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Simultaneous saccharification and ethanol fermentation of oxalic acid pretreated corncob assessed with response surface methodology

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ABSTRACT

Response surface methodology was used to evaluate optimal time, temperature and oxalic acid concentration for simultaneous saccharification and fermentation (SSF) of corncob particles by *Pichia stipitis* CBS 6054. Fifteen different conditions for pretreatment were examined in a 2^3 full factorial design with six axial points. Temperatures ranged from 132 to 180 °C, time from 10 to 90 min and oxalic acid loadings from 0.01 to 0.038 g/g solids. Separate maxima were found for enzymatic saccharification and hemicellulose fermentation, respectively, with the condition for maximum saccharification being significantly more severe. Ethanol production was affected by reaction temperature more than by oxalic acid and reaction time over the ranges examined. The effect of reaction temperature was significant at a 95% confidence level in its effect on ethanol production. Oxalic acid and reaction time were statistically significant at the 90% level. The highest ethanol concentration (20 g/l) was obtained after 48 h with an ethanol volumetric production rate of 0.42 g ethanol l^{-1} h^{-1}. The ethanol yield after SSF with *P. stipitis* was significantly higher than predicted by sequential saccharification and fermentation of substrate pretreated under the same condition. This was attributed to the secretion of β-glucosidase by *P. stipitis*. During SSF, free extracellular β-glucosidase activity was 1.30 pNPG U/g with *P. stipitis*, while saccharification without the yeast was 0.66 pNPG U/g.

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1. Introduction

A role for oxalic acid in the degradation of wood and other lignocellulosics has long been recognized (Schmidi et al., 1981; Munir et al., 2001; Shimada et al., 1994; Dutton and Evans, 1996; Green et al., 1991). Brown rot fungi are known to secrete oxalic acid, and more recently white-rot fungi have been shown to produce this dicarboxylic acid as well (Evans et al., 2006). Oxalate, which can chelate Mn^{3+}, Fe^{3+}, Fe^{2+}, and other divalent cations, is thought to play a role in degradation of lignin and cellulose by these fungi by stabilizing the reactive metal ion (Hofrichter, 2002).

Aside from this role, oxalic acid can catalyze the hydrolysis of hemicellulose and cellulose directly. It is one of the strongest organic acids known, with ionization constants of 6.5×10^{-2} and 6.1×10^{-5} corresponding to pKa's of 1.27 and 4.28, respectively. This is considerably stronger than acetic acid and somewhat stronger than H_2SO_3 as well. At the same time, oxalic acid is less toxic to yeasts and other microbes than acetic or sulfurous acids because its lower pKa restricts diffusion of the ionized form across cellular membranes. In comparison to H_2SO_3, it does not inhibit glycolysis and does not produce noxious odors (Mosier et al., 2001).

For these reasons, oxalic acid has been developed for the direct acid hydrolysis of wood and other lignocellulosics (Torget et al., 2002). One apparent advantage of pretreating wood with oxalic acid is that it is possible to remove a portion of the hemicellulose without significantly degrading the cellulosic fibers. Researchers have reported that this pretreatment saves significant energy in the refining process while providing a stronger paper product. The hemicellulose can thereby be used for fermentation and the fibers for papermaking (Akhtar et al., 2002; Swaney et al., 2003; Kenealy et al., 2007).

Agricultural residues are considerably less recalcitrant than wood due to their lower lignin contents, higher hemicellulosic contents and low bulk densities. A mild pretreatment process that will not cause extensive degradation of the arabinose and xylose, while still preparing the cellulose for enzymatic saccharification would be very useful. We were interested in knowing whether oxalic acid would be an appropriate pretreatment for corn (maize) cob residue.

Corncobs are important by-products of the sweet corn processing industry. Traditionally, they have been used as an animal bedding or returned to the field (Inglett, 1970). Corncobs contain

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approximately of 70% carbohydrate, 10% lignin and 2% protein (Barl et al., 1991). They can, therefore can serve as a potential source of sugars.

