylose units in hemicelluloses¹⁹. These peaks in the alkali pretreated sample had the highest absorbance suggesting increase in cellulose and hemicellulose content. The peak at 1637 cm⁻¹

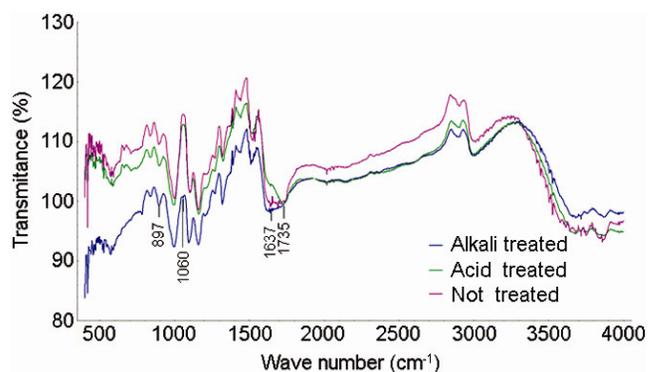


Fig. 1—FTIR spectra of untreated, acid treated and alkali treated biomass XRD Analysis.

represented the adsorbed water and this peak was enhanced in the acid and alkali treated WHB. In the FTIR spectrum, the peaks observed at 1060 and 896 cm^{-1} were attributed to C–O stretching and C–H rocking vibration of cellulose structure. At these peaks, the alkali treated sample had the maximum absorbance, suggesting increase in the cellulose content.

XRD analysis—X-ray diffraction profile of untreated, acid treated, alkali treated and alkali-enzyme treated biomass is shown in Fig. 2. The crystallinity index, degree of crystallinity and crystallite size of the raw biomass were calculated to be 52.88%, 0.242 nm, 62.43% respectively. The crystallinity index of untreated sample came out to be less than the pre-treated samples, where as the crystalline size of the untreated biomass came out to be larger than treated one. It can be attributed to the reason of lignin removal of biomass by saccharification. The crystallinity degree was more for pretreated biomass than that of untreated. This implies that pretreatment affects more on amorphous zone than crystalline zone.

Biomass saccharification with enzymes—A crucial step in the bioconversion of lignocellulosic feed stocks to biofuels is to cost-effectively maximize the saccharification of the cellulose and hemicellulose components to fermentable sugars. One of the challenges is the still too high enzyme costs involved

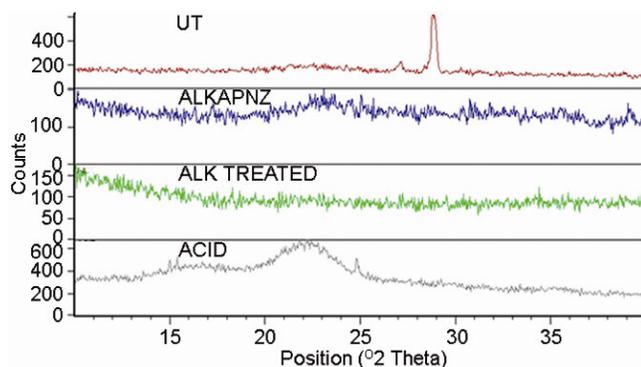


Fig. 2—XRD analysis of the biomass. (Untreated (UT), acid treated (ACID), alkali treated (ALK) and alkali-enzyme treated (ALKAPNZ) water hyacinth biomass)

in the saccharification of the cellulosic component and, to a lesser extent, the loss of some of the hemicellulosic sugars during pretreatment. Thus, in many pretreatment strategies such as steam explosion, mild severity conditions are often used to avoid, or at least minimize, sugar loss during pretreatment. Under these milder pretreatment conditions, some of the hemicellulose, mostly xylan in agricultural residues and hardwood, remains associated with the cellulose rich water insoluble fraction. However, this residual hemicellulose component is known to exert a significant influence on the effectiveness of enzymatic hydrolysis of its cellulosic component²⁰.

