BIOTECHNOLOGY LETTERS
Volume 14 No.5 (may 1992) pp.403-407
Received as revised 18th March

EVALUATION OF THE CELLOBIOSE-FERMENTING YEAST *Brettanomyces custersii* IN THE SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF CELLULOSE

Diane D. Spindler, Charles E. Wyman, Karel Grohmann[1], and George P. Philippidis*

Alternative Fuels Division
National Renewable Energy Laboratory (NREL)
Golden, CO 80401, USA

SUMMARY

Brettanomyces custersii (CBS 5512) was identified as a promising glucose- and cellobiose-fermenting yeast for the simultaneous saccharification and fermentation (SSF) of cellulose for ethanol production. In SSF studies with 75 g/L of cellulose, *B. custersii* produced 32 g/L of ethanol in just 3 days (75% of theoretical yield). This yield represents an increase of more than 16% over the yields of other fermentative yeasts and the time to achieve it is less than that with other organisms. In addition, the ethanol tolerance of *B. custersii* seems to be greater than that of other cellobiose-fermenting yeasts considered to date. Overall, the combination of higher yields, rates, and ethanol concentrations obtained with *B. custersii* improves the economics of ethanol production.

INTRODUCTION

The simultaneous saccharification and fermentation (SSF) process, which integrates cellulose hydrolysis to glucose with glucose fermentation to ethanol in a single step, enhances the kinetics and economics of cellulosic biomass conversion to ethanol (Takagi et al., 1977; Wright et al., 1988). During the SSF process, cellulose is hydrolyzed by the cellulase enzyme complex to cellobiose and eventually to glucose through the action of β-glucosidase. Glucose, in turn, provides a carbon/energy source for yeast cell growth and maintenance with concomitant production of ethanol and carbon dioxide. SSF requires less capital equipment than separate hydrolysis and fermentation, reduces the risk of contamination because of the presence of ethanol, and circumvents enzyme inhibition by hydrolysis products (cellobiose, glucose), resulting in improved ethanol yields, productivities, and associated economics.

Previous work in the areas of SSF and cellulase enzymes has allowed us to draw some conclusions regarding the choice of enzyme and yeast strain (Ghose, 1987; Gonde et al., 1984; Gosh et al., 1982; Howell, 1978; Lastick et al., 1984; Shoemaker, 1984; Spindler et al., 1988; Spindler et al., 1989a; Spindler et al., 1989b; Wyman et al., 1986). The proper choice of cellulase is critical to the performance of the SSF process, and a cellulase with well-balanced activities can result in improved SSF performance. In particular, the relative ratio of β-glucosidase activity in the cellulase mixture seems to affect ethanol yields and rates significantly (Gosh et al., 1982; Pemberton et al., 1980; Spindler et al., 1989b; Wyman et al., 1986). Supplementation of β-glucosidase reportedly increased the yields and rates of ethanol production significantly for *Saccharomyces cerevisiae*, but was not as beneficial when a co-culture of *S. cerevisiae*

[1] Present address: US Citrus and Subtropical Products, Box 1909, Winterhaven, Fl 33883-1909

and *Brettanomyces clausenii* was employed, presumably because of additional β-glucosidase activity provided by the cellobiose-fermenting yeast *B. clausenii*.

The employment of a cellobiose-fermenting yeast in SSF appears advantageous, because of the ability of the microorganism to ferment both cellobiose and glucose, the two cellulose hydrolysis products, into ethanol. Such yeast strains have been described in several reports (Cavazzoni and Adami, 1987; Freer and Detroy, 1983; Gonde et al., 1982; Lastick et al., 1983; Wyman et al., 1986). Based on the results reported in the literature, the most promising yeasts were selected for further analysis in the SSF process for conversion of lignocellulosic biomass to ethanol. In addition, glucose-fermenting yeasts were also tested. Although *B. custersii* strain CBS 5512 has been reported in the literature as a cellobiose-fermenting yeast (Blondin et al., 1982; Blondin et al., 1983; Gonde et al. 1982), it has not been evaluated to date in the SSF process.

