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Optimization of Ethanol Production from Enzymatic Hydrolysate of Maize Stover

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Abstract

For efficient bioethanol production from maize stover, fermentation of glucose and xylose both was attempted using *Saccharomyces cerevisiae* and *Pichia stipitis* sequentially from enzymatic hydrolysate of mild alkali treated maize stover. Enzymatic saccharification of mild alkali treated maize stover at high substrate (30%) loading using 13.0 FPU/g commercial cellulase (MAPs 450) and 74.42 U/g crude β-xylosidase (Inhouse produced) after 36 h, yielded 161.32 mg ml^{-1} reducing sugars. Ethanol production was optimized employing response surface methodology. Under optimized conditions *viz.* 5% glucose, 14.55% inoculum and Time 35.51 h; 90.65% glucose was utilized and produced 18.93 g l^{-1} ethanol with 0.53 g l^{-1} h^{-1} productivity by *Saccharomyces cerevisiae* NCIM 3524. Further attempts were made to produce ethanol from xylose present in enzymatic hydrolysate using *Pichia stipitis* NCIM 3497. However, xylose conversion was not satisfactory as only 71% xylose was utilized.

Keywords: *Aspergillus niger* ADH-11; High substrate saccharification; Response surface methodology; Ethanol; *Saccharomyces cerevisiae* NCIM 3524; *Pichia stipitis* NCIM 3497

Introduction

The progress of bioethanol industries could help to reduce dependence on fossil fuels and moderate global warming [1]. Bioethanol derived from lignocellulose can be a welcome technology due to its sustainability, lower-cost, and significantly larger availability of feedstock reserves as compared to starch-based ethanol [2,3]. Among various types of lignocellulosic biomass, maize stover is observed as one of the encouraging feedstock for the production of cellulosic bioethanol because of its high content of cellulose and hemicellulose [4]. However bioethanol production from lignocellulosic biomass is a challenging process because of its complex chemical structure and organization. Efficient bioconversion of lignocellulosic biomass in to fermentable sugars requires cooperative action of various cellulolytic and hemicellulolytic enzymes at appropriate levels [5]. Moreover, concentration of fermentable sugars should be high enough in enzymatic hydrolysate as at least 4% ethanol concentration is required to develop an economically viable distillation process [6]. Considering economic viability, high gravity ethanol fermentations are attempted at high sugar concentrations [7].

Another challenging aspect in bioethanol technology is bioconversion of hexoses and pentoses both to ethanol [8]. Unfortunately, there are limited microorganisms that can proficiently convert both of these sugars to ethanol. *Saccharomyces cerevisi*ae and *Zymomonas mobilis* have been reported for the glucose conversion while *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* have been reported for the xylose conversion [9]. Various recombinant yeasts and bacteria have been developed during the last few decades that can ferment both the sugars [10]. However, their use for large-scale industrial processes still needs improvements related to the stability of the entire process and to overcome the public concerns for recombinant microorganisms [11,12]. Attempts have been made to utilize both hexoses and pentoses by co-culturing of *Saccharomyces cerevisiae* and *Pichia stipitis* [13]. However, co-cultures of these yeasts do not always ensure the efficient xylose conversion which may be because of diauxic behavior of xylose-fermenting yeasts, inhibition by ethanol and oxygen competition between the xylose fermenters and glucose fermenters [14]. Generally microorganisms consume glucose over galactose followed by xylose and arabinose [15]. In co-culture process if fermentation time is not sufficiently long, pentoses remain unutilized in the medium. Hence to overcome the problems of co-culturing, sequential glucose fermentation by *Saccharomyces cerevisiae* and xylose fermentation by *Pichia stipitis* can be a logical approach. Such a strategy can help to overcome glucose repression, ethanol inhibition and oxygen competition on xylose fermenting *Pichia stipitis*. Sequential ethanol fermentation processes using hexose and pentose fermenting yeasts have been reported [15-17].