Comparative studies for acid, alkali and untreated WHB—Effect of chemical pretreatment with acid and alkali was studied followed by saccharification with commercial enzymes cellulase and xylanase was studied. The experiment was carried out for 48 h but it was observed that the maximum saccharification was at 36 h. Enzyme loadings of 15 U/g and 225 U/g were taken for cellulase and xylanase respectively. Table 1 show that alkaline treatment was the best pretreatment for enzymatic saccharification. This could be attributed to the fact that as lignin content decreases, accessibility of the substrate to the enzyme and, hence, digestibility increases²¹. The mechanism of alkaline hydrolysis is saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components, for example, lignin and other hemicelluloses. The porosity of the lignocellulosic materials increases with the removal of the cross links⁷. Alkaline hydrolysis has been reported to cause swelling, leading to increase in surface area, decrease in crystallinity and separation of structural linkages between lignin and carbohydrates, thus resulting in a more efficient pretreatment as the surface area available for reaction increases. It can also be seen that the reducing sugar yield was more than xylose yield when cellulase was used for saccharification, on the contrary when xylanase was used for saccharification, the trend was reversed. The alkaline pretreatment is known to solubilize hemicelluloses²². This effect may have resulted in the lesser amount of xylose released upon enzymatic hydrolysis. When

Table 1— Comparison of alkali and acid pretreatments for enzymatic hydrolysis.

Enzyme	Total reducing sugar (mg/g of WHB)			Xylose yield (mg/g of WHB)		
	Alkali treated WHB	Acid treated WHB	Untreated WHB	Alkali treated WHB	Acid treated WHB	Untreated WHB
Cellulase	151.545	41.864	18.955	88.43	40.815	25.18
Xylanase	27.654	26.675	27.275	61.35	32.45	32.975

xylanase was used for saccharification, the resultant sugars released was less as compared to those released by using cellulase. The cellulose content of the alkali treated WHB was higher than the hemicellulose content. Also xylanase released xylose as product. Thus the sugar yield was found to be less. Considering the results from the batch study alkali treated WHB was selected for further experiments of saccharification.

Individual effects of cellulase, xylanase and substrate loading—The hemicellulose-degrading enzyme activities detected in most of the commercially available cellulase preparations are too low, or are insufficiently active enough, to achieve significant conversion of the residual hemicelluloses. Therefore, supplementation of the cellulase enzymes with 'accessory enzymes'²² has been the most common approach to increase the overall fermentable sugar yields from pretreated hard woods and agricultural residues. Although accessory enzymes offer the potential to increase substrate digestibility, the high dosages of enzyme supplementation applied in many of the past studies can be difficult to justify because of the increased enzyme costs that are incurred²³. It is generally acknowledged that among the several factors that hamper our current ability to attain efficient lignocellulosic biomass conversion yields at low enzyme loadings, a major problem lies in our incomplete understanding of the cooperative action of the different enzymes acting on pre treated lignocellulosic substrates.

The synergistic action among the multiple forms of hemicellulose-degrading enzymes (for examples, enzymes acting on the xylan backbone and on xylan side chains) and also among the cellulose-degrading enzymes (such as exoglucanases and endoglucanases)

has been reported earlier²⁰. In the present study, the individual effects of cellulase, xylanase and substrate loading and the effect of the interaction between cellulase and xylanase enzymes and different substrate loadings were assessed to improve the hydrolysis efficiency.

Separate hydrolysis was studied using cellulase only on alkali treated WHB. A dosage range of 5 U/g to 70 U/g was used while keeping the substrate loading constant at 4%. It was observed that as the dosage of cellulase was increased the resultant sugar yield was also enhanced but there was not much of a difference in the sugars released at 50 U/g and 70 U/g cellulase concentrations (reducing sugar: 296.66 and 299.13 mg/g respectively and xylose: 167.66 and 149.08 mg/g. (Fig. 3). This could be attributed to the fact that as the concentration of enzyme was increased, the more freely it was available for reaction with the lignocellulosic substrate. When a dose of 70 and 100 U/g was applied, a plateau was reached and there was only marginal increase in the amount of sugars released. The yields of sugars released by using cellulase alone were low. This could most likely be because the readily accessible xylan was hydrolyzed in the first few hours of hydrolysis and that in order to access the xylan that is more closely associated with the cellulose and 'buried' within the fibre structure, the synergistic interaction with cellulases is required for more extensive xylan solubilization as reported earlier²⁰.