In this study, we applied oxalic acid as a pretreatment to a standardized corncob particulate substrate, and evaluated its effect on ethanol production by simultaneous saccharification and fermentation.

2. Methods

2.1. Pretreatment of the corncob

The pretreatment of corncobs by oxalic acid was based on the method developed by Kenealy et al. (2007). The reactor configuration has been described previously. Standardized corncob pellets (Pestell, New Hamburg, Canada) contained 10% moisture. The pretreatment condition was based on 2^3 full factorial design augmented with star design (six axial points) and three replicates in the central point (Table 1). Corncob pellets (1.5 kg dry matter) were impregnated under vacuum with different concentrations of oxalic acid solution in a semi-pilot reactor for 20 min at room temperature. The solid:liquid ratio during impregnation was 1:6. The residue took up 15% of the liquid, and excess oxalic acid solution was drained away. The impregnated pellets were treated at different temperatures and reaction times as shown in Table 1. The pretreated pellets were then washed to extract sugars and the resulting hemicellulose hydrolysate was stored at 4 °C. The solid residue was used as a substrate for simultaneous saccharification and fermentation (SSF), as described herein.

2.2. Yeast strain and inoculum medium

Pichia stipitis CBS 6054 was maintained on agar malt medium and stored at 4 °C then transferred to fresh plate to be used within 24 h of incubation at 30 °C. Cells were grown in 1000 ml Erlenmeyer flasks containing 400 ml of YPD (10 g/l yeast extract, 10 g/l peptone, and 20 g/l glucose) in an orbital shaker at 30 °C and 200 rpm. Following 24 h growth, cell cultures were harvested, centrifuged (4068*g*, 15 min at 4 °C) and decanted to yield cell pellets, which were washed once with sterile deionized water, and adjusted to a calculated concentration of 30 g dry cell weight (DCW) per liter via standard curves relating 600 nm absorbance to DCW/l concentration. An aliquot was transferred to fresh fermentation medium for an initial cell concentration of 2.0 DCW/l.

Table 1
The 2^3 full factorial design with six axial points and three replicates in the central point matrix employed for three independent variables.

Run no.	Variables			Coded levels		
	Temperature (°C) X_1	Oxalic acid* (g/g) X_2	Reaction time (min) X_3	X_1	X_2	X_3
1	132	0.015	26	−1	−1	−1
2	168	0.015	26	+1	−1	−1
3	132	0.032	26	−1	+1	−1
4	168	0.032	26	+1	+1	−1
5	132	0.015	74	−1	−1	+1
6	168	0.015	74	+1	−1	+1
7	132	0.032	74	−1	+1	+1
8	167	0.032	74	+1	+1	+1
9	120	0.024	50	−1.68	0	0
10	180	0.024	50	+1.68	0	0
11	150	0.010	50	0	−1.68	0
12	150	0.037	50	0	+1.68	0
13	150	0.024	10	0	0	−1.68
14	150	0.024	90	0	0	+1.68
15	150	0.024	50	0	0	0
16	150	0.024	50	0	0	0
17	150	0.024	50	0	0	0

* The oxalic acid concentration used during impregnation of corncob (solid liquid ratio of 1:6). Approximately 15% of the solution was taken up by the solids.

2.3. Fermentation medium preparation

The fermentation medium was prepared using oxalic acid pretreated corncob obtained as above. Initially 50 ml of 50 mM sodium citrate buffer (pH 6.0) was added to different oxalic acid pretreated corncob (5 g dry matter) in 125 ml Erlenmeyer flasks followed by supplementation with 5 g/l yeast extract, 5 g/l urea, 0.5 g/l $MgSO_4 \cdot 7H_2O$ and 1 g/l KH_2PO_4. The media were autoclaved at 120 °C for 15 min.