MATERIALS AND METHODS

The glucose- and cellobiose-fermenting yeasts *Candida lusitaniae* Y-5394, *Hansenula glucozyma* CBS-5766, *H. holstii* CBS-4069, *Torulopsis molischiana* Y-2234, and *Brettanomyces clausenii* strains Y-1414, CBS-4460, CBS-4462, CBS-4711, CBS-4712, and CBS-1939 were obtained from the Northern Regional Research Laboratory, U.S. Department of Agriculture (Peoria, IL), whereas *Brettanomyces anomalus* 10559, *B. intermedia* 34448, and *B. custersii* 34447 (CBS 5512) were obtained from the American Type Culture Collection (Rockville, MD). The glucose-fermenting yeasts *Saccharomyces diastaticus* #62, *Saccharomyces cerevisiae* #67, and *Kluyveromyces marxianus* #1510 were from the Labatt Brewing Company Ltd (London, Ontario), while *S. cerevisiae* D_5A was derived through genetic improvements of commercial Red Star bakers yeast at NREL. Chemicals, glucose, cellobiose, α-cellulose, and Sigmacell-50 cellulose were purchased from Sigma Chemical Company (St. Louis, MO). Yeast extract and peptone were ordered from Difco, (Detroit, MI). The cellulase enzyme, Genencor 150L (batch II), was purchased from Genencor Inc. (San Francisco, CA).

Shake flask SSF studies were carried out in 250-mL flasks outfitted with stoppers constructed to vent CO_2 through a water trap. These flasks contained 100 mL of fermentation broth and were agitated at 150 rpm in a shaker incubator at 37°C. A 10 g/L yeast extract and 20 g/L peptone medium (YP) was used with a substrate loading of 75 g/L Sigmacell-50 cellulose. A lipid mixture of ergosterol (5 mg/L) and oleic acid (30 mg/L) was added to the media for improved ethanol yield (Janssens, 1983). Penicillin and streptomycin at 10 mg/L each were used to prevent bacterial contamination. The organisms were inoculated in shake flasks with YP medium and 20 g/L glucose at 37°C. A 10% (v/v) inoculum was used for the fermentations. Large-scale SSF experiments were run in 3-L Biostat V fermenters (B. Braun Instruments, Burlingham, CA) at a working volume of 1 L in YP medium supplemented with 100 g/L Sigmacell-50 cellulose as substrate. Enzyme loadings of 26 IU/g cellulosic substrate and 19 IU/g cellulosic substrate were employed for small- and large-scale SSFs, respectively (IU: International Units of filter paper activity; Ghose et al., 1987). Ethanol concentrations in the supernatant were measured by gas chromatography with a Porapak Q80/100 column using 4% isopropanol as internal standard.

RESULTS AND DISCUSSION

Initial screening experiments were conducted on the 13 glucose- and cellobiose-fermenting yeasts listed above with 150 g/L of cellobiose in order to identify the best ethanol producers. Of these 13 strains, five were identified as the most promising: *B. custersii*, *B. clausenii* Y-1414, *H. holstii*, *H. glucozyma*, and *T. molischiana* Y-2234. Table 1 outlines the concentrations of ethanol produced by the five yeasts. *B. custersii* exhibited an increased ethanol yield of 60.3 g/L (74.5% of the theoretical yield) from 150 g/L of cellobiose in 5 days at 37°C, compared to 51.4 g/L of ethanol by the best of the other strains.

Table 1. Production of ethanol by various cellobiose-fermenting yeast strains cultivated in 150 g/L of cellobiose or 200 g/L of glucose. Ethanol concentration is expressed in g/L.

Microorganism (Yeast Strain)	Cellobiose (150 g/L)			Glucose (200 g/L)		
	Fermentation Time (h)			Fermentation Time (h)		
	48	72	120	48	72	120
B. custersii	20.5	45.7	60.3	52.8	68.0	80.5
H. glucozyma	24.0	28.0	42.8	26.7	32.0	48.9
T. molischiana	25.9	31.6	51.4	24.6	29.2	40.8
B. clausenii Y-1414	10.9	20.5	38.7	18.4	48.8	63.0
H. holstii	17.5	21.7	38.5	10.4	20.8	39.5

The ethanol yields of glucose fermentations (200 g/L) by the same five cellobiose-fermenting yeasts are also given in Table 1. An ethanol concentration of 80.5 g/L (79% of theoretical yield) was realized by *B. custersii*, which represents a 20% or more increase over the other yeasts tested. Ethanol yields as high as 94 g/L from 200 g/L of glucose have been reported for *B. custersii* (Blondin et al., 1982; Gonde et al., 1982). Such elevated yields indicate that *B. custersii* has a high ethanol tolerance and could potentially be employed in high cellulose concentration SSF studies.

The SSF potential of several yeast strains was examined with 75 g/L of Sigmacell-50 cellulose at 37°C at a cellulase loading of 26 IU/g cellulose. The results show that *B. custersii* excelled both in yield and ethanol productivity (Figure 1). It completed the fermentation in just 3 days with an ethanol yield of 75% of theoretical (32 g/L), a more than 16% improvement over the other fermentative yeasts.

