NOTE

Characterization of Cellobiose Conversion to Glucose and Ethanol by Immobilized *Candida wickerhamii*

G. ST. JULIAN, S. N. FREER, N. J. ALEXANDER, and R. J. BOTHAST[1]

Much research is currently being done on immobilized microbial cell technology. Such techniques are being used for production of antibiotics, enzymes, alcohol, and organic acids, denitrification and removal of heavy metals from waste water, and for electrode biological oxygen demand sensors (Kolot 1980, Hollo et al 1979, Kawabata and Demain 1979, Suzuki and Karube 1979, Karube et al 1977). Immobilization on an industrial scale is used significantly in three areas: production of high-fructose syrups (Chen 1980), amino acids (Chibata and Tosa 1976), and semisynthetic antibiotics (Carleysmith and Lilly 1979). Immobilized microbial cell techniques have the advantage of accelerated reaction rates owing to increased cell density, desirable product yield, fermentation on a continuous basis at a high dilution rate without washout, and less costly fermentor design and control, as compared with traditional fermentation techniques.

Freer (1985) completed a comprehensive study involving the purification and characterization of β-glucosidase produced by *Candida wickerhamii* growing in complex media, and further described the regulation of the enzyme's expression by the yeast (Freer and Detroy 1985). In part, those data showed that *C. wickerhamii* utilizes cellobiose by its production of β-glucosidase, that the enzyme has features that differentiate it from β-glucosidases of other yeasts and filamentous fungi, and that β-glucosidase activity produced by *C. wickerhamii* varies dramatically when cells are grown under different physiological conditions. Contrary to several other β-glucosidase producers, *C. wickerhamii* enzyme is not inhibited by substrate (cellobiose) or product (glucose) (Kilian et al 1983). The uniqueness of *C. wickerhamii*'s β-glucosidase prompted us to try to develop a stable environment in which intact, nongrowing yeast cells could be used to convert cellobiose to glucose and ethanol. This paper describes the conversion of cellobiose (9.0% aqueous solution) to glucose and ethanol by calcium-alginate-immobilized *C. wickerhamii* in a continuous-flow fermentation system.

MATERIALS AND METHODS

Microorganism and Culture Conditions

C. wickerhamii NRRL Y-2563 was obtained from the Agricultural Research Service Culture Collection (NRRL) and maintained in liquid YMG medium (pH 6.8), which contains (per liter) 5 g of yeast extract, 5 g of malt extract, 5 g of peptone, and 20 g of glucose. YMG medium and all other substrates (cellobiose feed solutions) were sterilized in an autoclave for 15 min at 121° C (15 lb/in^2). The yeast cells were grown in 2.8-L Fernbach flasks containing 1 L of YMG medium and were incubated at 28–30° C on a rotary shaker (200 rpm); a 10% (v/v) 16–18 hr-old exponential-growth-phase yeast culture was used as the inoculum. *C. wickerhamii* was incubated for four days, and then harvested by centrifugation at 1,800 × *g*.

Immobilization of Yeast Cells

Detailed explanation of the techniques used to immobilize *C. wickerhamii* can be found in the paper by McGhee et al (1982). Except in the research shown here, 20 g (wet wt paste) of 96-hr-old yeast cells were encapsulated in Ca-alginate beads.

Fermentation

The continuous-flow fermentor system used in these experiments was modified from that of McGhee et al (1982). The system, shown in Figure 1, consists of a reservoir (500-ml separatory funnel), a Multistaltic pump (Buchler Instruments), a B-D plastic syringe (2.5-cm inner diameter; 30-cm length), and a collecting flask (500-ml side-arm Erlenmeyer flask). In operation, the cellobiose feed solution was pumped from the reservoir into the bottom of the syringe through the bed of yeast cells immobilized in alginate beads and finally into the collecting flask. The syringe contained approximately 1,000 alginate beads. The cellobiose feed solution was pumped through the column at a rate of 2.0 ml/hr with a dilution rate of 0.066/hr. In full operation, the syringe containing the beads had a free liquid volume capacity of 30 ml. The fermentation time began as soon as the syringe was filled with beads and aqueous cellobiose solution.