We have already optimized saccharification of mild alkali treated maize stover using cocktail of in-house produced hemicellulases supplemented with commercial cellulases. This cocktail was effective at high substrate loading and preliminary experiments on ethanol production from enzymatic hydrolysate of maize stover by *Saccharomyces cerevisiae* 3524, yielded maximum 7.68 g/l ethanol at 3% reducing sugar concentration. In order to generate high concentration of ethanol, the present study was aimed at optimization of ethanol fermentation process using response surface methodology. In addition the research attempted sequential fermentation of hexoses followed by pentoses using *Saccharomyces cerevisiae* and *Pichia stipitis* respectively.

Materials and Methods

Materials

Throughout the research work analytical grade chemicals, media and reagents were used and were purchased from Qualigens, Hi-media, Merck, Loba from India. *P*-nitrophenyl-β-D-xylopyranoside *P*-nitrophenyl-α-L-arabinofuranoside, *P*-nitrophenyl-

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β-D-glucopyranoside and Ethyl ferlulate (Ethyl 4-hydroxy-3-methoxycinnamate) were obtained from Sigma-Aldrich, USA. Maps Enzyme Limited, India provided commercial cellulase MAPs 450.

Maize stover

Maize stover was obtained from local farmer from Panchmahal district, Gujarat. Maize stover was cut to 5 mm length, washed with tap water, air-dried and stored in air tight plastic bag at room temperature.

Microbial strains

For production of hemicellulases under solid state fermentation, *Aspergillus niger* ADH-11 (Accession no KF026012) was used. While for ethanol production standard yeast cultures, *Sacchromyces cerevisiae* NCIM 3524 and *Pichia stipitis* NCIM 3497 were procured from NCIM, Pune, India.

Enzymes

Crude β-xylosidase rich hemicellulases was indigenously produced by *Aspergillus niger* ADH-11 under solid state fermentation using previously optimized conditions [18]. Commercial cellulase was kindly provided by MAPS Enzymes Private Ltd., Gujarat, India. Enzyme doses were used as per necessity in the experiment. The crude β-xylosidase enzyme from *Aspergillus niger* ADH-11 and commercial cellulase (MAPs 450), were also having other cellulolytic and xylanolytic enzymes (Table 1).

Enzyme assays

β-Xylosidase activity was determined by quantifying *p*-nitrophenol release from *p*-nitrophenyl-β-D-xylopyranoside at 50°C. The reaction mixture containing of 2 mM *p*-nitrophenyl-β-D-xylopyranoside in 50 mM sodium citrate buffer (pH 5.3) was incubated with enzyme at 50°C for 30 min in a total volume of 0.5 ml. The reaction was ended by adding 1 ml of 2 M sodium carbonate. The amount of released *p*-nitrophenol was determined by measuring absorbance at 410 nm. One unit of β-xylosidase activity is defined as amount of enzyme required to release 1 µM of *p*-nitrophenol per minute under assay condition. α-L-Arabinofuranosidase activity was determined by measuring *p*-nitrophenol release from *p*-nitrophenyl-α-L-arabinofuranoside at 50°C. The reaction mixture consisting of 1 mM *p*-nitrophenyl-α-L-arabinofuranoside in 50 mM sodium citrate buffer (pH 5.3) was incubated with enzyme at 50°C for 10 min in total volume of 0.5 ml. The reaction was terminated by adding 1 ml of 2 M sodium carbonate. The amount of *p*-nitrophenol released was determined by measuring absorbance at 410 nm. One unit of α-L-arabinofuranosidase activity is defined as amount of enzyme required to release 1 µM of *p*-nitrophenol per minute under assay condition. Xylanase activity was determined using birch wood xylan solution (1%) as a substrate [19]. The substrate was prepared in 50 mM sodium citrate buffer (pH 5.3). The enzyme reaction was carried out for 10 min at 50°C and was ended by adding 1 ml of DNS reagent. The xylanase activity was determined by quantifying the release of reducing sugar using xylose as standard [20]. One unit of xylanase activity is defined as amount of enzyme required to release 1 µM of xylose per minute under assay condition. According to the IUPAC recommendation, Filter paper activity was measured using Whatmann filter paper no. 1 as a substrate [21]. The reducing sugars, release after 60 min in 50°C, at pH 4.8 (50 mM sodium citrate buffer) was measured as glucose equivalent by DNS method. One unit of filter paper activity is defined as amount of enzyme required to release 1 µM of glucose per minute under assay condition. Endo-glucanase activity was determined by measuring reducing sugars release from carboxymethylcellulose (CMC) at 55°C. The reaction mixture consisting of 2% carboxymethylcellulose in 50 mM sodium citrate buffer (pH 4.8) was incubated with enzyme at 55°C for 30 min in total volume of 1.0 ml. The reaction was terminated by adding 1 ml of DNS reagent. The enzyme activity was determined by measuring the release of reducing sugar using glucose as standard. One unit of endo-glucanase activity is defined as amount of enzyme required to release 1 µM of glucose per minute under assay condition. β-Glucosidase activity was determined by measuring *p*-nitrophenol release from *p*-nitrophenyl-β-D-glucopyranoside at 50°C. The reaction mixture consisting of 2 mM *p*-nitrophenyl-β-D-glucopyranoside in 50 mM sodium citrate buffer (pH 4.8) was incubated with enzyme at 50°C for 30 min in total volume of 0.5 ml. The reaction was terminated by adding 1 ml of 2 M sodium carbonate. The amount of *p*-nitrophenol released was determined by measuring absorbance at 410 nm. One unit of β-glucosidase activity is defined as amount of enzyme required to release 1 µM of *p*-nitrophenol per minute under assay condition. Feruloyl esterase activity was determined by measuring ferulic acid release from ethyl ferlulate (Ethyl 4-hydroxy-3-methoxycinnamate) at 50°C. The reaction mixture consists of 2 mM ethyl ferulate in 50 mM sodium citrate buffer (pH 5.3) was incubated with enzyme at 50°C for 30 min in total volume of 1.0 ml. The reaction was terminated by heating in boiling water bath for 10 min. The amount of ferulic acid released was determined by HPLC using ferulic acid as standard. One unit of feruloyl esterase activity is defined as amount of enzyme required to release 1 µM of ferulic acid per minute under assay condition.

