Optimization of Simultaneous Saccharification and Fermentation for the Production of Ethanol from Lignocellulosic Biomass

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Simultaneous saccharification and fermentation (SSF) of alkaline hydrogen peroxide pretreated *Antigonum leptopus* (Linn) leaves to ethanol was optimized using cellulase from *Trichoderma reesei* QM-9414 (Celluclast from Novo) and *Saccharomyces cerevisiae* NRRL-Y-132 cells. Response surface methodology (RSM) and a three-level four-variable design were employed to evaluate the effects of SSF process variables such as cellulase concentration (20−100 FPU/g of substrate), substrate concentration (5−15% w/v), incubation time (24−72 h), and temperature (35−45 °C) on ethanol production efficiency. Cellulase and substrate concentrations were found to be the most significant variables. The optimum conditions arrived at are as follows: cellulase = 100 FPU/g of substrate, substrate = 15% (w/v), incubation time = 57.2 h, and temperature = 38.5 °C. At these conditions, the predicted ethanol yield was 3.02% (w/v) and the actual experimental value was 3.0% (w/v).

Keywords: *Simultaneous saccharification and fermentation (SSF); Antigonum leptopus leaves; alkaline H_2O_2 pretreatment; cellulase; ethanol; lignocellulose*

INTRODUCTION

Cellulosic biomass is an abundant renewable resource on earth and includes various agricultural residues, fruit and vegetable wastes, woods, municipal solid waste, waste from the pulp and paper industry, and herbaceous energy crops. The degradation of cellulosic material has gained increasing research attention due to its worldwide availability and immense potential for its transformation into sugars, alternative fuels, and chemical feedstocks. Ethanol is the one of the prime alternatives to gasoline and can also be used to supplement gasoline (as gasohol: 80% gasoline + 20% ethanol) (Flickinger, 1980). Apart from an alternative to fuel, alcohol is also used as a solvent and is a versatile feedstock to obtain various organic chemicals and their derivatives. The basic raw materials for fermentative production of ethanol include saccharine, starchy, and cellulosic materials. Of these, cellulosic raw materials are receiving the major research thrust mainly because most of them are agrowastes and are abundantly available. Various steps involved in the production of fuels and chemicals from lignocellulosic biomass are feedstock preparation, pretreatment, fractionation, enzymatic hydrolysis (saccharification), fermentation, product recovery, and waste treatment. There are substantial research inputs in the areas of pretreatments (Begum, 1988; Barl et al., 1991; McMillan, 1994; Pinto and Kamden, 1996; Esteghlalian et al., 1997) and saccharification (dePadilla and Hoskins, 1968; Coughlan, 1991; Kong et al., 1992; Hari Krishna et al., 1997; Medve et al., 1998; Mooney et al., 1998; Meunier-Goddik and Penner, 1999).

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Many studies on the saccharification of cellulose established product inhibition on cellulase prohibiting complete conversion, due to which only low substrate concentrations were utilized. To overcome the inhibition exerted by the saccharification products, a simultaneous fermentation step was undertaken (Takagi et al., 1977). Among the systems for ethanol production from cellulose, the simultaneous saccharification and fermentation (SSF) process has attracted many investigators (Deshpande et al., 1983; Spangler and Emert, 1986; Philippidis et al., 1993; South et al., 1995; Gregg and Saddler, 1996; Vinzant et al., 1997; Hari Krishna et al., 1998, 1999). The SSF process offers benefits such as improved ethanol yields by reducing the product inhibition exerted by saccharification products and also eliminates the need for separate reactors for saccharification and fermentation, which results in cost reductions.

Screening several lignocellulosics to select the ideal substrate is the key for economic production of ethanol. *Antigonum leptopus* (Linn), a weedy creeper, is found abundantly in regions not utilized for conventional agriculture. This biomass has not been put to commercial use and was found to contain low lignin content and less crystalline cellulose (Hari Krishna et al., 1997). In this context, it has been suggested that the rate of saccharification decreases with increase in cellulose crystallinity. In a highly crystalline substrate (hydrocellulose or cotton), the closely packed, hydrogen-bonded cellulose molecules might be less accessible to enzyme attack than the loosely organized microcrystalline or amorphous (swelled) cellulose. The lignin content also plays a significant role in substrate susceptibility. Accordingly, saccharification of the cellulose from *A. leptopus* leaves was completed in short time, compared to other lignocellulosic substrates (Hari Krishna et al., 1997). In an another paper (Hari Krishna et al., 1999), we have also shown that *A. leptopus* leave's cellulose

10.1021/jf991296z CCC: $19.00
Published on Web 04/19/2000

can be converted to ethanol (with high yields in a shorter time) by *Trichoderma reesei* cellulase and *Saccharomyces cerevisiae* cells in an SSF system. Transforming this biomass would provide a new source of energy and chemicals.

