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Bio-ethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy

U.S. Aswathy, Rajeev K. Sukumaran *, G. Lalitha Devi, K.P. Rajasree, Reeta Rani Singhania, Ashok Pandey

Biotechnology Division, National Institute for Interdisciplinary Science and Technology (CSIR), Trivandrum 695019, India

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ABSTRACT

Biomass feedstock having less competition with food crops are desirable for bio-ethanol production and such resources may not be localized geographically. A distributed production strategy is therefore more suitable for feedstock like water hyacinth with a decentralized availability. In this study, we have demonstrated the suitability of this feedstock for production of fermentable sugars using cellulases produced on site. Testing of acid and alkali pretreatment methods indicated that alkali pretreatment was more efficient in making the sample susceptible to enzyme hydrolysis. Cellulase and β-glucosidase loading and the effect of surfactants were studied and optimized to improve saccharification. Redesigning of enzyme blends resulted in an improvement of saccharification from 57% to 71%. A crude trial on fermentation of the enzymatic hydrolysate using the common baker's yeast *Saccharomyces cerevisiae* yielded an ethanol concentration of 4.4 g/L.

1. Introduction

Cellulosic ethanol has gained tremendous importance over the last decade due to it's projection as a feasible alternative to petroleum based transportation fuels. The choice of feedstock for bio-ethanol production is a major concern since the biomass requirements either directly or indirectly competes with the food crops for arable land resources. Agro residues when used for ethanol production may address this problem to an extent, but the operation of large scale plants for cellulosic ethanol production still have several limitations including high capital investment, high technical know how, and above all the high transportation costs of feedstock. A distributed biomass-ethanol production system may be far more efficient, especially in countries where agriculture is decentralized and the biomass availability is distributed. Such a system would allow the use of regionally available biomass types as feedstock; shall include economic and social benefits for the rural community and will be less capital intensive and may have easy to operate technologies requiring fewer skills.

Water hyacinth (*Eichhornia crassipes*) biomass (WHB) was proven to be suitable for bio-ethanol production (Abraham and Kurup, 1996; Mishima et al., 2008). The biomass from water hyacinth has about 48% hemicellulose, 18% cellulose 3.5% lignin (Nigam, 2002). Though there is a significant amount of variability in composition reported by different labs, in general the biomass is considered to be rich in hemicellulose and with very less lignin content. The biomass productivities of this plant is very high (Mitchell, 1976), and there is abundant availability of this plant in certain parts of the world making it a suitable feedstock for distributed ethanol production. Also aquatic biomass has the added advantage of not being a competition to food crops for arable land resources (Mishima et al., 2008). The plant is a typical menace infesting the backwaters of Kerala state in southern part of India (Praveena and Naseema, 2004). Effective technologies for ethanol production from water hyacinth biomass can serve the purpose of weed removal as well as generation of employment and income in rural areas. Large scale plants for water hyacinth biomass processing may not be economical due to the problems in collection, transportation and infrastructural requirements. Also, technical challenges exist in the pretreatment of biomass, and the enzymatic saccharification, especially the sourcing of enzymes. Adopting cost effective and simpler methods of pretreatment, detoxification, saccharification and fermentation, and generation of enzymes on site are necessary for an effective distributed production technology. Though there are several reports on the use of WHB for ethanol production (Abraham and Kurup, 1996; Kahlon and Kumar, 1987; Mishima et al., 2008; Nigam, 2002), these reports had sparsely addressed the above aspects in the larger perspective of a distributed biomass-ethanol production strategy. In the present study we had tried to follow less technologically intensive methods for on-site production of enzyme, biomass pretreatment and saccharification to generate reducing sugar that can be fermented to ethanol. We had also done preliminary studies on the suitability of the crude

* Corresponding author. Tel.: +91 471 2515368.
E-mail address: rajvaryaveedu@gmail.com (R.K. Sukumaran).

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hydrolysate as substrate for ethanol production using *Saccharomyces cerevisiae*. The data indicated that the saccharification efficiency using optimized enzyme cocktails was good, and the hydrolysate could be fermented to produce ethanol.