Analysis

Ethanol concentrations were determined by gas-liquid chromatography. Glucose and residual cellobiose in the product solution were determined by the high-pressure chromatography procedures of Black and Bagley (1978). The β-1,4-glucosidase activity was measured by the method described by Freer and Detroy (1983). In separate experiments, the β-1,4-glucosidase content of *C. wickerhamii* cells was measured before their encapsulation, immediately after encapsulation, and on days 1, 2, 6, and 15 of the continuous-flow fermentation. The cells in each fermentor were examined for β-1,4-glucosidase content. The

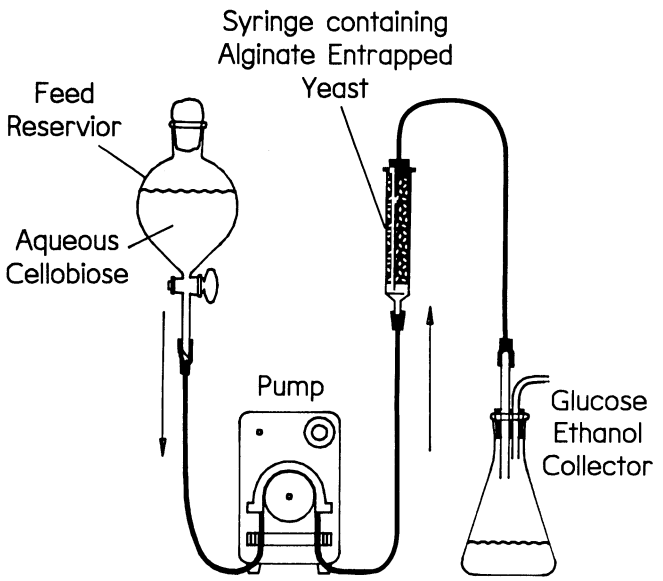

Fig. 1. The continuous-flow fermentor system for cellobiose fermentation to glucose and ethanol via calcium-alginate entrapped *Candida wickerhamii*.

[1]Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

TABLE I
Continuous-Flow[a] Conversion of Cellobiose (90 g/L sol) to Glucose and Ethanol by Immobilized[b] *Candida wickerhamii* NRRL Y-2563

Fermentation Age (days)[c]	Total Cellobiose Recovered[d] (g/L)	Total Free Glucose Recovered[e] (g/L)	Total Ethanol Recovered (g/L)
1	6	10	34
2	34	4	25
3–14	67	1	11
15	90	0	0

[a] Flow rate was 2 ml/hr (dilution rate 0.066/hr).
[b] *C. wickerhamii* cells (20 g, wet wt) were entrapped in calcium-alginate beads.
[c] Total product accumulation was analyzed daily.
[d] All data represent averages from at least six separate sets of experiments. Experimental variation was 0.001–0.05 g/L.
[e] Theoretical ethanol yield is 0.51 g per gram of glucose.

β-1,4-glucosidase content of the 20 g (wet wt) of free yeast cells averaged 21.83 units (U)/ml (range 16.1–27.9 U/ml). After 20 g of cells was entrapped in the Ca-alginate gel and subsequently dissolved away from the gel, the enzyme content remained nearly the same (16–27 U/ml). Cellobiose, glucose, and ethanol analyses were made daily.

RESULTS AND DISCUSSION

A high initial cellobiose conversion was followed by several days of relatively less conversion, and ended after 14 days. As expected, the duration of each fermentation was dependent on the available β-1,4-glucosidase. The β-1,4-glucosidase content in the 9% cellobiose reactor was an average of 10.5 U/ml on day 1. The enzyme content on day 2 was about 5.6 U/ml, and on day 6 it was 3.5 U/ml. In none of the experiments could β-1,4-glucosidase be detected at the time the conversions ceased. Apparently, the rapid conversion of cellobiose during the first fermentation day results from the initial abundant supply of β-1,4-glucosidase. On subsequent days, in each of the fermentations, as the quantity of the enzyme diminished less cellobiose was converted. As mentioned earlier, the immobilized yeast is not growing and, therefore, incapable of producing additional β-1,4-glucosidase. Why the enzyme falls apart or loses its activity is unknown.