2.4. Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation was performed in 125 ml Erlenmeyer flasks containing 50 ml of fermentation medium prepared as above. For saccharification, appropriate amounts of cellulase (500 CMC U/g) and β-glucosidase (80 pNPG U/g) of Acellerase 1000 from Genenor (Danisco, Rochester, NY) were added. *P. stipitis* was then inoculated at a cell concentration of 2 DCW/l. SSF was started by adding enzymes and *P. stipitis*, and then incubated at 30 °C in orbital shaker at 150 rpm. Samples were taken at 24, 48, 72 h, and the amounts of monosaccharides remaining and ethanol produced were analyzed by high performance liquid chromatography (HPLC) (see below). All determinations were performed in triplicate biological replications.

2.5. Determination of sugars and ethanol

Concentrations of D-glucose, D-xylose, L-arabinose and ethanol were determined by following separation by HPLC using a BioRad (Hercules, CA) Aminex HPX-87H column (300 × 7.8 mm) at 55 °C with 5 mM H_2SO_4 as eluant, at a flow rate of 0.3 ml/min and an injection volume of 20 µl. A refractive index detector (Hitachi High-Technologies Corporation model L-2490, Japan) was used to quantify the products. Samples were appropriately diluted in deionized water, and then filtered through PrepSEP C18 (Fisher Scientific) filters prior to injection.

2.6. β-Glucosidase activity

β-Glucosidase activity was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (Sigma Chemical Co., USA). The enzyme solution was incubated for 10 min at 50 °C with 1 mM *p*-nitrophenyl-β-D-glucopyranoside in a 10 mM sodium acetate buffer (pH 5.0). The enzymatic reaction was stopped by the addition of 100 µl of 2 M sodium carbonate. The amount of *p*-nitrophenol released by β-glucosidase was determined colorimetrically at 400 nm (Igarashi et al., 2003).

2.7. Surface response analysis

A 2^3 full factorial design was used in the optimization of oxalic acid corncob pretreatment to improve its simultaneous saccharification and fermentation for ethanol production. Temperature (X_1, °C), oxalic acid concentration during impregnation of corncob (X_2, g/l) and reaction time (X_3, h) were chosen for the independent variables. Ethanol concentration (Y_1, g/l) and ethanol volumetric productivity (Y_2, g/l per h) were used as the dependent output variables.

For statistical calculation the variables X_i were coded as x_i according to the following equation.

$$X_i = (X_i - \bar{x}_i)/(\Delta x_f/2) \quad (i = 1, 2, 3, \ldots, k) \tag{1}$$

where x_i is dimensionless value of an independent variable, X_i is real value of an independent variable, $\bar{x}_i$ is real value of the independent variable at the central point and Δx_j is step change. A 2^3 full factorial design with six axial points ($\alpha = \sqrt{3}$) and three replication at the central points leading to a total number of 17 experiments was employed for the optimization of the condition of oxalic acid corncob pretreatment to improve the simultaneous saccharification and fermentation for ethanol production. The second-degree polynomials were calculated with Design-Expert Version 6.0.6 software (Stat-Ease Inc., USA) to estimate the response of the dependent variables (Eq. (2)). The response and contour plot was performed using Statistica for Windows™ release 5.1 software (StatSoft Inc., USA).

$$Y_i = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-2} \beta_{ii} x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \tag{2}$$

where Y_i is the predicted value, x_i and x_j are the coded values of the factors, β_0 is a constant coefficient, β_i are the linear coefficients, β_{ij} (i and j) are the interaction coefficients and β_{ii} are the quadratic coefficients.

3. Results and discussion

3.1. Simultaneous saccharification and fermentation: ethanol production and ethanol volumetric productivity

The composition of pretreated corncob depended on the pretreatment condition. The most important physical factors that affect the corncob pretreatment are temperature, oxalic acid concentration and reaction time. Suitable levels for these parameters were determined using a statistical 2^3 full factorial design. The experimental design matrix is given in Table 1. Seventeen experiments were performed to evaluated different pretreated oxalic acid corncob to ethanol production by *P. stipitis* CBS 6054. The simultaneous saccharification and fermentation process was used to achieve this goal.