Although the SSF process was observed to be effective, a recent economic analysis of ethanol production from biomass (Hinman et al., 1992) still identifies the SSF operation as the major contributor to the cost of ethanol (>20%), thereby raising the need for optimization of the SSF performance. In the present study an attempt was made to identify optimum operating conditions for the production of ethanol from *A. leptopus* leaves through SSF by analyzing the relationships among the parameters (cellulase concentration, substrate concentration, incubation time, and temperature) affecting the process by the use of response surface methodology (RSM).

MATERIALS AND METHODS

Enzymes. Cellulase from *T. reesei* QM-9414 (Celluclast) and β-glucosidase (Novozym 188) from *Aspergillus niger* were generous gifts from Novo Nordisk (Bagsvaerd, Denmark). It should be noted that although, in general, cellulase enzyme complex consists of 1,4-β-D-glucanohydrolases (endoglucanases), 1,4-β-D-glucan cellobiohydrolases (exoglucanases), and β-D-glucoside glucohydrolase (β-glucosidase or cellobiase), in this study, the enzyme complex is treated as two distinct entities: (i) cellulase that hydrolyzes cellulose to cellobiose through the combined action of endo- and exoglucanases and (ii) β-glucosidase that hydrolyzes cellobiose to glucose. Celluclast contained 80 FPU/mL and 80 mg of protein/mL. Novozym 188 contained 792 cellobiase units/mL and 73 mg of protein/mL.

Substrate and Pretreatments. Substrate material, *A. leptopus* (Linn), was collected from the university campus, Andhra University (Visakhapatnam, India). Substrate leaves were cut into small pieces (2−3 mm) and air-dried before use. Dried biomass was ground to a 10-mesh size and used after alkaline hydrogen peroxide ($NaOH + H_2O_2$) pretreatment (Gould and Freer, 1984).

Enzymatic Saccharification. Experiments were carried out in stoppered conical flasks (100 mL) in the presence of 0.01% (w/v) sodium azide. The pH was adjusted to 4.5 with 0.05 M citrate buffer, and cellulase (8 FPU/g of substrate) was added to the pretreated substrate (2.5%, dry basis) in a total working volume of 50 mL. The flasks were incubated at 50 °C on an orbital shaker agitated at 150 rpm. Sample aliquots (2 mL) were taken periodically and centrifuged, and the supernatants were analyzed for reducing sugars. The percentage of saccharification was calculated as follows:

$$\%\ \text{saccharification} = \frac{\text{reducing sugars} \times 0.9}{\text{carbohydrates in substrate}} \times 100$$

Simultaneous Saccharification and Fermentation (SSF). SSF reaction mixtures contained alkaline peroxide pretreated substrate (previously autoclaved for 15 min at 121 °C), cellulase, 10% (v/v) yeast inoculum, and basal medium (Takagi et al., 1977) to make up the volume to 100 mL. The medium pH was adjusted to 5.1 with 0.05 M citrate buffer. Experiments were carried out in 250 mL flasks with 100 mL working volume. Reaction mixtures were incubated on an orbital shaker at 150 rpm. Samples were taken aseptically and assayed for ethanol content. Owing to low activities of β-glucosidase in *T. reesei* enzyme complex, it was supplemented in the reaction mixture at a concentration of 50 units/g of substrate.

Preparation of Yeast Inoculum. The yeast, *S. cerevisiae* NRRL-Y-132, was obtained from NCIM, National Chemical Laboratories, Pune, India. Inoculum was prepared by transferring the organisms maintained on MGYP (malt extract, glucose, yeast extract, peptone) medium into 100 mL of the basal medium (Takagi et al., 1977) having 4% (w/v) sugar, contained in a 500 mL flask. Growth was carried out at 30 °C on an orbital shaker for 10 h. The amount of inoculum added was 10% (v/v) of the SSF medium, which did not contain sugar but did contain cellulose.