2. Methods

2.1. Biomass feedstock and pretreatment

Water hyacinth plants were collected from unpolluted backwaters in Kottayam district in Kerala, India. The leaves and stalks were dried in sun, brought to the lab and further dried overnight at 70 °C in a hot air oven to remove residual moisture. The dried biomass was milled to reduce the size to approximately 1–2 mm prior to pretreatment. Pretreatment was done with either dilute acid or alkali using conditions optimized in our laboratory. Briefly, the samples at 10% biomass loading were reacted with 0.88% H_2SO_4 or 2% NaOH for 1 h at 95 °C. After cooling, the samples were washed several times in tap water to neutralize the pH followed by a final rinse in distilled water, after which they were air dried at room temperature by spreading on paper. The pretreated feedstock were either used immediately for hydrolysis experiments or stored in airtight containers at 4 °C until used.

2.2. Microorganisms, inoculum preparation and enzyme production

The fungus *Trichoderma reesei* RUT C30 was a kind gift from Prof. George Scakacs, Technical University of Budapest. *Aspergillus niger* MTCC 7956 was isolated from decaying wood in our lab and was identified at the Microbial Type Culture Collection (MTCC), Chandigarh, India. Cellulase and β-glucosidase (BGL) for biomass saccharification was produced by solid state fermentation employing *T. reesei* and *A. niger*, respectively as described previously (Sukumaran et al., 2008). Common baker's yeast *Saccharomyces cerevisiae* purified from a commercial preparation was used for alcohol production. The yeast culture was grown in YEP broth for 12 h with 180 rpm agitation on rotary shaker and the culture was used at 5% v/v as inoculum for alcohol fermentation of hydrolysate.

2.3. Biomass saccharification

Enzymatic saccharification of biomass was done by incubating 1 g dry weight of pretreated WHB with different blends of *T. reesei* cellulase and *A. niger* BGL at 45 °C, in stoppered 150 ml flasks with gentle agitation (100 rpm) in a shaking water bath. The hydrolysis was performed in 50 mM citrate buffer (pH 4.8), and the volumes were adjusted to achieve required biomass loading (5% dry weight/volume for acid treated water hyacinth (WHB-Ac) and 6.5% for alkali treated water hyacinth (WHB-Al) were used since these were the maximum possible loadings that allowed presence of free water necessary for enzyme action). Different combinations of cellulase and BGL loading and incubation times were tried for biomass hydrolysis. Sugar release was monitored as total reducing sugar using DNS method (Miller, 1959). Saccharification efficiency wherever mentioned was calculated as percentage conversion of pretreated biomass to reducing sugars assuming a theoretical yield of 1.12 g sugar/g pretreated biomass.

2.4. Optimization of biomass saccharification

The loading of cellulase, BGL, and the concentration of surfactant (Tween 80) was optimized for enhancing the reducing sugar yield 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, BGL concentration, and the concentration of surfactant were coded as X_1 and X_2 and X_3, respectively. Incubations were carried out as described above for 48 h.

The model, constructed as a response function of the variables on cellulase production was a second-order polynomial as follows:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j \quad (1)$$

where Y is the measured response (reducing sugar yield); β_0, β_i and β_{ii} are the regression coefficients, and X_1–X_3 are the factors under study.

For three variable systems the model equation is given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

Regression analysis and estimation of the coefficients were performed using Design Expert® software (Stat ease Corp, USA). 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®.

2.5. Ethanol production using WHB hydrolysate

Ethanol production was studied using the enzymatic hydrolysate of WHB-Al. The pretreated biomass was hydrolyzed under the conditions optimized for higher yield of sugars. The hydrolysate was concentrated by evaporation to a reducing-sugar concentration of 10%. This was inoculated with 5% v/v of a 12 h old seed culture of *Saccharomyces cerevisiae*. Incubation was carried out in stoppered flasks at room temperature (28 ± 2 °C) without agitation. 1 ml samples were withdrawn at regular intervals and centrifuged for 10 min at 4 °C at 13,000 rpm. The supernatant was filtered using 0.45 μm filters and the ethanol content was analyzed by gas chromatography.