A temperature tolerance study was performed on *B. custersii* in 80 g/L of α-cellulose to determine the optimal temperature for cellulose bioconversion to ethanol. It was found that the rate of ethanol production by *B. custersii* reached a maximum between 38.5° and 39.5°C (data not shown). Although higher temperatures enhance the activity of the cellulase enzyme, which exhibits an optimum at about 45°C, the viability of the microorganism drops at temperatures above 40°C.

In earlier SSF studies, we had examined *B. clausenii* strains and found that strain Y-1414 excelled in conversion of Sigmacell-50 cellulose to ethanol (Lastick et al., 1983; Lastick et al., 1984). Continued evaluations of yeasts in the SSF process disclosed a mixed culture of *B. clausenii* Y-1414 and *S. cerevisiae* D_5A to be the best choice for high productivity and yield of ethanol (Spindler et al., 1989a; Spindler et al., 1989b). The performance of *B. custersii* was compared to that of the mixed culture in the presence of 100 g/L cellulose (Sigmacell-50) and 19 IU cellulase/g cellulose. The ethanol production and yield of *B. custersii* were identical to those of the mixed culture throughout the course of the SSF process (Figure 2). Both produced about 40 g/L of ethanol (70% of theoretical yield).

CONCLUSION

Brettanomyces custersii (CBS 5512) is a promising glucose- and cellobiose-fermenting yeast for cellulose conversion into ethanol by the SSF process. The *B. custersii* production rates and yields of ethanol are similar to those of a mixed culture of *S. cerevisiae* and *B. clausenii*, previously considered the

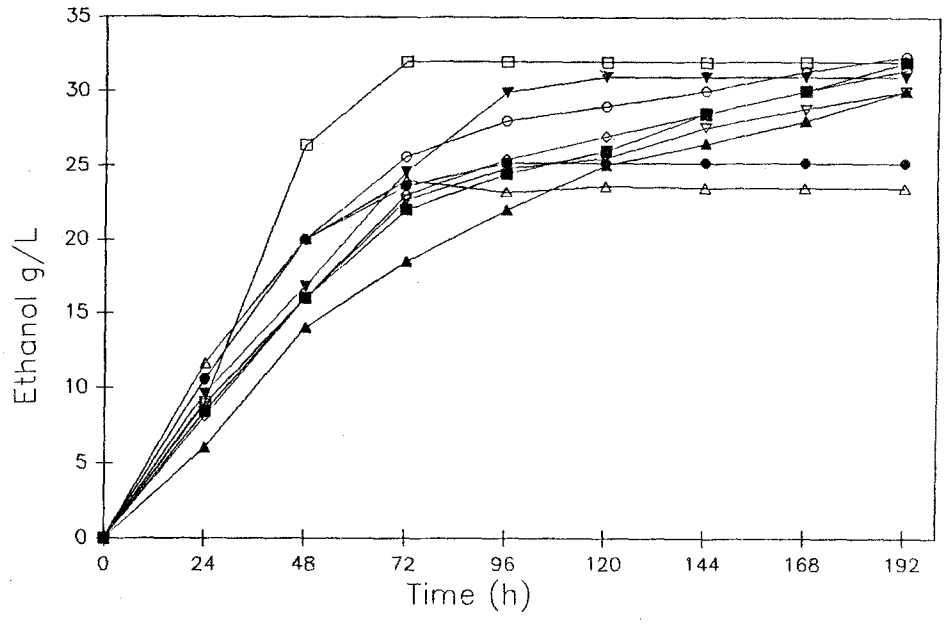

Figure 1. Comparison of ethanol production by various yeast strains during the SSF of 75 g/L Sigmacell-50 cellulose: (□) *B. custersii*; (○) *T. molischiana*; (•) *H. glucozyma*; (△) *H. holstii*; (▲) *B. anomalus*; (■) *S. diastaticus*; (▽) *S. cerevisiae* #67; (▼) *K. marxianus*; (◊) *S. cerevisiae* D_5A.