Table 1. Experimental Design Showing Both Coded and Actual Values of Variables and Experimental and Predicted Responses[a]

sample	X_1	X_2	X_3	X_4	Y_{exp}	Y_{pred}
1	1 (100)	1 (15)	0 (48)	0 (40)	3.0	2.913
2	1 (100)	−1 (5)	0 (48)	0 (40)	1.8	1.829
3	−1 (20)	1 (15)	0 (48)	0 (40)	1.3	1.279
4	−1 (20)	−1 (5)	0 (48)	0 (40)	1.1	1.196
5	0 (60)	0 (10)	1 (72)	1 (45)	0.5	0.663
6	0 (60)	0 (10)	1 (72)	−1 (35)	1.9	1.863
7	0 (60)	0 (10)	−1 (24)	1 (45)	0.4	0.446
8	0 (60)	0 (10)	−1 (24)	−1 (35)	1.7	1.646
9	0 (60)	0 (10)	0 (48)	0 (40)	2.3	2.300
10	1 (100)	0 (10)	0 (48)	1 (45)	0.8	0.933
11	1 (100)	0 (10)	0 (48)	−1 (35)	2.0	2.133
12	−1 (20)	0 (10)	0 (48)	1 (45)	0.2	0.000
13	−1 (20)	0 (10)	0 (48)	−1 (35)	1.0	1.000
14	0 (60)	1 (15)	1 (72)	0 (40)	2.6	2.392
15	0 (60)	1 (15)	−1 (24)	0 (40)	2.1	2.175
16	0 (60)	−1 (5)	1 (72)	0 (40)	1.7	1.808
17	0 (60)	−1 (5)	−1 (24)	0 (40)	1.6	1.592
18	0 (60)	0 (10)	0 (48)	0 (40)	2.3	2.300
19	1 (100)	0 (10)	1 (72)	0 (40)	2.6	2.504
20	1 (100)	0 (10)	−1 (24)	0 (40)	2.4	2.288
21	−1 (20)	0 (10)	1 (72)	0 (40)	1.2	1.371
22	−1 (20)	0 (10)	−1 (24)	0 (40)	1.1	1.154
23	0 (60)	1 (15)	0 (48)	1 (45)	0.6	0.821
24	0 (60)	1 (15)	0 (48)	−1 (35)	1.9	2.021
25	0 (60)	−1 (5)	0 (48)	1 (45)	0.4	0.238
26	0 (60)	−1 (5)	0 (48)	−1 (35)	1.6	1.438
27	0 (60)	0 (10)	0 (48)	0 (40)	2.3	2.300

[a] Average absolute relative deviation = 11.58%. X_1 = cellulase concentration (FPU/g of substrate); X_2 = substrate concentration (% w/v); X_3 = incubation time (h); X_4 = temperature (°C). Y_{exp} and Y_{pred} are the responses (ethanol, % w/v) of experimental and predicted origin, respectively.

Analytical Techniques. Cellulose and hemicellulose in the dried substrate were estimated according to the gravimetric detergent fiber procedure of Goering and Van Soest (1970). Lignin and ash contents were determined by the trigol activated method (Edwards, 1973). Potential glucose and sugars in the substrate were determined after total hydrolysis with sulfuric acid. Residual cellulose after hydrolysis was measured by using the anthrone reagent method (Updegraff, 1969). Cellulase activity was assayed as filter paper units (FPU) (Mandels et al., 1976), and reducing sugars were estimated according to the dinitrosalicylic acid (DNS) method (Miller, 1959). Ethanol was estimated by gas chromatography (GC-15A; Shimadzu, Tokyo, Japan) in which a flame ionization detector and stainless steel column (2.0 m length, 3.0 mm i.d.) packed with Porapak-Q (50−80 mesh) were used. The column oven was operated isothermally at 150 °C, and the detector and injection port were kept at 170 °C. Nitrogen was used as carrier gas at a flow rate of 30 mL/min, and the combustion gas was a mixture of hydrogen and air. Ethanol was also estimated by using a chemical oxidation method (Caputi and Wright, 1969). Quantifications of ethanol by both GC analysis and the chemical method were found to be in good agreement, indicating the accuracy of the present results.