2.6. Analytical methods

Cellulase activities in the crude enzyme preparation were determined according to IUPAC method (Ghose, 1987). β-Glucosidase activity was determined using *p*-nitrophenyl β-D-glucopyranoside as substrate according to the IUPAC method (Ghose and Bisaria, 1987). Estimation of total reducing sugar in the enzymatic hydrolysate of biomass was done by DNS method (Miller, 1959) and the estimation of ethanol was done by gas chromatography as outlined in NREL Laboratory Analytical protocol # 011 (Templeton, 1994).

3. Results and discussion

3.1. Production of enzymes

A significant part of the cost for lignocellulosic ethanol production is the cost of cellulase enzyme used for biomass hydrolysis. On site production of cellulase using agro-industrial wastes can reduce the cost of enzyme production and that of transportation. Crude cellulase and BGL preparations used in this study were produced by solid state fermentation in an effort to reduce the cost of ethanol production. Solid state fermentation needs lesser infrastructure and relatively less skilled manpower besides being able to use cheaper raw materials for enzyme production (Pandey et al., 2000; Raimbault, 1998). SSF also produces a more concentrated

product, which in this case is very much advantageous. Crude cellulase was produced employing *T. reesei* RUT C30 on wheat bran as substrate as described previously (Singhania et al., 2007; Sukumaran et al., 2008). BGL was produced using *A. niger* MTCC 7956 using conditions optimized in our laboratory (unpublished results). *T. reesei* enzyme had a cellulase activity of 0.77 FPUs/ml, BGL activity of 82 U/ml and xylanase activity of 1024 U/ml. The *A. niger* enzyme had high BGL and xylanase activities of 984 U/ml and 249.7 U/ml, respectively while it had only 0.23 U of filter paper activity. We had also found in an earlier study that the crude BGL preparation from *A. niger* contain multiple BGL proteins differentially expressed in response to growth conditions and at least one of them is moderately tolerant to glucose with a 20% activity retention at 0.5 M glucose (unpublished results). It has been reported earlier that *T. reesei* produces lesser amount of BGL compared to *A. niger* (Reczey et al., 1998) and the latter is capable of glucose tolerant BGL production (Gunata and Vallier, 1999; Yan and Lin, 1997).

3.2. Biomass saccharification

Six different combinations of cellulase and BGL loading was tried for saccharification of either acid or alkali pretreated WHB. Each sample was incubated for 48 h with shaking at 45 °C. Samples taken at 12 h intervals were analyzed for reducing sugar content and the total reducing sugar yield per gram of biomass was calculated. The conditions used for WHB hydrolysis and the reducing sugar yields are presented in Table 1.

It may be seen from the results that acid pretreatment of WHB was not efficient in improving the reducing sugar yield. The maximal reducing sugar yield obtained from hydrolysis of WHB-Ac was only about 136 mg/g where as this value for WHB-Al was 639.42 mg/g. This amounts to saccharification efficiency of approximately 57% assuming the pretreated sample to be mostly hemicellulose and cellulose. The data clearly indicate the superiority of alkali pretreatment in making the substrate susceptible to enzymatic hydrolysis. It may be considered here that WHB is a feedstock which contains more hemicellulose than cellulose (Nigam, 2002).

Dilute acid pretreatment of biomass is known to hydrolyze hemicellulose leaving most of the cellulose intact while alkali pretreatment preserves at least part of the hemicellulose and acts mainly to remove the lignin content (Sun and Cheng, 2002). WHB-Al therefore contained more hemicellulose compared to WHB-Ac and the hydrolysis of both cellulose and hemicellulose in WHB-Al would have resulted in a better reducing sugar yield. The cellulase preparations used for saccharification contain a significant amount of xylanase activity which might act on the hemicellulose part of the biomass and release sugars. The best combination of cellulase and BGL seemed to be 12 FPUs and 2400 U, respectively per gram of WHB-Al. In the trials which used 12 FPU of cellulase loading, there was an increase in reducing sugar yield with increase in BGL loading indicating that the major factor limiting hydrolysis could be the amount of available beta glucosidase. It may be also noted that the increase in reducing sugar yield was not linear indicating product inhibition. The maximal reducing-sugar concentration attained was 4.4% which is close to 0.25 M if the reducing sugar is assumed to be glucose. Since *T. reesei* BGL is inhibited by 0.01 M glucose concentration (Woodward and Arnold, 1981) it can be assumed that the supplementation of *A. niger* BGL could significantly enhance the maximum attainable sugar concentration. Use of a more glucose tolerant preparation could have improved the sugar yield further without having to

Table 1
Effect of pretreatment method, enzyme loadings and incubation time on WHB saccharification.