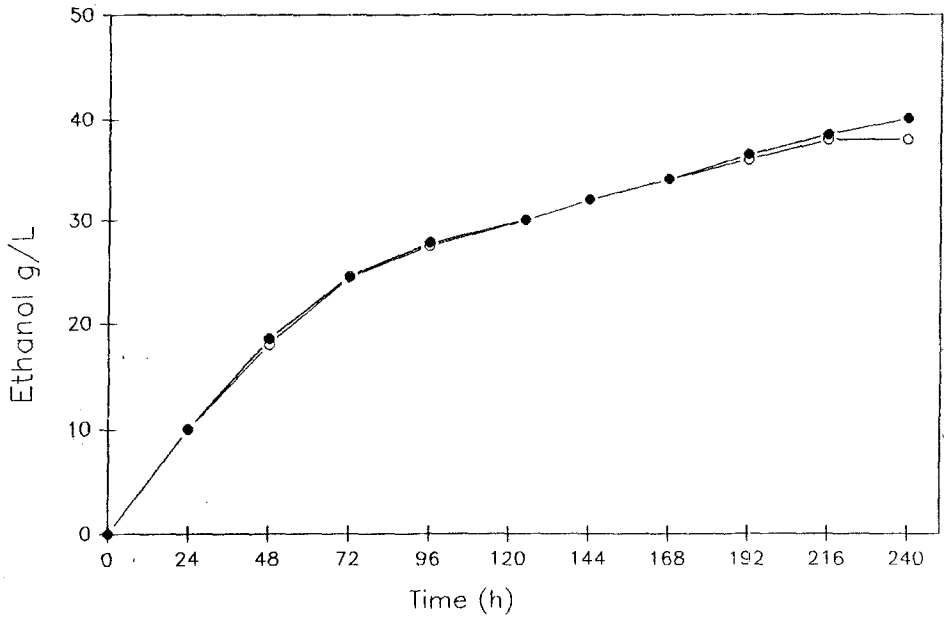

Figure 2. Comparison of ethanol production by *B. custersii* and a mixed culture of *S. cerevisiae* and *B. clausenii* during the SSF of 100 g/L Sigmacell-50 cellulose: (•) *B. custersii*; (○) Mixed culture.

frontrunner for ethanol production from cellulosic biomass. Substitution of the single organism, *B. custersii*, for the mixed culture will simplify the ethanol production process and facilitate control of the SSF unit operation.

REFERENCES

Blondin, B., Ratomahenina, R., Arnaud, A., and Galzy, P. (1982). *Biotechnol. Bioeng.* **25**, 2031.

Blondin, B., Ratomahenina, R., Arnaud, A., and Galzy, P. (1983). *Appl. Microbiol. Biotechnol.* **17**, 1.

Cavazzoni, V., and Adami, A. (1987). *Ann. Microbiol.* **37**, 127.

Freer, S.N. and Detroy, R.W. (1983). *Biotechnol. Bioeng.* **25**, 541.

Ghose, T.K. (1987), Biochemical Engineering Centre, Indian Institute of Technology, vol. 59, pp. 257-268 IUPAC.

Gosh, P., Pamment, N.B., and Martin, W.R.B. (1982). *Enzyme Microb. Technology* **4**, 425.

Gonde, P., Blondin, B., Ratomahenina, R., Arnaud, A., and Galzy, P. (1982). *J. Ferm. Technol.* **60**, 579.

Gonde, P., Blondin, B., Leclerc, M., Ratomahenina, R., Arnaud, R., and Galzy, P. (1984). *Appl. Environ. Microbiol.* **48**, 265.

Howell, J.A. (1978). *Biotechnol. Bioeng.* **20**, 847.

Janssens, J.H., Burris, N., Woodward, A., and Bailey, R.B. (1983). *Appl. Environ. Microbiol.*, **45**, 598.

Lastick, S.M., Spindler, D.D., and Grohmann, K. (1983). *Wood and Agricultural Residues*, J. Soltes, ed., p. 239.

Lastick, S.M., Spindler, D.D., Terrel, S., and Grohmann, K. (1984). *Biotech 84*, 277.

Pemberton, M.S., Brown, R.D., Jr., and Emert, G.H. (1980). *Can. J. Chem. Eng.* **58**, 723.

Shoemaker, S.P. (1984). *Biotech 84*, 593.

Spindler, D.D., Wyman, C.E., Mohagheghi, A., and Grohmann, K. (1988). *Appl. Biochem. Biotechnol.* **17**, 279.

Spindler, D.D., Wyman, C.E., and Grohmann, K. (1989a). *Biotechnol. Bioeng.* **34**, 189.

Spindler, D.D., Wyman, C.E., Grohmann, K., and Mohagheghi, A. (1989b). *Biotechnol. Bioeng.* **20/21**, 19.

Takagi, M., Abe, S., Suzuki, S., Emert, G.H., and Yata, N. (1977). *Bioconversion Symposium Proceedings*, IIT, Delhi, pp. 551-571.

Wright, J.D., Wyman, C.E., and Grohmann, K. (1988). *Appl. Biochem. Biotechnol.* **18**, 75.

Wyman, C.E., Spindler, D.D., Grohmann, K., and Lastick, S.M. (1986). *Biotechnol. Bioeng.* **17**, 221.