The depolymerisation action of endo-xylanase results in the conversion of the polymeric substance into xylooligosaccharides and xylose. The effect of xylanase loading was studied on alkali pretreated WHB. Xylanase loading was used in the range of 10 to 300 U/g. As shown in the graphs (Fig. 3), the

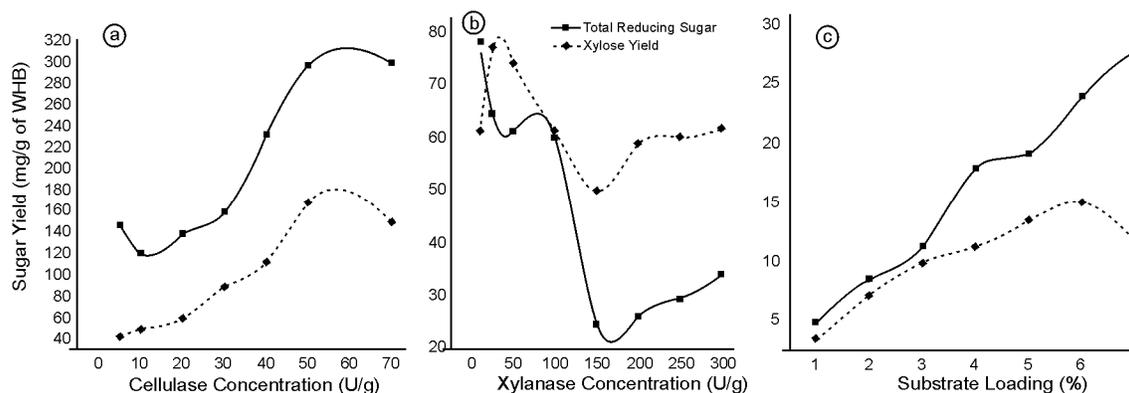


Fig. 3—Variation of sugar released based on the cellulase, xylanase and substrate loading.

sugars released decreased with increase in xylanase loading.

A range of 1-7% was chosen, as 7% was the maximum loading possible for water hyacinth so as to allow free aqueous solution for reaction. As the substrate loading was increased, the resultant sugars released also increased (Fig. 3). There was little difference between the amount of sugars released at 6 and 7% loading (reducing sugar: 23.9 and 27.78 mg/g respectively and xylose 14.98 and 11.6 mg/g respectively).

Design of experiment—Based on the batch studies above, a modelling experiment was performed on the above mentioned parameters. To optimize these parameters, a Box Behnken design was chosen to perform the experiment. Based on the batch experimental results the levels of each of the independent parameter were selected as given in Table 2. The different combinations of Cellulase, Xylanase and

Table 2—Values of independent variables at different levels in the study of enzymatic saccharification of alkali treated WHB using commercial enzymes.

Variable	Level		
	Low	Mid	High
Cellulase (U/g) - (A)	20	35	50
Xylanase (U/g) - (B)	150	225	300
Substrate Loading (%) - (C)	2	4	6

Substrate Loading tried for saccharification of alkali treated WHB and the corresponding reducing sugars yields are given in Table 3.