Experimental Design. A three-level four-factor fractional factorial design was adopted in this study (Box and Behnken, 1960; Shieh et al., 1995). The variables considered to be important [on the basis of our earlier papers: Hari Krishna et al. (1998, 1999)] in ethanol production were cellulase concentration (20−100 FPU/g of substrate), substrate concentration (5−15% w/v), incubation time (24−72 h), and temperature (35−45 °C). The independent variables (X_i) and their levels are presented in Table 1. To avoid bias, 27 runs were performed in a totally random order.

Statistical Analysis. The experimental data (Table 1) were analyzed according to the response surface regression procedure to fit the following second-order polynomial equation (SAS, 1990) in which the level of significance (P value) of all coefficients was <0.05:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_5X_1^2 + A_6X_2^2 + A_7X_3^2 + A_8X_4^2 + A_9X_1X_2 + A_{10}X_1X_3 + A_{11}X_1X_4 + A_{12}X_2X_3 + A_{13}X_2X_4 + A_{14}X_3X_4 \quad (1)$$

Y is the response (ethanol, % w/v), A_0 is the intercept, A_1-A_4 are the linear coefficients, A_5-A_8 are the quadratic coefficients, A_9-A_{14} are the cross-product coefficients, and X_i are the coded independent variables.

The regression analyses, statistical significances, and response surfaces were carried out using Microsoft Excel software (version 5.0; Microsoft Corp., Redmond, WA). Optimization of the reaction parameters for maximum ethanol yield were obtained through the software package Microsoft Excel–Solver program, which used Newton's search method.

RESULTS AND DISCUSSION

Evaluation of Saccharification Conditions. The dried substrate leaves, prior to pretreatment, contained 38.7% cellulose, 36.3% hemicellulose, 13.4% lignin, and 11.6% ash. Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment mainly because the lignin in plant cell walls forms a barrier against enzyme attack (Sewalt et al., 1997). An ideal pretreatment would reduce the lignin content and crystallinity of the cellulose and increase the surface area. In the present study, alkaline hydrogen peroxide pretreated substrate was employed as it was identified in our previous studies to be a suitable one (Hari Krishna et al., 1997, 1998, 1999), resulting in quantitative yields of reducing sugars. The alkaline peroxide pretreatment was effective in providing fractionation of the hemicellulose and lignin components and resulted in efficient hydrolysis.

The optimum reaction time was found to be 24 h, and extending the reaction time to ≥48 h had no significant effect on saccharification. A temperature of 50 °C was found to be optimum. However, in the range of 35–50 °C only a negligible decrease (maximum 8% for 35 °C) in sugar yields was observed (Hari Krishna et al., 1997). This result is useful for the SSF process, as high temperature is the limiting factor for yeast growth as well as ethanol yield.

Although pH 4.5 was optimum, the pH range 3.5–5.5 did not affect sugar yield significantly. The slight decrease in sugar yield at lower pH is favorable for SSF. It is known that the pH optimum for yeast growth is between 5.0 and 5.5, but during fermentation pH decreases even below 4.0. The results show that such changes in pH during fermentation cannot bring about a great effect on saccharification efficiency. Thus, an initial pH of 5.1 was selected for SSF as in the case of several SSF studies.

A cellulase concentration of 120 FPU/g of substrate was found to be optimum, but using a very high (120 FPU) cellulase concentration per gram of substrate is not economically feasible. It is also observed that the increase in enzyme content from 40 to 120 FPU increased the yield only by 10%. A substrate concentration of 2.5% (w/v) was found to be optimum for saccharification. An increase in the substrate concentration (5–25% w/v) limited the saccharification yield, due to difficulties in stirring and enzyme inhibition exerted by saccharification products. At the optimum conditions for saccharification (substrate = 2.5%, cellulase = 40 FPU/g of substrate, 50 °C, and pH 4.5), >90% saccharification was obtained.

Table 2. Values of Coefficients and ANOVA

Coefficients

coefficient	coefficient value	standard error	t stat	P value
A_0	2.3	0.08024	28.665	7.807 E−16
A_1	0.567	0.04012	14.125	8.005 E−11
A_2	0.292	0.04012	7.270	1.309 E−06
A_3	0.108	0.04012	2.700	0.0152
A_4	−0.6	0.04012	−14.956	3.245 E−11
A_5	−0.329	0.0602	−5.47	4.150 E−05
A_6	−0.167	0.06018	−2.77	0.01312
A_7	−0.142	0.06018	−2.354	0.0309
A_8	−1.004	0.06018	−16.687	5.643 E−12
A_9	0.25	0.0695	3.598	0.00222

ANOVA[a]

	degrees of freedom	sum of squares	mean sum of squares	F value	P value
regression	9	15.308	1.700885	88.066	1.77 E−12
residual	17	0.328	0.019314		
total	26	15.636			

[a] Coefficient of determination (R^2) = 0.979; standard error = 0.139. Description of variables as given for Table 1.