The ethanol yield obtained depended on the pretreatment condition as shown in Table 2. After 48 h incubation, glucose was directly converted into ethanol and close to zero while xylose remained. The highest ethanol concentration was 20 g/l on the pretreated corncob at 168 °C for 74 min with 0.032 g/g of oxalic acid, corresponding to an ethanol volumetric production rate of 0.42 g ethanol l^{-1} h^{-1}. The SSF process was completed after 72 h (data not shown). By that time, the concentrations of glucose and xylose were close to zero. However the Q_p value was lower than at 48 h. Experimental ethanol yield increased with treatment severity, which decreased the amount of xylan in the pretreated corncob (data not shown). This result is in agreement with the report of Sassner et al. (2008).

The effects and standard errors for the ethanol production and ethanol volumetric productivity from the experimental matrix were estimated. From a general analysis it was possible to select the factors and second-order interactions that were significant in the 90–95% confidence range. For both cases, the first-order effects of temperature, oxalic acid concentration and reaction time were significant at the 95% confidence level. However, the interaction oxalic acid and reaction time was statistically significant at 90% of confidence level. Also for both cases, ethanol production and ethanol volumetric productivity, the residual analysis (plot of residuals versus estimated values) of the proposed model showed that the residuals varied randomly, which means they did not have unexplained systematic tendencies, so the curve represented the points satisfactorily (data not shown). An ANOVA of the significant values from Student's *t* distribution was made (Tables 3 and 4) to determine if the regression equation was statistically significant.

Table 2
Sugar and product accumulation during cellulose saccharification and simultaneous saccharification and fermentation (SSF).

Run no.	Glucose	Xylose	Experimental ethanol yield[b]	Ethanol (g/l)	Q_p[c] (g/l h)
1	0.1 (7.7)[a]	0.2 (4.0)	0.167	1.9	0.04
2	0.1 (20.3)	6.6 (11.2)	0.548	13.6	0.283
3	0 (17.1)	3.8 (10.2)	0.502	11.8	0.246
4	0.1(19.7)	3.5 (11.1)	0.665	18.1	0.377
5	0 (15.5)	1.6 (7.9)	0.463	10.1	0.210
6	0 (22.2)	3.6 (10.7)	0.569	16.7	0.348
7	0 (18.9)	3.7 (11.1)	0.521	13.7	0.285
8	0 (20.2)	0.9 (9.8)	0.687	20.0	0.417
9	0 (13.8)	0.8 (7.4)	0.475	9.7	0.202
10	0.1 (20.9)	2.2 (10.3)	0.692	20.0	0.417
11	0 (17.8)	2.7 (8)	0.424	9.8	0.204
12	0.1 (21.3)	4.5 (12.9)	0.514	15.2	0.317
13	0 (16.7)	2.8 (8.4)	0.488	10.9	0.227
14	0.1 (21.5)	4.1 (11.2)	0.533	15.2	0.317
15	0.1 (21.5)	6.2 (12.8)	0.468	13.1	0.273
16	0 (20.6)	5 (12.4)	0.504	14.1	0.294
17	0 (19.4)	7.7 (13.1)	0.476	11.8	0.246

[a] The values show the amounts of sugars following enzymatic saccharification using the same condition with SSF. Values in parentheses are at 30 °C, 150 rpm for 48 h without the yeast strain.
[b] Experimental ethanol yield was calculated as ethanol production/(total sugar − remaining sugar).
[c] Q_p = ethanol volumetric productivity.

Table 3
Analysis of variance (ANOVA) for ethanol production (Y_1) by *P. stipitis* CBS 6054 during simultaneous saccharification and fermentation as a function of temperature (X_1), oxalic acid concentration (X_2), reaction time (X_3) used during pretreatment of corncob.