The data were analyzed by multiple regression analysis and a second-order polynomial equation was derived as follows (eq-4 and eq-5):

$$\begin{aligned} \text{Total reducing sugar} = & -913.52136 + 10.33205 \\ & * \text{Cellulase} + 10.47430 \\ & * \text{Xylanase} + 56.44398 \\ & * \text{Substrate Loading} - 0.012853 \\ & * \text{Cellulase} * \text{Xylanase} + 0.36658 \\ & * \text{Cellulase} * \text{Substrate Loading} \\ & - 0.24807 * \text{Xylanase} * \text{Substrate} \\ & \text{Loading} - 0.092464 * \text{Cellulase}^2 \\ & - 0.023635 * \text{Xylanase}^2 - 3.69234 \\ & * \text{Substrate Loading}^2 \quad \dots(4) \end{aligned}$$

$$\begin{aligned} \text{Xylose yield} = & + 723.48225 + 14.26160 * \text{Cellulase} - \\ & 11.45672 * \text{Xylanase} + 107.80816 \\ & * \text{Substrate Loading} - 3.10222\text{E-}003 \\ & * \text{Cellulase} * \text{Xylanase} - 0.49472 \\ & * \text{CELLULASE} * \text{Substrate Loading} - \\ & 0.34422 * \text{Xylanase} * \text{Substrate Loading} \\ & - 0.13462 * \text{Cellulase}^2 + 0.036378 * \\ & \text{Xylanase}^2 - 4.07410 * \\ & \text{Substrate Loading}^2 \quad \dots (5) \end{aligned}$$

Testing of the model was performed by the Fisher's statistical test for the analysis of variance (ANOVA)

Table 3— Box Behnken experimental matrix for optimization of alkali treated biomass based on the responses of Total reducing sugar and xylose yields.

Run no.	Coded values			Real values			Total reducing sugars mg/g of WHB	Xylose yield mg/g of WHB
	A	B	C	A	B	C		
1	-1	-1	0	20	150	4	302.96	166.7
2	+1	-1	0	50	150	4	407.1	237.38
3	-1	+1	0	20	300	4	330.2	221.4
4	+1	+1	0	50	300	4	405.4	285.1
5	-1	0	-1	20	225	2	362	125.0
6	+1	0	-1	50	225	2	425.4	224.14
7	-1	0	+1	20	225	6	312.38	125.927
8	+1	0	+1	50	225	6	419.67	165.7
9	0	-1	-1	35	150	2	355.8	201.135
10	0	+1	-1	35	300	2	405.6	306.51
11	0	-1	+1	35	150	6	366.5	228.4
12	0	+1	+1	35	300	6	341.9	230.51
13	0	0	0	35	225	4	415.316	206.51
14	0	0	0	35	225	4	410.12	216.116
15	0	0	0	35	225	4	420.51	198.48
16	0	0	0	35	225	4	416.88	204.32
17	0	0	0	35	225	4	414.48	208.56

using Design Expert software and the results are shown in Tables 4 and 5, for reducing sugar yield and xylose yield, respectively.

ANOVA of the quadratic regression model suggested that the model was significant for both reducing sugar and xylose with a computed F value of 315.52 and 150.64, respectively and a $P >$ lower than 0.05. The value of multiple correlation coefficients (R^2) for both the response was 0.9999 indicating a better correlation between the observed and predicted values. A lower value for the coefficient of variation suggested higher reliability of the experiment and in this case the obtained CV value of 0.81 and 2.23%, for the responses studied, demonstrated a greater reliability of the trials. Tables 4 and 5 also give the P values of each of the parameters and their quadratic and interaction terms. The significance of individual variables could be evaluated from their P values, the more significant terms having a lower P value. The values of $P > F$ less than 0.05 indicated that the

model terms were significant and the quadratic and interaction effects of all the tested parameters were found to be significant. The predicted and adjusted R^2 for response surface curves were plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal sugar yields. For reducing sugar, the R^2 was 0.9975, and the predicted R^2 of 0.9907 was in reasonable agreement with the adjusted R^2 of 0.9944. For xylose, the R^2 was 0.9949, and the predicted R^2 of 0.9857 was in reasonable agreement with the adjusted R^2 of 0.9883. The interactions between cellulase and xylanase showed increase in reducing sugar as compared to separate hydrolysis with cellulase and xylanase, respectively. The increased sugar yields may be a resultant of both cellulose and hemicellulose being hydrolyzed due to the action of cellulase and xylanase. Therefore, it can be said that a boosting effort was observed when both cellulase and xylanase both were combined.