Optimization of SSF. On the basis of the saccharification results, alkaline H_2O_2 treatment, 20–100 FPU of cellulase/g of substrate, 5–15% (w/v) substrate, 5.1 initial pH, 35–45 °C, and 24–72 h were chosen as the conditions for optimizing the SSF process.

The coefficients of the response surface model as given in eq 1 were evaluated. Student's t test indicated that all of the linear terms, all of the quadratic terms, and only the enzyme–substrate interaction term were highly significant ($P < 0.05$). The analysis of variance (ANOVA) and the values of coefficients are presented in Table 2, which indicate that the model is highly significant as the F_{model} value (88.07) is very high compared to the tabular $F_{9,17}$ value (3.68 at $P = 0.01$). The coefficient of determination (R^2) of the model was 0.979, which further indicates that the model is suitable to adequately represent the real relationships among the selected reaction variables. The average absolute relative deviation was 11.58%. The final second-order polynomial predictive equation, after the elimination of insignificant terms from eq 1, is

$$Y = 2.3 + 0.567(X_1) + 0.2917(X_2) + 0.1083(X_3) - 0.6(X_4) - 0.3292(X_1^2) - 0.167(X_2^2) - 0.1417(X_3^2) - 1.0042(X_4^2) + 0.25(X_1X_2) \quad (2)$$

At the highest temperature (45 °C) with the lowest enzyme concentration (20 FPU/g of substrate) no ethanol yield was observed (Figure 1) when substrate and incubation time were fixed at 15% (w/v) and 72 h, respectively. With an increase in cellulase concentration, yields increased gradually even at the highest temperature. However, the increase was marginal at high temperature compared to the steep increase at moderate temperatures (35–40 °C). When observed in terms of temperature, yields increased with increase in temperature up to 40 °C and decreased thereafter at all cellulase concentrations. Maximum ethanol yield (3.0% w/v) was observed at a cellulase concentration of

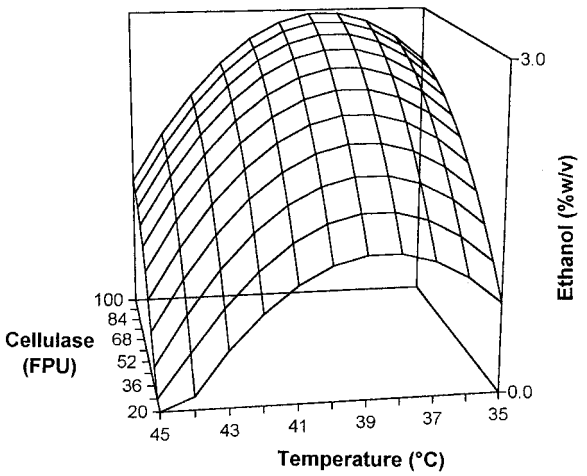

Figure 1. Response surface of ethanol production as a function of cellulase concentration and temperature at 15% (w/v) substrate and 72 h of incubation time.

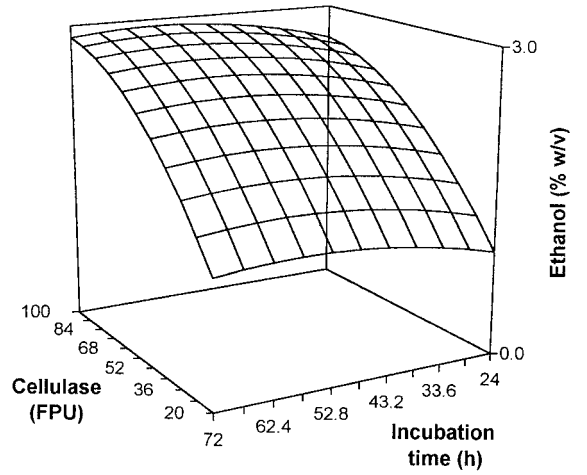

Figure 2. Response surface of ethanol production as a function of cellulase concentration and incubation time at 15% (w/v) substrate and 40 °C.