Time (h)	Cellulase (FPU/g)	BGL (U/g)	Reducing sugar Conc. (mg/ml)		Total Reducing sugar (mg/g)	
			WHB-Ac	WHB-Al	WHB-Ac	WHB-Al
0	6	300	0.00	0.00	0.00	0.00
	6	600	0.00	0.00	0.00	0.00
	6	1200	0.00	0.00	0.00	0.00
	12	600	0.00	0.00	0.00	0.00
	12	1200	0.00	0.00	0.00	0.00
	12	2400	0.00	0.00	0.00	0.00
12	6	300	0.00	7.04	0.00	104.88
	6	600	1.96	4.31	38.83	64.17
	6	1200	1.06	7.34	21.01	109.37
	12	600	2.94	32.78	58.25	488.39
	12	1200	4.21	35.38	83.27	527.21
	12	2400	6.88	36.98	136.32	551.02
24	6	300	ND	5.68	ND	81.83
	6	600	0.34	4.66	6.73	67.16
	6	1200	0.16	10.6	3.07	152.63
	12	600	ND	35.99	ND	518.26
	12	1200	2.54	38.55	49.79	555.09
	12	2400	1.77	44.4	ND	639.42
36	6	300	ND	5.78	ND	80.31
	6	600	2.44	4.97	47.28	69.12
	6	1200	1.3	2.56	25.2	35.55
	12	600	0.99	36.44	19.22	506.55
	12	1200	5.73	37.1	111.13	515.64
	12	2400	7.03	41.86	136.33	581.83
48	6	300	0.3	5.16	5.75	69.13
	6	600	1.1	3.95	21.21	52.93
	6	1200	0.56	3.89	10.78	52.13
	12	600	1.3	30.85	24.96	413.4
	12	1200	2.3	39.74	44.2	532.48
	12	2400	2.65	42.29	50.87	566.64

ND – Not determined.
Biomass loading: WHB-Ac: 5% w/v, WHB-Al: 6.5%.

add more BGL. The effect of incubation time was also studied and it can be seen from the data that the highest yield of reducing sugars (639.42 mg/g) was obtained after 24 h of incubation. The yields for 36 h and 48 h were also comparable (581.83 and 566.64 mg/g, respectively) though lesser than that at 24 h and this reduction may be attributed to variation between flasks.

3.3. Optimization of biomass saccharification

Trials done on WHB saccharification indicated that the yield of reducing sugar is better with higher cellulase and BGL loading and it was speculated that an increase in enzyme loading might improve the saccharification efficiency. A Box Behnken design was used for optimization of the cellulase and BGL concentrations. Surfactant addition to the hydrolysis mixture is known to increase the efficiency of saccharification (Eriksson et al., 2002; Sun and Cheng, 2002; Kristensen et al., 2007). Tween 80 was added to the hydrolysis mixture and the effect of the addition of this surfactant was studied. The different combinations of Cellulase, BGL and Tween 80 tried for WHB saccharification and the corresponding reducing sugars yields are given in Table 2.

The data was analyzed by multiple regression analysis and a second-order polynomial equation (Eq. (3)) was derived to represent the cellulase production as a function of the independent variables tested.

$$Y = 577.21 + 21.04X_1 + 79.26X_2 + 70.38X_3 + 34.97X_{12} - 65.56X_{22} - 52.46X_{32} - 89.31X_1X_2 - 120.18X_1X_3 + 40.21X_2X_3 \quad (3)$$

where Y is the predicted response (reducing sugar yield), X_1, X_2 and X_3 are coded values for Cellulase, BGL and Tween 80 concentrations, respectively. Testing of the model was performed by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software and the results are shown in Table 3.