Maize stover pretreatment

Pretreatment of maize stover was carried out by soaking maize stover in 2% NaOH solution (10% slurry (w/v)) at 30°C for 12 h. The substrate was neutralized with 12 N HCl, filtered with wet muslin cloth and then dried in oven at 80°C. Using Goering and Van Soest [22] method, composition (cellulose, hemicellulose and lignin) of untreated and pretreated maize stover were determined.

Enzymatic saccharification of pretreated maize stover at high substrate loading using cocktail of crude β-xylosidase and commercial cellulase (MAPs 450)

Maize stover saccharification was carried out using 13 FPU/g commercial cellulase (MAPs 450) supplemented with 74.42 U/g in-house produced β-xylosidase rich crude enzyme from *Aspergillus niger* ADH-11. Enzymatic sacccharification was carried out in 250 ml plastic bottle containing 30% pretreated maize stover and diluted enzyme in 50 mM sodium citrate buffer (pH 4.8) having 0.1% Tween-80 (v/v) in a total volume of 40 ml. Controls were taken for each reaction in which the active enzyme was substituted with heat inactivated enzyme. The reaction system was fortified with 10 mg % sodium azide. The reaction was performed at 50°C in hybridization oven at 100 rpm. After 36 h,

Enzyme	β-Xylosidase (U ml^{-1})	α-L-rabinofuranosidase (U ml^{-1})	Feruloyl esterase (U ml^{-1})	Xylanase (U ml^{-1})	Filter paper activity (U ml^{-1})	Endo-glucanase (U ml^{-1})	β-Glucosidase (U ml^{-1})
Crude β-xylosidase	30.0	1.57	1.0	20.0	0.64	42.98	11.40
Commercial cellulase	ND	ND	ND	300.0	43.9	2798.0	43.0

ND: Not Detected

Table 1: Composition of enzymes in each source.

samples were withdrawn and heated in boiling water bath (100°C) for enzyme inactivation. Then filtered using wet muslin cloth by thorough squeezing followed by centrifuged to collect the clear supernatant, which was used for further analysis to estimate reducing sugars by DNS method [20] and glucose, xylose and arabinose by HPLC method.