100 FPU/g of substrate and 39 °C, which was also verified to be true experimentally.

The effects of varying cellulase concentration and incubation time at constant substrate (15% w/v) and temperature (40 °C) are shown in Figure 2. Increases in cellulase concentration led to increased conversions irrespective of the incubation time. However, incubation time was found to have no significant impact on yields throughout the range of enzyme concentrations employed. This is an important finding as it was found that the long residence times added significantly to the capital and operating costs and, consequently, to the overall biomass to ethanol process (Nguyen and Saddler, 1991). The time course of ethanol production (not shown) indicated that the increase in ethanol concentration was linear up to 12−18 h, reaching a maximum at 24−48 h and remaining almost unchanged thereafter.

Figure 3 depicts the interactive effect of substrate concentration on enzyme requirement. Increases in enzyme loading resulted in improved conversions at all substrate concentrations used. However, at low substrate concentrations, an increase in cellulase concentration from 80 to 100 FPU/g of substrate showed saturation in yields. This could be due to the depletion of the substrate and the presence of excess enzyme. At low enzyme concentrations (20−44 FPU/g of substrate),

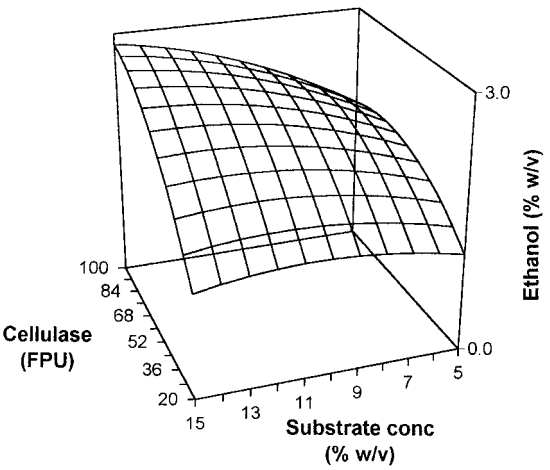

Figure 3. Response surface of ethanol production as a function of cellulase and substrate concentrations at 40 °C and 72 h of incubation time

increases in substrate concentration showed no significant improvement in terms of conversions. However, at high enzyme concentrations (50−100 FPU/g of substrate), increases in substrate concentration resulted in improved yields, the effect being more pronounced at a cellulase concentration of 100 FPU/g of substrate.

Temperature is a crucial factor for SSF because of the differences in saccharification optima (50 °C) and that of the yeast (35 °C). The saccharification rate was slow at 35 °C, but the ethanol yield was better. At 45 °C, ethanol production was low and a significant amount of sugars was found to remain unmetabolized. A temperature range of 39−40 °C was observed to be optimum for maximum ethanol yields. Although the optimum incubation time for saccharification was found to be between 24 and 48 h (Hari Krishna et al., 1997), incubation time has shown no significant effect on SSF, as can be seen from Figure 2. Therefore, cellulase and substrate concentrations were the most important variables for fermentative production of ethanol. Although an increase in substrate concentration decreased saccharification yield, it was not the same in the case of SSF, for which an increase in substrate concentration resulted in better ethanol production (Figure 3). This is evidently due to the removal of sugars (formed during saccharification) by the yeast.

The most efficient conditions for the present system could be to use economical quantities of enzyme to achieve maximum conversions at high substrate loading (>10%) in minimal incubation time (<72 h) at ambient temperature (<40 °C). For a given optimum temperature (38.5 °C; i.e., −0.3 coded level) and incubation time (57.2 h; i.e., 0.38 coded level) (found through the Solver program), the enzyme and substrate concentrations required to achieve a known extent of ethanol concentration can be calculated using eq 2. Figure 4 shows the contour plot predicting ethanol yields for different enzyme and substrate concentrations at a fixed temperature (38.5 °C) and incubation time (57.2 h). Whereas several combinations of enzyme and substrate concentrations were shown to give the same conversion, from an economic point of view it is desirable to choose the lowest possible enzyme concentration and highest possible substrate concentration from Figure 4.