ANOVA of the quadratic regression model suggests that the model is significant with a computed F value of 27.22 and a $P > F$ lower than 0.05. The value of multiple correlation coefficient (R) was 0.9860 indicating a better correlation between the observed and predicted values. A lower value for the coefficient of variation suggests higher reliability of the experiment and in this case the obtained CV value of 5.62% demonstrated a greater reliability of the trials. Table 3 also gives the P values of each of the parameters and their quadratic and interaction terms. The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P value. The values of $P > F$ less than 0.05 indicates that the model terms are significant and this case BGL and Tween 80 concentrations and the quadratic and interaction effects of all the three tested parameters were found to be significant. Response surface curves were plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal cellulase yield. Figs. 1A–C represent the response surfaces obtained for the three tested parameters on saccharification efficiency.

All the three interaction effects were significant. The effect of interaction between cellulase and BGL is shown in Fig. 1A. It can be seen that at lower cellulase loading, the saccharification efficiency increased with increase in BGL loading, while at higher cellulase loading, though the yield of reducing sugars increased with increase in BGL levels initially, it became lesser with further increase in levels of BGL indicating a probable saturation effect. The highest reducing sugar yields were obtained with a cellulase loading of around 8 FPU/g biomass and with a BGL loading of more than 2400 U/g. Similarly, the interaction effect of Cellulase loading and Tween 80 concentration is given in Fig. 1B. At lower cellulase loading, the efficiency of saccharification increased with increase in Tween 80 levels, while at higher cellulase loading, the yield of reducing sugars increased initially with increase in Tween 80 con-

Table 2
Box Behnken experiment design matrix for optimization of saccharification with obtained responses (reducing sugar yield) for different trials.

Std order	Cellulase (FPUs/g)	BGL (U/g)	Tween (% v/v)	Reducing sugar yield (mg/g)
1	8	1600	0.1	329.19
2	16	1600	0.1	583.57
3	8	3200	0.1	688.31
4	16	3200	0.1	585.44
5	8	2400	0.05	379.69
6	16	2400	0.05	628.46
7	8	2400	0.15	731.33
8	16	2400	0.15	499.40
9	12	1600	0.05	346.03
10	12	3200	0.05	402.14
11	12	1600	0.15	435.81
12	12	3200	0.15	652.78
13	12	2400	0.1	570.48
14	12	2400	0.1	568.61
15	12	2400	0.1	598.53
16	12	2400	0.1	553.64
17	12	2400	0.1	594.79

Table 3
Analysis of variance for the selected quadratic model.

Source	Sum of squares	DF	Mean square	F Value	Prob > F
Model	224279.10	9	24919.9	27.23	0.0001
X_1	3542.71	1	3542.72	3.87	0.0898
X_2	50255.60	1	50255.60	54.91	0.0001
X_3	39621.13	1	39621.13	43.29	0.0003
X_1^2	5150.16	1	5150.16	5.63	0.0495
X_2^2	18095.25	1	18095.25	19.77	0.0030
X_3^2	11589.24	1	11589.24	12.66	0.0092
X_1X_2	31906.89	1	31906.89	34.86	0.0006
X_1X_3	57768.12	1	57768.12	63.11	<0.0001
X_2X_3	6468.99	1	6468.99	7.07	0.0325
Residual	6407.20	7	915.31		
Lack of fit	4968.81	3	1656.27	4.61	0.0871
Pure error	1438.40	4	359.60		
Cor. total	230686.30	16			

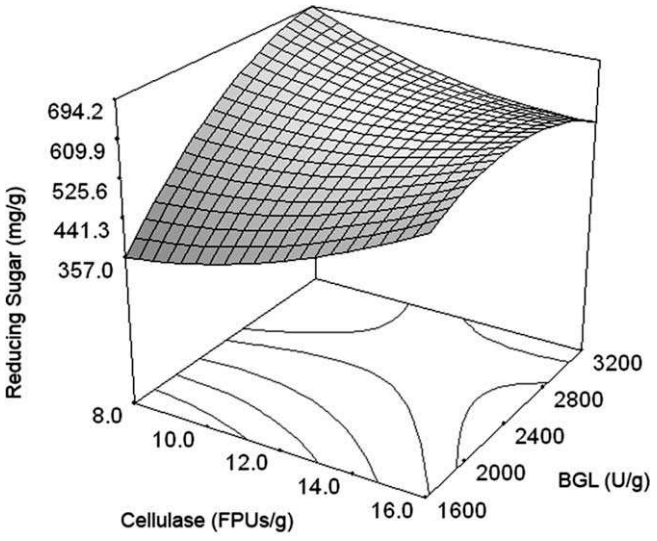

Fig. 1A. Response surface showing interaction effect of cellulase and BGL.