Effects	Sum of squares	Degrees of freedom	Mean square	*F*-ratio	*p*-value
X_1	170.294	1	170.294	76.43	0.0000
X_2	67.6051	1	67.6051	30.34	0.0001
X_3	36.7239	1	36.7239	16.39	0.0016
X_2X_3	7.03125	1	7.03125	3.16	0.1010
Total error	26.7365	12	2.22804		
Total	308.191	16			

R-squared = 0.9133.
R-squared (adjusted for d.f.) = 0.8843.

Tables 3 and 4 also show that the main factors were significant at a 95% confidence level – excluding the interaction between oxalic acid and reaction time, which was significant at 90% – indicating that the estimated *F* was higher than the tabular *F*. So, it is evident that there was a quadratic relationship between the independent variables (factors and their interactions) and the response variable (ethanol production and ethanol volumetric productivity), indicating that all the factors were statistically significant, with a good confidence interval. The *p*-value is the probability of the test statistic being at least as extreme as the one observed given that the null hypothesis is true. A small *p*-value is an indication that the null hypothesis is false. In this study $p \leqslant 0.1$ and $p \leqslant 0.01$ were considered to be significant. The significance of the data is judged by its *p*-value being closer to zero (0.00). For a 90%, 95% and 99% confidence level the *p*-value should be 0.1, 0.05 and 0.01, respectively.

Tables 5 and 6 show the ANOVA of the quadratic model adjustment to data from Tables 3 and 4, where the total error was dismembered into lack-of-fit and pure error. The *F* values estimated with the use of the experimental data corresponded to total residual and lack-of-fit, respectively, and were lower than the tabular *F* values, both found in the *F* distribution, indicating that the models were significant in the region studied. For both models the *p*-val-

Table 4
Analysis of variance (ANOVA) for ethanol volumetric productivity (Y_2) by *P. stipitis* CBS 6054 during simultaneous saccharification and fermentation as a function of temperature (X_1), oxalic acid concentration (X_2), reaction time (X_3) used during pretreatment of corncob.

Effects	Sum of squares	Degrees of freedom	Mean square	*F*-ratio	*p*-value
X_1	0.0739	1	0.0739	76.43	0.0000
X_2	0.0293	1	0.0293	30.34	0.0001
X_3	0.0159	1	0.0159	16.40	0.0016
X_2X_3	0.0031	1	0.0031	3.16	0.1010
Total error	0.0116	12			
Total	0.1338	16			

R-squared = 0.9133.
R-squared (adjusted for d.f.) = 0.8843.

Table 5
Analysis of variance for the adjusted model for ethanol production by *P. stipitis* CBS 6054 during simultaneous saccharification and fermentation based on oxalic acid pretreatment of corncob.

Statistical parameters	Sum of squares	Degrees of freedom	Mean square	*F*-ratio	*p*-value
Model	281.41	4	70.35	31.52	<0.0001
Residual	26.78	12	2.23		
Lack-of-fit	24.12	10	2.41	1.81	0.4073
Pure error	2.66	2	1.33		

R-squared = 0.9133.

Table 6
Analysis of variance for the adjusted model for ethanol volumetric productivity by *P. stipitis* CBS 6054 during simultaneous saccharification and fermentation based on oxalic acid pretreatment of corncob.

Statistical parameters	Sum of squares	Degrees of freedom	Mean square	*F*-ratio	*p*-value
Model	0.12	4	0.031	31.53	<0.0001
Residual	0.012	12	0.0010		
Lack-of-fit	0.010	10	0.0010	1.81	0.4074
Pure error	0.0012	2	0.0006		

R-squared = 0.9133.

ues were <0.0001 (Tables 5 and 6), which shows that the models are strongly significant at 99% of confidence level.