Optimization of ethanol production using hexose fermenting *Saccharomyces cerevisiae* NCIM 3524

Inoculum was prepared using 12 h grown culture (*Saccharomyces cerevisiae* NCIM 3524) at 30°C in a medium containing glucose (1%), yeast extract (0.3%), malt extract (0.3%) and peptone (0.5%) having pH 6.5 ± 0.2. The fermentation experiment was carried out in 50 ml screw cap tube at 30 ± 2°C without agitation. The samples were centrifuged for 10 min at 4°C at 15,000 rpm, filtered using 0.2 µ filter and analyzed using HPLC for the presence of sugars and ethanol.

BBD for optimization of ethanol fermentation from hexose sugar: A Box- Behnken factorial design with three factors and three levels was used for optimization of ethanol production. The independent variables were % glucose (X_1), % inoculum (X_2) and time (X_3). Final ethanol yield (g g^{-1}) was the dependent response variable. Selected independent variables were studied at three levels (Table 2) employing BBD with total 15 experimental runs (Table 3). The variables were taken at a central code value considered as zero. The temperature was kept constant at 30°C throughout the experiments runs.

Quadratic models considered as response surface model for predicting the optimal points were expressed according to Eq. (1) and (2).

For statistical calculations the independent variables were coded as:

$$x_i=(X_i-X_o)/\delta X_i \quad (1)$$

Where x_i is the experimental coded value of the variable; X_o is the middle value of X_i and δX_i is the step change.

Variable	Symbols	Coded level of variable		
		-1	0	1
% Glucose	X_1	1	3	5
% Inoculum	X_2	5	10	15
Time (h)	X_3	12	24	36

Table 2: Coded values and experimental range of process variables used in experimental design.

Run	Sugar concentration (X_1)	Inoculum size (X_2)	Time (X_3)	Ethanol yield (g g^{-1})	
				Predicted	Experimental
1	-1	-1	0	0.33	0.34
2	-1	1	0	0.34	0.36
3	1	-1	0	0.11	0.14
4	1	1	0	0.20	0.22
5	-1	0	-1	0.38	0.36
6	-1	0	1	0.30	0.31
7	1	0	-1	0.08	0.07
8	1	0	1	0.18	0.2
9	0	-1	-1	0.15	0.16
10	0	-1	1	0.33	0.31
11	0	1	-1	0.26	0.24
12	0	1	1	0.30	0.33
13	0	0	0	0.28	0.29
14	0	0	0	0.28	0.29
15	0	0	0	0.29	0.31

Table 3: Experimental plan and the results of the Box-Behnken design.

Ethanol yield (response Y) was explained as a second order response model on three independent variables given by

$$Y=\beta_o+\Sigma\beta_i X_i+\Sigma\beta_{ii} X^2_i+\Sigma\beta_{ij} X_i X_j \quad (2)$$

Where Y is the predicted response variable, β_o, β_i, β_{ii}, β_{ij} are fixed regression coefficients of the model, x_i, x_j (i=1, 2, 3, i ≠ j) represents independent variables in the form of original values.

Interpretation and data analysis

The experimental design results were evaluated and interpreted by MINITAB 16 (PA, USA) statistical software. Optimum fermentation parameters prediction and response contour plot created by the model was also evaluated by the same software. ANOVA was used to establish the significance of the model parameters.

Recovery of ethanol from fermented broth

Ethanol produced by hexose fermenting yeast was recovered using Rota-evaporator under vacuum at 45°C with 120 rpm for 60 min.

Ethanol production using pentose fermenting Pichia stipitis NCIM 3497

Ethanol fermentation using *Pichia stipitis* NCIM 3497 from fermented broth after distillation was carried out by preparing medium containing 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone and adjusting xylose concentration at 1.58% with pH 6.5 ± 0.2. The medium was sterilized at 10 lbs for 20 min. After cooling the medium to 30°C, it was inoculated aseptically with 5.40% (v/v) of 24 h old culture inoculum (*Pichia stipitis* NCIM 3497 with Absorbance of 0.6 at 660 nm). All the closed screw caps tubes were incubated at 30°C in static condition. The samples were withdrawn after 24 h and analyzed for ethanol production by HPLC and reducing sugars by DNS method [20].