In each of the experiments, a one-day lag period existed between the glucose production and its subsequent fermentation to ethanol. Such a lag has been described by Lee and Woodward (1983) to be caused by the formation of a significant concentration gradient between the bulk feed solution and the cellobiose within the Ca-alginate bead. As can be seen in Table I, only about 86% of the total carbohydrate was recovered on day 1 of the conversion. We assume that the cellobiose was adsorbed or utilized by the yeast cells in some unknown manner. On day 2 and days 3 through 14, about 96 and 100% of the carbohydrate could be accounted for, respectively. Several concentrations of cellobiose (not shown here), ranging from 1 to 15%, gave similar results as those shown in Table I.

Apparently, the rate and extent of cellobiose conversion is dependent upon the amount of active β-glucosidase present in the reactor. To improve the performance of the reactor, a method by which immobilized yeast could be induced to continuously produce β-glucosidase, or a means to stabilize the enzyme initially present in the reactor would be desirable.

ACKNOWLEDGMENTS

We extend a special thanks to Renee I. Seppa and W. L. Orton for their excellent technical assistance.

LITERATURE CITED

BLACK, L. T., and BAGLEY, E. B. 1978. Determination of oligosaccharides in soybeans by high pressure liquid chromatography using internal standards. J. Am. Oil Chem. Soc. 55:228.

CARLEYSMITH, S. W., and LILLY, M. D. 1979. Deacylation of benzylpenicillin by immobilized penicillin acylase in a continuous four-stage stirred tank reactor. Biotechnol. Bioeng. 21:1057.

CHEN, W. P. 1980. Production of high fructose syrups. Process Biochem. 20:30.

CHIBATA, I., and TOSA, T. 1976. Industrial applications of immobilized enzymes and immobilized microbial cells. Appl. Biochem. Bioeng. 1:329.

FREER, S. N. 1985. Purification and characterization of the extracellular β-glucosidase produced by *Candida wickerhamii*. Arch. Biochem. Biophys. 243:515.

FREER, S. N., and DETROY, R. W. 1983. Characterization of cellobiose fermentation to ethanol by yeasts. Biotechnol. Bioeng. 25:541.

FREER, S. N., and DETROY, R. W. 1985. Regulation of β-1,4-glucosidase expression by *Candida wickerhamii*. Appl. Environ. Microbiol. 50:152.

HOLLO, J. T., TENGERDY, R. P., and JOHNSON, J. E. 1979. Denitrification and removal of heavy metals from waste water by immobilized microorganisms. ACS Symp. Ser. 106:73.

KOLOT, F. B. 1980. New trends in yeast technology immobilized cells. Process Biochem. 15:2.

KAWABATA, Y., and DEMAIN, A. L. 1979. Enzymatic synthesis of pantothenic acid by *Escherichia coli* cells. ACS Symp. Ser. 106:133.

KARUBE, I., MATSUNAGA, T., MITSUDA, S., and SUZUKI, S. 1977. Microbial electrode BOD sensors. Biotechnol. Bioeng. 19:1535.

KILIAN, S. G., PRIOR, B. A., and LATEGAN, P. M. 1983. Diauxic utilization of glucose-cellobiose mixtures by *Candida wickerhamii*. Eur. J. Appl. Microbiol. Biotechnol. 18:369.

LEE, J. M., and WOODWARD, J. 1983. Properties and application to immobilized β-D-glucosidase coentrapped with *Zymomonas mobilis* in calcium-alginate. Biotechnol. Bioeng. 25:2441.

McGHEE, J. E., ST. JULIAN, G., DETROY, R. W., and BOTHAST, R. J. 1982. Ethanol production by immobilized *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, and *Zymomonas mobilis*. Biotechnol. Bioeng. 24:1155.

SUZUKI, S., and KARUBE, I. 1979. Production of antibiotics and enzymes by immobilized whole cells. ACS Symp. Ser. 106:59.

[Received February 26, 1987. Revision received August 12, 1987. Accepted August 14, 1987.]