The determination coefficients (*R*-squared) in Tables 3 and 4 indicate that 91.33% of the total variation around the average could be explained by the regression. The models calculated for ethanol production (Y_1) and ethanol volumetric productivity (Y_2) were:

$$Y_1 = 16.81125 + 2.22702X_2 + 1.63695X_3 - 0.93950X_2X_3 \quad (3)$$

$$Y_2 = 0.350232 + 0.046396X_2 + 0.034106X_3 - 0.019573X_2X_3 \quad (4)$$

where Y_1, Y_2, X_2 and X_3 are ethanol production, ethanol volumetric productivity, oxalic acid concentration during the vacuum impregnation of the corncob, and reaction time, respectively.

The response surfaces were drawn as three-dimensional plots of two factors (reaction time and oxalic acid concentration) while the other factor (temperature) was kept constant at higher level (+1) (Fig. 1A and B). Temperature (X_1) was kept constant at the coded level of +1. Fig. 1 represents the surface of Eqs. (3) and (4). The analysis of response surface (Fig. 1A and B) shows that the highest values of temperature (+1), oxalic acid solution (+1) during impregnation of corncob and reaction time (+1) used during the corncob pretreatment tended to enhance ethanol production and ethanol volumetric productivity.

Also, the response surface showed that the interaction of oxalic acid concentration and reaction time had a significant effect on both ethanol production and ethanol volumetric productivity. The highest value for ethanol production (19.73 g/l) and ethanol volumetric productivity (0.41 g l^{-1} h^{-1}) were at the highest values for temperature (168 °C), oxalic acid concentration (0.032 g/g) and reaction time (74 min) (Fig. 1A and B). By reducing the oxalic acid concentration or the reaction time at the lowest values (−1) both ethanol production and ethanol volumetric productivity were reduced to 17.16 and 18.33 g/l and 0.36 and 0.38 g/l h, respectively. The lowest values for both responses were obtained by reducing the oxalic acid concentration during the corncob impregnation. In most cases it is easier to visualize these results from the contour plot (Fig. 1A and B). The lowest values for ethanol production (4.95 g/l) and ethanol volumetric productivity (0.1 g/l h) were found at lowest values for all main factors. These data are not shown in the response surfaces because the temperature value in these figures was kept constant at highest level (+1).

The proposed model for measuring the ethanol production and ethanol volumetric productivity was validated under the condi-

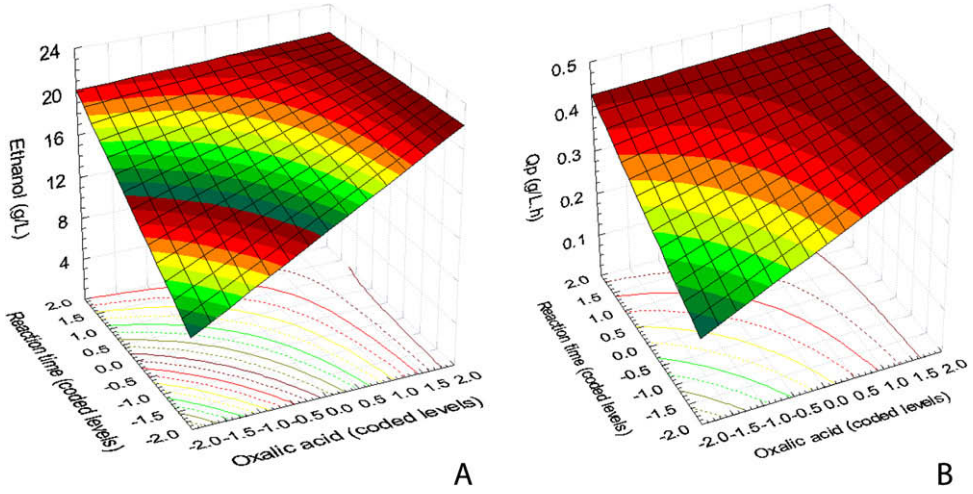

Fig. 1. Response surface and contour plot of oxalic acid concentration used during corncob impregnation vs. pretreatment reaction time on ethanol production (A) and ethanol volumetric productivity (B) by *P. stipitis* CBS 6054 during simultaneous saccharification and fermentation of the pretreated corncob (temperature was kept constant at coded level of +1).