Table 4—Analysis of variance for the selected quadratic model for total reducing sugar.

Source	Sum of squares	dF	Mean square	F Value	P value
Model	27309.66	9	3034.41	315.52	<0.0001
A-Cellulase (U/g)	15304.6	1	15304.6	159.39	<0.0001
B- Xylanase (U/g)	321.31	1	321.31	33.41	0.0007
C- Substrate Loading (%) (w/v)	1469.63	1	1469.63	152.81	<0.0001
AB	209.09	1	209.09	21.74	0.0023
AC	483.77	1	483.77	50.30	0.0002
BC	384.58	1	384.58	143.92	<0.0001
A ²	1822.40	1	1822.40	189.50	<0.0001
B ²	4651.32	1	4651.32	483.65	<0.0001
C ²	918.46	1	918.46	95.50	<0.0001
Residual	67.32	7	9.62	-	-
Lack of Fit	10.32	3	3.43	0.24	0.8640
Pure Error	57.02	4	14.25	-	-
Total	27376.98	16	-	-	-

Table 5— Analysis of variance for the selected quadratic model for xylose yield.

Source	Sum of squares	dF	Mean square	F Value	P value
Model	35019.27	9	3891.03	150.64	<0.0001
A-Cellulase (U/g)	9336.16	1	9336.16	361.44	<0.0001
B-Xylanase (U/g)	5507.51	1	5507.51	213.22	<0.0001
C- Substrate Loading (%) (w/v)	1411.09	1	1411.09	54.63	0.0002
AB	12.18	1	12.18	0.47	0.5144
AC	881.10	1	881.10	34.11	0.0006
BC	2665.92	1	2665.92	103.21	<0.0001
A ²	3863.12	1	3863.12	149.56	<0.0001
B ²	11019.09	1	11019.09	426.59	<0.0001
C ²	1118.20	1	1118.20	43.29	0.0003
Residual	180.81	7	25.83	-	-
Lack of Fit	15.41	3	5.14	0.12	0.941
Pure Error	165.40	4	41.35	-	-
Total	352000.08	16	-	-	-

All the interactions were significant. The interaction between xylanase and cellulase is shown in Fig. 4(a). It can be seen that as both cellulase and xylanase loadings were increased, the reducing sugar yield also increased. Thus, this interaction showed the synergistic action of cellulase and xylanase, as reported earlier²⁰. The interaction between substrate loading and cellulase (Fig. 4b) also depicts a synergistic relation between them. The reducing sugar response increased with increased cellulase and substrate loading. The effects of substrate loading and xylanase loading on the hydrolysis of alkali treated WHB are shown in Fig. 4(c). At low levels of xylanase loading and low levels of biomass loading, the reducing sugar yield was low. Middle level of biomass loading and middle level of enzyme loading showed maximum reducing sugar yield. At high biomass loading, the amount of available free water became less, which in turn decreased the hydrolysis efficiency. High biomass loading is associated with difficulties in mixing as well as end-product inhibition. Similar results have been reported by sindhu *et al*²⁴.

Similar synergism was observed between cellulase and xylanase for xylose yield (Fig. 5a). The interaction between cellulase and substrate loading for xylose (Fig. 5b.) were similar to that observed for reducing sugar. The xylanase and substrate loading interaction (Fig. 5c.) showed that at lower substrate loadings the increase in xylanase loading corresponded to increase in xylose response. But at higher substrate loadings, the increase in xylose was comparatively lesser. It could be due to saturation of catalytic sites for reaction arising out of excess of substrate present. The maximum reducing sugar was obtained when a cellulase loading of 50 U/g, xylanase loading of 225 U/g and 2% substrate loading, while for maximum xylose was 35 U/g cellulase, 300 U/g xylanase and 2% substrate loading. The conditions for maximal reducing sugar yield and maximal xylose yield were optimized using the numerical optimization function in Design Expert. Among the multiple solutions suggested, the one which gave maximal reducing sugar and xylose yield was a cellulase and xylanase loading of 46.12 and 289.98 U/g and 2.26% substrate loading. The

