©Copyright 1990 by The Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/90/2302—0171$02.00

Ethanol Production from Jerusalem Artichoke by Inulinase and *Zymomonas mobilis*

C. H. KIM AND S. K. RHEE*

Genetic Engineering Center, Korea Institute of Science and Technology, PO Box 131, Dongdaemun, Seoul, Korea

Received May 10, 1989; Accepted July 21, 1989

ABSTRACT

Ethanol production from Jerusalem artichoke was studied using inulinase and Z. *mobilis* by simultaneous saccharification and fermentation (SSF) process. The SSF process showed higher ethanol yield and productivity than the acid or enzymatic prehydrolyzed two-step process. The optimum temperature and inulinase concentration for SSF were 35°C and 0.25% (v/w, 4.4 units/g of sugar), respectively. In order to operate the SSF process in a continuous mode, inulinase and Z. *mobilis* cells were coimmobilized in alginate beads, using chitin as a matrix for enzyme immobilization. The maximum ethanol productivity of the continuous SSF process was 55.1 g/L/h, with 55% conversion yield. At the conversion yield of 90%, the productivity was 32.7 g/L/h. The continuous SSF system could be operated stably over 2 wk with an ethanol concentration of 48.6 g/L (95% of theoretical yield).

Index Entries: Jerusalem artichoke; inulinase; *Zymomonas mobilis;* ethanol fermentation; simultaneous saccharification and fermentation.

INTRODUCTION

Among the various sources of biomass, Jerusalem artichoke (JA) has drawn much attention in recent years as a potential energy crop. JA possesses several agricultural advantages, including significantly high carbohydrate yields (1000–13,600 lb/acre/y), tolerance to frost and plant diseases,

*Author to whom all correspondence and reprint requests should be addressed.

and no or little fertilizer requirement (*1*). The major carbohydrate of JA tuber, inulin, can be hydrolyzed to free sugars by mild acid or an enzyme, inulinase (EC 3.2.1.7), treatment (*2-6*). The conventional techniques to produce ethanol from JA consist of the acid or enzymatic hydrolysis of inulin, followed by fermentation of the resulting free sugars into ethanol (*7*). These processes, however, have some disadvantages, including byproduct formation, product inhibition during hydrolysis, and subsequently, high production cost. Although some yeast strains with inulinase activity were used to convert inulin to ethanol directly (*8-12*), the yeast strains had shown low ethanol yield and fermentation rate owing to the weak inulinase activity.

Zymomonas mobilis, a gram negative bacterium, was proven as a good alternative to yeast strains (*13,14*). Since *Zymomonas* strains, however, can not dissimilate inulin directly as a carbon source, inulin should be hydrolyzed to fructose and glucose prior to fermentation. In the present investigation, these problems could be overcome by employing the simultaneous saccharification and ethanol fermentation (SSF) process. We immobilized inulinase and Z. *mobilis* cells separately or coimmobilized the enzyme and cells to evaluate various SSF processes for the one-step production of ethanol from JA tubers.

MATERIALS AND METHODS

Microorganism and Culture Media

The bacterial strain used in this study was Z. *mobilis* strain ZM4 (ATCC 31821). It was cultured and maintained in a liquid medium, as previously described (*6*).

Preparation of Jerusalem Artichoke Juice

The JA tubers were washed, sliced, and crushed out to homogenized juice with a blender. The acid hydrolysis was carried out at pH 1.5 with concentrated H_2SO_4, and enzymatic hydrolysis was carried out at pH 4.5 and 60°C for 48 h. The pHs of the crude and hydrolyzed juice were adjusted to 5.0 using concentrated H_2SO_4. The resulting juice was autoclaved at 121°C for 15 min and filtered through tissue papers (Kimwipe).