Analytical techniques

Ferulic acid was analyzed using high performance liquid chromatography system (Shimadzu, Japan) equipped with photo diode detector (PDA) using a Luna 5u C18 (2) column with dimension of 250 mm × 4.6 mm. Samples were eluted using Water: Acetonitrile : Formic acid (7:2:1) with the flow rate of 0.6 ml min^{-1}. Column was kept at room temperature throughout analysis. Ferulic acid (1 mg ml^{-1}) was used as the standards. Appropriately diluted samples were filtered through 0.2 µ filters to remove any residual debris before injecting in the column. Ferulic acid was quantified using average peak areas of standards.

Monosaccharide sugars were analyzed using high performance liquid chromatography system (Shimadzu, Japan) equipped with a refractive index detector (RID). Sugars were analyzed using a Phenomenex, Rezex ROA-Organic acid H+(8%) column with dimension of 300 mm × 7.8 mm. Samples were eluted using 5 mM H_2SO_4 with the flow rate of 0.6 ml min^{-1}. Column temperature was kept at 65°C throughout analysis. Glucose, xylose arabinose and ethanol were used as the standards. Appropriately diluted samples were filtered through 0.2 µ filters to remove any residual debris before injecting in the column. Sugars and ethanol were quantified using average peak areas of standards.

Results and Discussion

Enzymatic hydrolysis of mild alkali treated maize stover using in-house produced hemicellulases supplemented with commercial cellulases was previously optimized in our laboratory at low substrate loading and the cocktail was also found effective at high substrate

loading. In order to achieve higher ethanol concentration enzymatic hydrolysis of maize stover was carried out at 30% substrate loading. Effective industrial production of bioethanol from maize stover requires utilization of both hexose and pentose sugars. Hence, the sequential ethanol fermentation was carried out for bioethanol production from enzymatic hydrolysate of maize stover.

Enzymatic saccharification of pretreated maize stover at high substrate loading using cocktail of crude β-xylosidase and commercial cellulase (MAPs 450)

Enzymatic saccharification at high substrate loading potentially offers many advantages. High substrate load have positive effect on economy of the process as it allows use of smaller reactor, produce concentrated sugar syrup and eventually lower distillation cost. The developed cocktail using in-house produced hemicellulases with commercial cellulases was used for saccharification of maize stover at high substrate loading (30%). The result clearly showed that the cocktail was highly effective at high substrate loading and liquefaction of solid residues was observed after 36 h (Figure 1) which contained maximum 161.32 mg ml^{-1} reducing sugar. This sugar rich hydrolysate mainly contained 67.84 mg ml^{-1} glucose, 34.62 mg ml^{-1} xylose and 6.14 mg ml^{-1} arabinose. Such a concentrated sugar syrup is highly advantageous for commercial ethanol fermentation process as concentration step can be avoided.

Optimization of ethanol production from hexose sugar using RSM

Three variables *viz.* % glucose, % inoculum and time (h) were selected for response surface optimization and ethanol yield g g^{-1} was selected as response variable. The results of all the experimental runs (15 run) are shown in Table 3. The experimental results suggest that the variable used in the present study had strong effect on ethanol yield. On the basis of these experimental values, statistical testing was carried out using MINITAB 16. The analysis of the model was tested by Fisher's 'F' and Student's t-test. Analysis of variance (ANOVA) of ethanol yield showed that the model was significant (P=0.002) as shown in Table 5, mainly due to the square portion of the regression model. A P-value below 0.05 indicates the test parameter are significant.

The fitted second-order response surface model as specified by Equation 2 for ethanol yield in coded process variables is as follows:

$$Y=0.419688-0.081875x_1+0.029750x_2+0.001146x_3-0.006562x_1^2-0.001450x_2^2-0.000043x_3^2+0.003250x_1x_2+0.001563x_1x_3-0.000208x_2x_3 \quad (3)$$

Where, Y is ethanol yield, and x_1, x_2 and x_3 are uncoded values of % glucose, % inoculum and Time h, respectively. A comparison of the experimentally obtained values with the predicted values indicated that these data are in reasonable agreement as shown in Table 3. The parameter estimates and the corresponding P-value showed that selected variable % glucose and % inoculum had significant square effect (0.024 and 0.052) on ethanol yield (Tables 4 and 5). Also interaction between % glucose and % inoculum, % glucose and time and % inoculum and time had significant square effect (0.042, 0.026 and 0.043) on ethanol yield (Table 4). The R^2 value provides a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. Joglekar and May [23] have suggested that for a good fit of the model, R^2 should be at least 80%. Coefficient R^2 for reducing sugar yield was observed to be 97.5%.