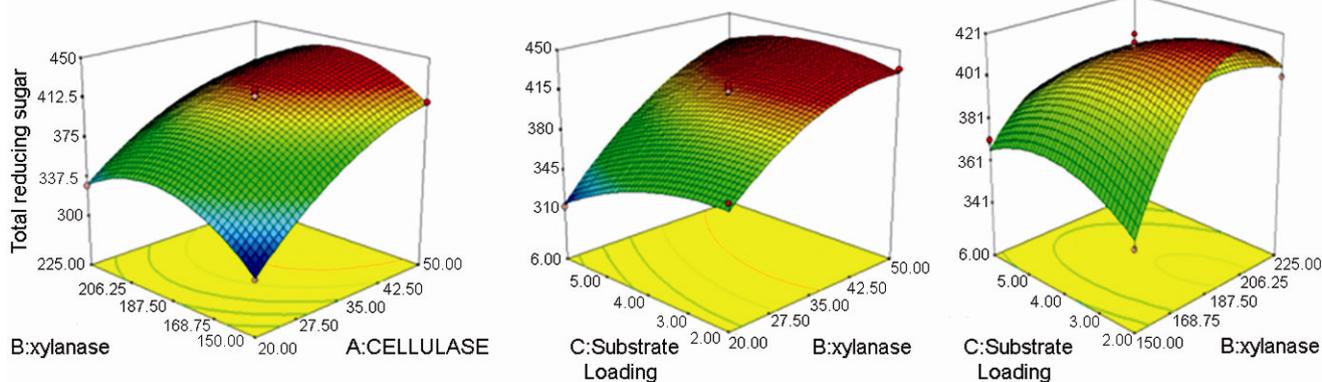


Fig. 4—Response surfaces showing interactions for reducing sugar response between a. Cellulase, and Xylanase, b. Substrate Loading and Cellulase, c. Substrate Loading and Xylanase.

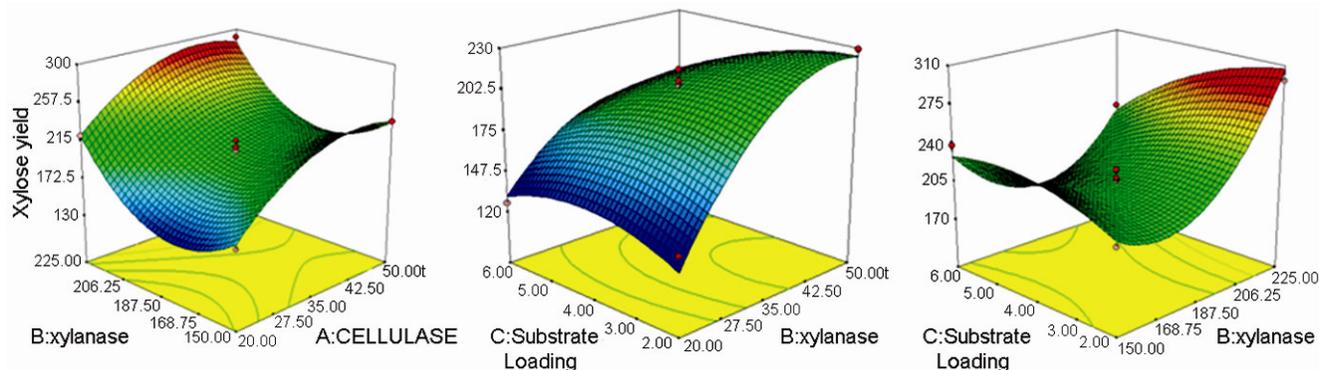


Fig. 5—Response surfaces showing interactions for xylose yield response between a. Cellulase and Xylanase, b. Substrate loading and Cellulase and c. Substrate loading and Xylanase.

predicted yield for this combination was 388.751 and 269.55 mg/g for reducing sugar and xylose yield, respectively, and trials conducted at this concentration yielded reducing sugar in the range of 378-432 mg/g and xylose in the range of 267-275 mg/g.