Enzyme and Chemicals

A commercial inulinase preparation from *Aspergillus ficuum* was supplied by Novo Industri A/S (Seoul, Korea). Sodium alginate was from Junsei Chemical Co. (Tokyo, Japan). Chitin (powder type) from crab shells, inulin from dahlia, and glutaraldehyde were obtained from Sigma Chemical Co. (St. Louis, MO).

Immobilization Procedure

The procedure of immobilization of *Z. mobilis* cells in sodium alginate gels was described previously (*15*). Inulinase and *Z. mobilis* cells were coimmobilized by two different methods: (1) colloidal chitin was prepared using the procedure of Hsu et al. (*16*) and used for coimmobilization. The enzyme solution was added to the chitin with 3% glutaraldehyde in 0.1*M* acetate buffer, pH 5.0 and allowed to stand at room temperature for 1 h, followed by standing overnight at 4°C. The resulting products were washed with distilled water and 0.1*M* acetate buffer, pH 5.0; and (2) colloidal chitin was treated with 3% glutaraldehyde at room temperature for overnight and washed with 0.1*M* acetate buffer, pH 5.0. The enzyme solution was added to the chitin treated with glutaraldehyde and allowed to stand at room temperature for 1 h, followed by standing overnight at 4°C. The resulting products were used to coimmobilize without washing.

Exponentially growing *Z. mobilis* cells (6 g dry cell weight) were harvested by centrifugation and resuspended with the inulinase immobilized on chitin in 50 mL of physiological saline. The suspension was mixed with 50 mL of 4% sodium alginate solution. The mixture was then added dropwise using a syringe pump (Cole-Parmer, Chicago, IL) equipped with 10 mL syringe to 0.05*M* $CaCl_2$ solution with continuous stirring. Beads of 3–4 mm diameter were formed in this solution and stored at 4°C.

Batch and Continuous Fermentation

The batch fermentation was carried out in a 1 L Bioflow fermentor (New Brunswick Scientific Co., Edison, NJ) using 400 mL of JA juice containing 154.2 g/L of total carbohydrate at pH 5.0. The continuous fermentation was performed in a water-jacketed packed bed reactor of 2.2 cm diameter and 40 cm long using 100 g/L of JA juice at pH 5.0 and 35°C. The dilution rate and ethanol productivity were calculated on the void volume basis of the reactor. The void volume of the reactor was 23 mL and corresponded to 26% of the total working volume.

ANALYTICAL METHODS

Total sugar, reducing sugar, and glucose were determined by the Anthrone method (*17*), dinitrosalicylic acid method (*18*), and glucose oxidase—peroxidase method (*19*), respectively. D-Fructose was determined as the difference between the amount of total reducing sugars and D-glucose (*20*). Ethanol was determined by gas chromatography (*15*).

Inulinase activity was assayed by the following procedure. The reaction mixture, composed of 100 mL of 2.5% inulin solution (dissolved in 0.1*M* acetate buffer, pH 5.0) and a proper portion of soluble or immobilized enzyme preparations, was incubated at 50°C for 20 min. One unit of inu-

linase was defined as the amount of enzyme that liberates 1 μmol reducing sugar/min at 50°C and pH 5.0.

RESULTS AND DISCUSSION

Immobilization of Inulinase

In order to increase the operational efficiency, the coimmobilization of inulinase and *Z. mobilis* cells was tried. For coimmobilization, the enzyme was immobilized on chitin before entrapment in alginate gel beads with cells. Glutaraldehyde was used as a crosslinking agent to provide both chitin and inulinase with active aldehyde groups. When the inulinase was immobilized on chitin with a simultaneous treatment of 3% glutaraldehyde, the activity retained about 26% of the total activity of the soluble enzyme initially added. The remaining activity was decreased to only 9.4% when the enzyme was entrapped with *Z. mobilis* cells into alginate gel beads. However, the activity retention of the enzyme was increased to 16.4% in cases that the enzyme was immobilized on the chitin, which was pretreated with 3% glutaraldehyde (Table 1). This increase in the activity retention is likely owing to the differences in preparation of the immobilized inulinase on chitin. In the first method, a large portion of the enzyme was removed together with the excess glutaraldehyde during the washing process. In contrast, the glutaraldehyde washing was carried out before the immobilization in the second method to entrap more enzyme molecules.