Application of RSM by Box-Behnken design, predicted that maximum ethanol yield occurred at values of process parameters as 5% glucose, 14.55% inoculum and Time 35.51 h. The model predicted highest (optimum) ethanol yield was 0.364 g g^{-1} (Figure 2).

Validation of the experimental model

A repeat fermentation for ethanol production from hexoses using *Saccharomyces cerevisiae* NCIM 3524 under optimal conditions was carried out for the validation of optimized parameters. The enzymatic hydrolysate was effectively utilized by hexose fermenting yeast and under optimized condition 90.65% glucose was utilized. Ethanol yield was 0.374 g g^{-1} (18.93 mg ml^{-1}) with 0.53 g l^{-1} h^{-1} productivity (Table 6). Before optimization, ethanol yield was only 7.68 mg ml^{-1} at 3% total reducing sugars which contained only 1.2% glucose. By applying response surface methodology ethanol production was enhanced up to 2.46 fold at high sugar concentration (9% total reducing sugars which contained 5% glucose).

In current scenario, cost-competitive ligno-cellulosic biofuel production can be intended by operating the process at high gravity [7]. Li et al. reported maximum 11.8 mg ml^{-1} ethanol production with 0.110 g l^{-1} h^{-1} productivity using *Saccharomyces cerevisiae* [17]. As compared to this, the productivity of the ethanol from hexose sugar in the present study was higher. A comparison of ethanol production by *Saccharomycere cerevisiae* from various lignocellulosic biomass is shown in Table 7. It can be seen that in the present study ethanol concentration is comparable or even higher as compared to other reports.

Term	Coef	SE Coef	T	P
Constant	0.419688	0.101719	4.126	0.009
% Glucose	-0.081875	0.025498	-3.211	0.024
% Inoculum	0.029750	0.011716	2.539	0.052
Time	0.001146	0.004882	0.235	0.824
% Glucose X % Glucose	-0.006562	0.003106	-2.113	0.088
% Inoculum X % Inoculum	-0.001450	0.000497	-2.918	0.033
Time X Time	-0.000043	0.000086	-0.503	0.636
% Glucose X % Inoculum	0.003250	0.001194	2.723	0.042
% Glucose X Time	0.001563	0.000497	3.141	0.026
% Inoculum X Time	-0.000208	0.000199	-1.047	0.043

Table 4: Regression coefficients for ethanol yield g g^{-1}.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	0.110683	0.110683	0.011298	21.58	0.002
Linear	3	0.093300	0.011789	0.003930	6.89	0.032
Square	3	0.006908	0.006908	0.002303	4.04	0.083
Interaction	3	0.010475	0.010475	0.003492	6.13	0.040
Residual Error	5	0.002850	0.002850	0.000570		
Lack-of-Fit	3	0.002650	0.002650	0.000883	8.83	0.103
Pure Error	2	0.000200	0.000200	0.000100		
Total	14	0.113533				

Table 5: Analysis of variance for ethanol yield g g^{-1}.

Parameters	
Initial reducing sugar (g l^{-1})	90.0
Initial glucose (g l^{-1})	50.0
Ethanol (g l^{-1})	18.93
% Reducing sugar consumed	55.55%
% Glucose consumed	90.65%
Ethanol yield g g^{-1} glucose	0.374
Ethanol yield g g^{-1} consumed reducing sugar	0.378
Productivity (g l^{-1} h^{-1})	0.53

Table 6: Ethanol fermentation from enzymatic hydrolysate of maize stover by *Saccharomycere cerevisiae* under optimized conditions.