Production of ethanol—Concentrated enzymatic hydrolysate of WHB which contained 10% reducing sugars and 6-7% xylose was used as substrate for ethanol fermentation. From the results of ethanol fermentation study shown in Fig. 6, it became clear that the efficiencies of ethanol production from the WHB hydrolysate were very low and the maximal ethanol yield was 3.4969 g/L when *P. stipitis* was used, followed by 3.4496 and 3.1349 g/L for

C. shehatae and *S. cerevisiae*. *Saccharomyces cerevisiae* can ferment only hexoses, probably accounting for the low ethanol production. The overall low ethanol production may have basis in the initial sugar loss during the alkaline hydrolysis step. Pretreatment of lignocellulosic biomass may produce degradation products with an inhibitory effect on the fermentation process. Major types of inhibitors are sugar and lignin degradation products.

Maximum ethanol production was reported by *P. stipitis* to be because it can ferment both hexoses and pentoses, thus increasing the efficiency of sugar to ethanol conversion. Similar observations have been reported earlier²³.

Summarised conclusions

A comparative study between acid and alkali pretreatment of the WHB for the removal of lignin was done. The alkali pretreatment was found to be best suited for biomass saccharification with enzymes, both crude enzymes and commercial enzymes. It was more effective in enhancing the cellulose and hemicellulose content of WHB by removing lignin. For enzymatic saccharification, the alkali pretreatment was the best suited. The use of crude enzyme extracts could make the process of conversion of lignocellulose to sugars more cost effective. Optimization of cellulase, xylanase and substrate loading by the Box-Behnken Design showed substrate loading as the most important factor influencing the sugar yield. It was observed that enzymatic saccharification was more effective than saccharification with whole cell biocatalysts. Further strategies can be developed for using whole cell biocatalysts, instead of enzymes, as their stability is high from industrial point of view. It was also concluded that water hyacinth can be used as a potential source for bioethanol production. The production of ethanol can be enhanced by using both hexose and pentose fermenting yeasts simultaneously.

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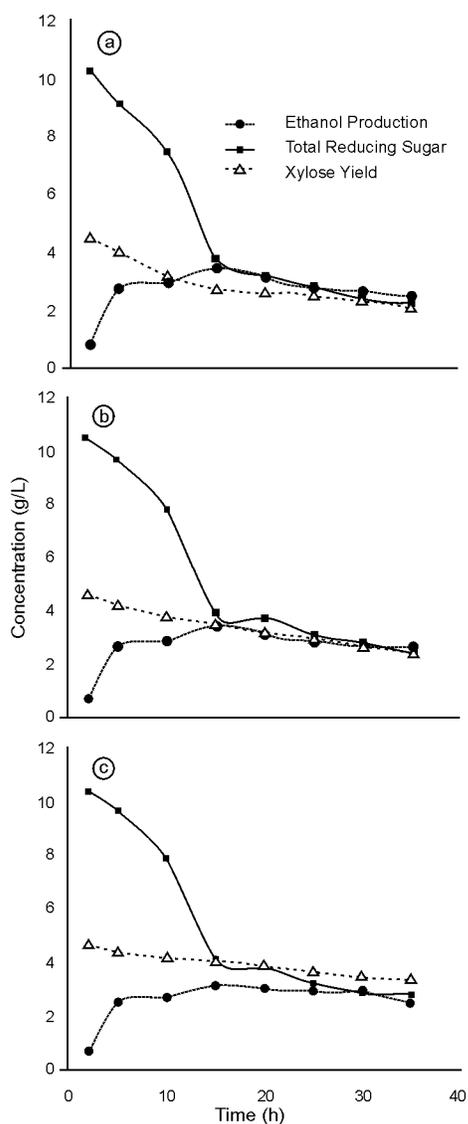


Fig. 6—Ethanol production from WHB hydrolysate by (a) *Candida shehatae* (b) *Pichia stipitis* and (c) *Saccharomyces cerevisiae*

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