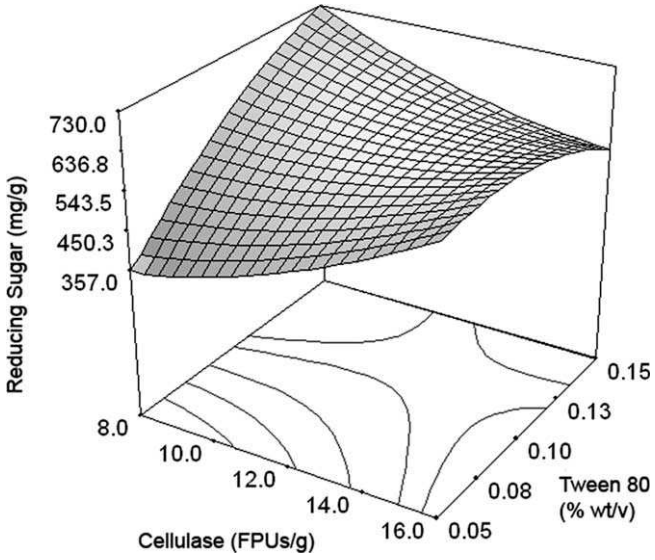

Fig. 1B. Response surface plot showing interaction effects of Cellulase& Tween 80.

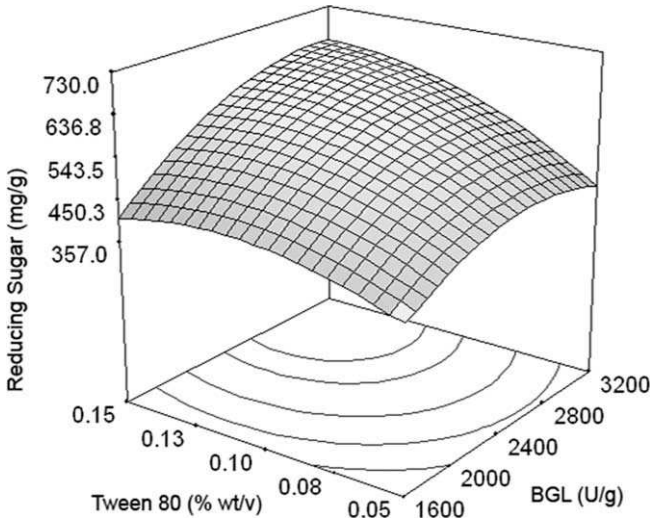

Fig. 1C. Response surface plot showing interaction effects of BGL and Tween 80.

centration up to 0.1% v/v and decreased thereafter. This might indicate a better availability of enzyme in presence of surfactant.

The results on interaction effects of BGL loading and Tween 80 concentration also showed a similar trend with the reducing sugar yield increasing with increase in Tween 80 concentration to a certain level initially and either decreasing or remaining stable thereafter for all the BGL loadings tried. The mechanism by which surfactants increase saccharification efficiency of cellulases is largely unknown, but it has been speculated that the addition of surfactants decreases non-productive binding of cellulases on the biomass surface and thereby increase the availability of the enzyme (Eriksson et al., 2002; Kristensen et al., 2007). This would have been the reason for the requirement of lesser enzyme loading to achieve similar levels of hydrolysis in comparison with trials in which Tween 80 was not added. So, the maximal reducing sugar yield of 731 mg/g WHB was attained with an enzyme loading of only 8 FPUs and 2400 BGL Units per gram biomass in comparison with the previous highest value of 639.42 mg/g reducing sugar attained with an enzyme loading of 12 FPUs and 2400 U of cellulase and BGL, respectively. Apparently, the addition of cost effective surfactants at low levels in the saccharification mixture might improve the process economics due to the savings on enzyme costs. The role of beta glucosidase is also highlighted. It may be observed that with increased BGL loading, there is definitely an improvement in hydrolysis but to a threshold most probably defined by the glucose concentration at which the enzyme gets inhibited. Use of a more glucose tolerant BGL can probably improve the system drastically. The conditions for maximal reducing sugar yield were optimized using the numerical optimization function in Design expert. Among the multiple solutions suggested, the one which gave maximal reducing sugar yield was a cellulase and FPU loading of 8 and 3130 U/g, respectively and a Tween 80 concentration of 0.14%. The predicted yield for this combination was 825 mg/g WHB and trials conducted at this concentration yielded reducing sugar in the range of 790–850 mg/g (817 + 23 mg/g).