The adequacy of the model was further examined at additional independent conditions that were not employed to generate the model. It was observed that the

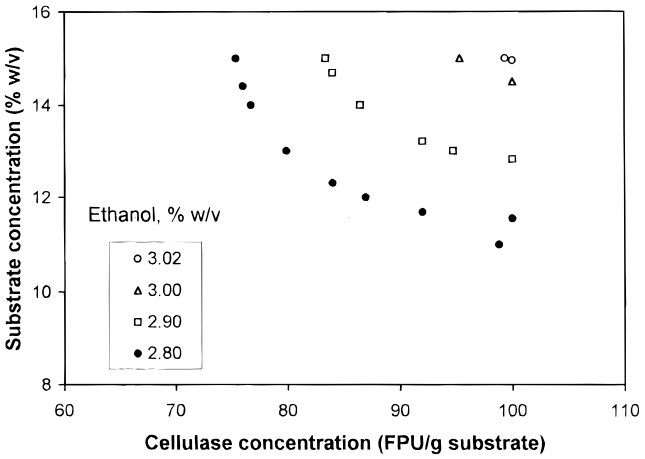

Figure 4. Contour plot showing the effects of cellulase and substrate concentrations to obtain maximum ethanol yields at 38.5 °C and 57.2 h of incubation time.

Table 3. Validation of the Model[a]

X_1	X_2	X_3	X_4	Y_{exp}	Y_{pred}
99.4	15.0	57.2	38.5	3.0	3.02
100.0	15.0	57.2	38.5	3.0	3.02
95.3	15.0	57.2	38.5	3.0	3.0
100.0	14.5	57.2	38.5	3.0	3.0
83.4	15.0	57.2	38.5	2.8	2.9
86.5	14.0	57.2	38.5	2.8	2.9
100.0	12.8	57.2	38.5	2.9	2.9
75.4	15.0	57.2	38.5	2.7	2.8
86.9	12.0	57.2	38.5	2.7	2.8
100.0	11.6	57.2	38.5	2.8	2.8

[a] Average absolute relative deviation = 1.5364%. Description of variables as given in Table 1.

experimental and predicted values of ethanol concentrations showed good correlation (Table 3). The optimum conditions predicted for synthesizing 3.02% (w/v) ethanol were as follows: cellulase = 100 FPU/g of substrate; substrate = 15% (w/v); incubation time = 57.2 h; and temperature = 38.5 °C. The actual experimental value obtained at these predicted conditions was 3.0% (w/v), which was in good agreement with the predicted value. At this ethanol concentration, its yield corresponded to 0.353 g of ethanol/g of substrate, which was calculated by dividing the ethanol produced (30 g/L) by the difference (85 g/L) between the cellulose degraded (135 g/L) and the total reducing sugars accumulated (50 g/L) at 90% saccharification.

In this context, Saddler et al. (1982) reported 2.0% (w/v) ethanol from 5% Solka floc or steam-exploded wood after 6 days using *T. reesei* cellulase and *S. cerevisiae* cells in an SSF system. Most of the other studies (Takagi et al., 1977; Deshpande et al., 1983; Spangler and Emert, 1986) on SSF reported the conversions took place for 4−6 days. In the previous studies, we reported ethanol concentrations in the range of 2.5−3.0% (w/v) from sugar cane leaves (Hari Krishna et al., 1998) and *A. leptopus* leaves (Hari Krishna et al., 1999). In these studies, we have evaluated the effects of saccharification parameters as applied to SSF and the effectiveness of SSF over saccharification and subsequent fermentation. It was identified that cellulase and substrate concentrations, incubation time, and temperature were the four important parameters affecting the SSF process, which were optimized in the present investigation to obtain high ethanol yields. In this work, conversion was completed in a shorter time mainly due to the fine microcrystalline cellulose that is present in the leaves of *A. leptopus*.

It was possible to obtain relatively high ethanol production efficiency by using optimized conditions together with the microcrystalline nature of the substrate. The presented results also show good compatibility of *T. reesei* cellulases with *S. cerevisiae* cells in the SSF system. It was also possible to convert substrate concentrations as high as 15% (w/v). An overall economic process (Bothast and Saha, 1997) must include achieving a high ethanol yield (≅3.5% w/v) at high substrate loading (>10% w/v) over short residence times (<4 days), most of which were achieved in the present study.

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Received for review November 30, 1999. Revised manuscript received March 3, 2000. Accepted March 8, 2000.

JF991296Z