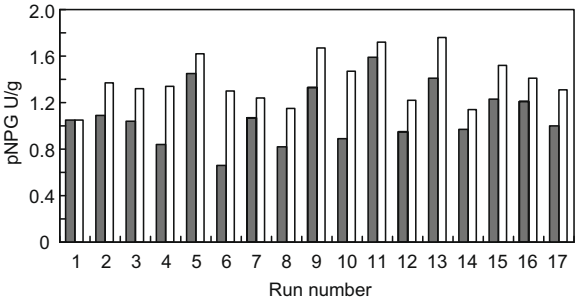

Fig. 2. Activity of β-glucosidase as a function of pretreatment condition after SSF reaction (blank: after 48 h of SSF reaction with *P. stipitis*, gray: enzymatic saccharification without *P. stipitis* at the same condition with SSF).

tions determined by the statistical methodology. For that purpose, the corncob pretreatment was conducted at 168 °C for 74 min after vacuum impregnation with 0.032 g/g of oxalic acid solution (solid liquid ratio of 1:6). The values of ethanol production and ethanol volumetric productivity estimated by Eqs. (3) and (4) were 19.74 g/l and 0.41 g/l h, respectively. The result of the experimental assay was 20 g/l of ethanol and 0.42 g/l h, indicating that the models can mathematically represent the ethanol production and volumetric ethanol productivity for the simultaneous saccharification and fermentation of pretreated corncob by *P. stipitis* CBS 6054.

This study using the technique of response surface methodology (RSM) enables us to find the accurate values of the oxalic acid corncob pretreatment for the maximum ethanol and ethanol volumetric productivity using simultaneous saccharification and fermentation with enzyme such as cellulase (500 CMC U/g) and β-glucosidase (80 pNPG U/g) and *P. stipitis* CBS 6054.

3.2. Simultaneous saccharification and fermentation: relationship between ethanol yield and β-glucosidase

During SSF with *P. stipitis*, the experimental ethanol yield was higher than the theoretical yield of ethanol that could be obtained by complete fermentation of the sugars released by the cellulase complex under the same condition but in the absence of *P. stipitis*. In addition to relieving cellulase inhibition by consuming glucose, *P. stipitis* could increase the yield by secreting β-glucosidase. The reaction catalyzed by β-glucosidase is the most important step in the degradation of cellulose because it limits the efficiency of hydrolysis, and β-glucosidase can relieve cellobiose-mediated inhibition of exoglucanase and endoglucanase (Yan et al., 1998). Therefore, SSF using *P. stipitis* – along with the β-glucosidase it produced – effectively accelerated ethanol production. The β-glucosidase activities following SSF are shown in Fig. 2. In comparing the activities, β-glucosidase was higher when *P. stipitis* was used in the SSF reaction than when only exogenous enzyme was used. The level of β-glucosidase depended on the pretreatment condition. The difference in activity was particularly apparent when high temperatures and long reaction times were used. Under this condition, the pretreated corncob contained more lignin than xylan (data not shown). Here, it is very important to distinguish the separate interferences of lignin and xylan during SSF. The β-glucosidase works better with low lignin. However, the glucose released during saccharification was higher in the pretreated corncob with low xylan – even though the lignin content was relatively higher. So, the highest ethanol yield was not obtained in the condition exhibiting the highest β-glucosidase activity. In general, the β-glucosidase activity found in all experiments was enough to improve ethanol production; SSF improved ethanol production even when the cells formed relatively little β-glucosidase (Jeffries et al., 2007).

The β-glucosidase activity after SSF with *P. stipitis* and corncob pretreated at 180 °C for 90 min was 1.30 pNPG U/g, while that of without strain was 0.66 pNPG U/g. The β-glucosidase produced by *P. stipitis* increased the ethanol yield because β-glucosidase degrades cellobiose to glucose, which was in turn fermented to ethanol.

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