3.4. Ethanol production using WHB hydrolysate

Concentrated Enzymatic hydrolysate of WHB which contained 10% reducing sugars was used as substrate for ethanol fermentation. From the results of ethanol fermentation study shown in Fig. 2, it becomes clear that the efficiencies of ethanol production from the WHB hydrolysate are very less and the maximal ethanol yield was 4.25 g/L (0.54% v/v). However it may be noted that WHB contains about 48% hemicellulose and only about 18% cellulose as estimated by Nigam (2002) and though the enzymatic hydrolysis would have released both hexoses and pentoses due to the presence of high xylanase activity of both *T. reesei* and *A. niger* enzyme preparations, *Saccharomyces cerevisiae* can ferment only hexoses probably accounting for the low ethanol production. Up to 18 g/L ethanol has been produced by pentose fermenting yeasts on water hyacinth acid hydrolysate (Nigam, 2002) and the use of pentose fermenters might help in improving the yields of ethanol. Nevertheless, the saccharification efficiency is approximately 73% (reducing sugar yield = 817 mg/g pretreated biomass). Reports have indicated about 60% hydrolysis of degradable sugars in water hyacinth (Mishima et al., 2008) and 62% in woody biomass (Lee et al., 2009) while 71% efficiency for cellulose to sugar conversion is reported for cellulose (Ghose, 1972). The saccharification efficiency in the present study is calculated as percentage conversion of pretreated biomass to reducing sugars assuming a theoretical yield of 1.12 g sugar/g pretreated biomass. Actual values can be even higher, since any residual lignin or other compounds were not analyzed and the maximum theoretical yield will be lesser than the assumed value.

Ethanol production was attempted in this study only as a preliminary effort and significant improvement in ethanol yields may

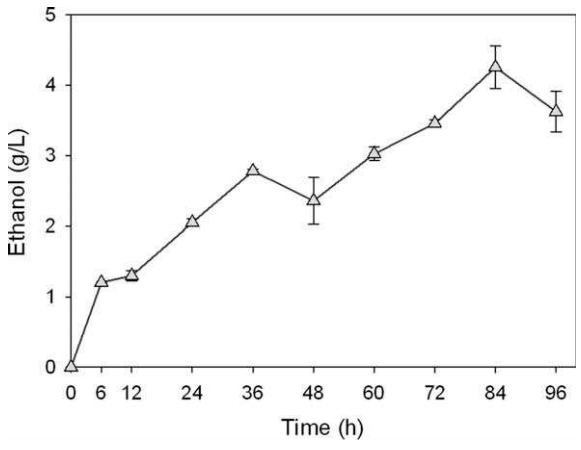

Fig. 2. Ethanol production from WHB hydrolysate by *Saccharomyces cerevisiae*.

be brought about by co-fermentation using pentose fermenting yeasts along with *Saccharomyces*. We now plan to conduct studies for saccharification using more glucose tolerant BGL for saccharification and to use pentose fermenting yeasts for alcohol fermentation.

4. Conclusions

Water Hyacinth is plentiful in the water bodies in the tropics and the biomass is rather distributed in its geographical occurrence. Ethanol production from this feedstock has several technical challenges including biomass collection and preprocessing and decentralized plants for ethanol production may be more appropriate for such feedstock. Here we had demonstrated that it is possible to achieve almost 71% efficiency in saccharification of water hyacinth biomass with very crude and cost effective methods for pretreatment and onsite enzyme production. Also we had demonstrated that the hydrolysate of WHB can be fermented to ethanol using the common bakers yeast *Saccharomyces cerevisiae* albeit with lesser efficiency. Use of more suitable organisms for fermentation can improve the yield of ethanol. Almost all of the technologies what we had employed in production of bio-ethanol from WHB were less technically intensive and may be operated by non-skilled workers making the strategy suitable for small scale distributed production of fuel ethanol.

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