Lignocellulosic Biomass	Fermentation condition	Ethanol concentration (mg/ml)	Ethanol Yield (g/g)	Reference
Wheat straw	Reducing sugar 6.5%, inoculum 3.3% at 30°C, 36 h	15.6	0.480	[25]
Rice straw	Reducing sugar 6%, inoculum 10% at 28°C, 24 h	12.34	0.093	[26]
Sweet sorghum	Sorghum juice, containing 19.9% sucrose and 1.9% (w/w) glucose at 33°C, 24 h	-	0.400	[27]
Corn cobs	95.3 g l^{-1} glucose, 5 g l^{-1} peptone, 3 g l^{-1} yeast extract at 30°C, 24 h	45.7	0.480	[28]
Maize stover	50 g l^{-1} glucose (90 g l^{-1} reducing sugars), 14.55% inoculum at 30°C, 35.51 h	18.93	0.374	Present study

Table 7: Ethanol yield from various lignocellulosic biomass by *Saccharomycere cerevisiae*.

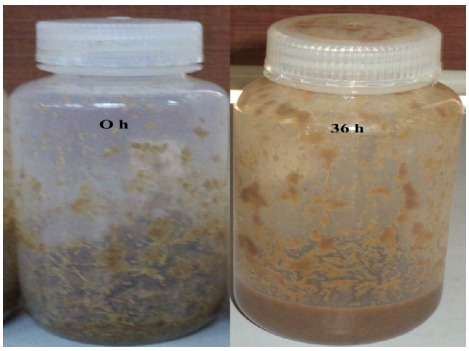

Figure 1: Liquefaction of solid residues at high substrate loading (30%) using developed cocktail of 74.42 U/g crude β-xylosidase and 13 FPU/g commercial cellulase at 50°C after 36 h.

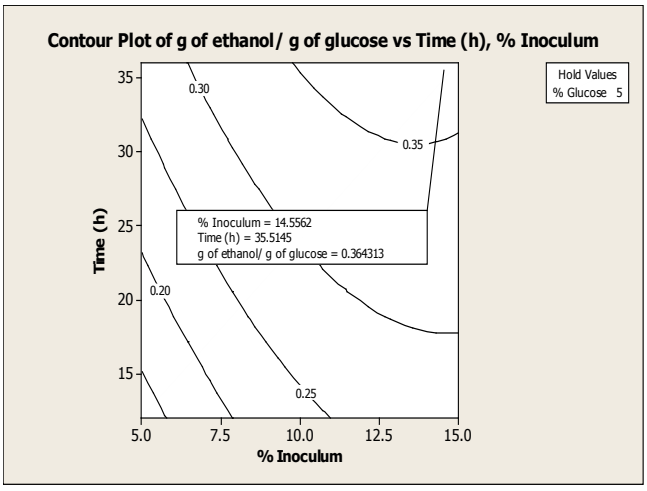

Figure 2: Contour plot showing interaction effect of % inoculum and time on ethanol yield (g/g) at hold value of % glucose.

Ethanol production by *Pichia stipitis* NCIM 3497

After ethanol recovery, hydrolysate mainly contained xylose, hence ethanol production using *Pichia stipitis* NCIM 3497 was carried out and only 71% xylose was utilized by yeast with maximum ethanol yield of 0.38 g g^{-1} (2.23 mg ml^{-1}) with productivity 0.09 g l^{-1} h^{-1}. Agbogbo and Wenger [24] reported that *Pichia stipitis* produced ethanol yield 0.37 g g^{-1} (10.4 mg ml^{-1}) from enzymatic hydrolysate of corn stover. In the current investigation it was observed that production of ethanol from the remaining pentose sugar was lesser which may be due to existence of fermentative inhibitors produced by the hexose utilizing yeast. Hence, further studies are needed for effective utilization of pentose sugars.

Conclusion

It can be concluded from the present study that enzymatic hydrolysate obtained after saccharification of maize stover at high substrate loading was effectively utilized by *Saccharomyces cerevisiae*. By statistical optimization of ethanol fermentation process, production was enhanced up to 2.46 fold and ethanol concentration reached to 18.93 mg ml^{-1}. However utilization of pentoses by sequential fermentation was not so effective. Hence, further studies are needed to increase ethanol production from pentoses.

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