Effect of the Hydrolysis Process on Ethanol Fermentation from JA

The results of ethanol fermentation, using acid or enzymatic hydrolyzed JA juice, are shown in Table 2, compared with the simultaneous saccharification of inulin and ethanol fermentation. The total carbohydrate content of the jucie used was 154.2 g/L. In the prehydrolysis process, the degrees of hydrolysis were 81.2 and 92.5% for acid and enzymatic hydrolysis, respectively. Both fermentations were almost completed in 24 h at 30°C by employing free *Z. mobilis* cells.

The reducing sugars produced by hydrolysis were converted to ethanol with a similar yield in both cases. Although the degree of acid hydrolysis could be increased by an increase of reaction time (*6,7*), this resulted in the formation of byproducts that may inhibit growth of cells and ethanol fermentation (*8*). Consequently, it was believed that enzymatic hydrolysis was more desirable method than acid hydrolysis under these conditions. The addtion of yeast extract (10 g/L) did not influence the kinetics and yield of ethanol fermentation, indicating that the juice from JA tuber contains almost all nutrients needed for *Z. mobilis* cells for ethanol fermentation. The SSF was carried out using JA juice containing

Table 1
Immobilization of Inulinase

		Method I[a]		Method II[b]	
	Initial Activity	Activity on chitin	Activity in beads	Activity on chitin	Activity in beads
Activity, unit	1683	434.2	157.5	745.7	275.9
Activity retention, %	100	25.8	9.4	44.3	16.4

[a]Inulinase was immobilized on chitin with 3% glutaraldehyde, washed with acetate buffer, and then entrapped into alginate gel beads with dead cells of *Z. mobilis*.
[b]Chitin was treated with 3% glutaraldehyde and washed with acetate buffer, and then inulinase was added and entrapped into alginate beads with dead cells of *Z. mobilis*.

Table 2
Effect of Hydrolysis Process on Ethanol Fermentation

System	Ethanol Concentration, g/L	Ethanol Yield,[a] %
Prehydrolysis Process		
Acid Hydrolysis	61.5	78.0 (96.1)[b]
Enzymatic Hydrolysis		
Yeast Extract, 10 g/L	69.6	88.3 (95.5)[b]
None	69.7	88.5 (95.6)[b]
Simultaneous Saccharification and Fermentation Process	73.1	92.8

[a]Ethanol yield was calculated on the total carbohydrate basis.
[b]Ethanol yield was calculated on the reducing sugar basis, considering the hydrolysis yields.

154.2 g/L of total sugar, 0.25% (v/w, 4.4 U/g sugar) of inulinase and free *Z. mobilis* cells (5% inoculum size) at pH 5.0 and 30°C. The SSF process showed higher ethanol and cell mass yield than obtained in the prehydrolysis process. This result is similar to that reported previously in an SSF using starch as a substrate (*21*). The SSF process seems to eliminate the product inhibition by rapidly utilizing reducing sugars being produced.

Optimization of Operating Conditions for SSF Process

Since the optimum reaction temperature of inulinase from *Aspergillus ficuum* lies at approximately 60 and 65°C (*22*), whereas at 30 and 35°C for ethanol fermentation by *Z. mobilis* (*23*), it is important to optimize the operating temperature in the first place. To optimize the operating temperature for the SSF process, the experiment was initiated using JA juice (154.2 g/L), 0.25% (v/w) of inulinase and exponentially growing *Z. mobilis* cells. The kinetics of ethanol fermentation at various temperatures are shown in Fig. 1. The most beneficial result was obtained at 35°C with 96% ethanol yield.

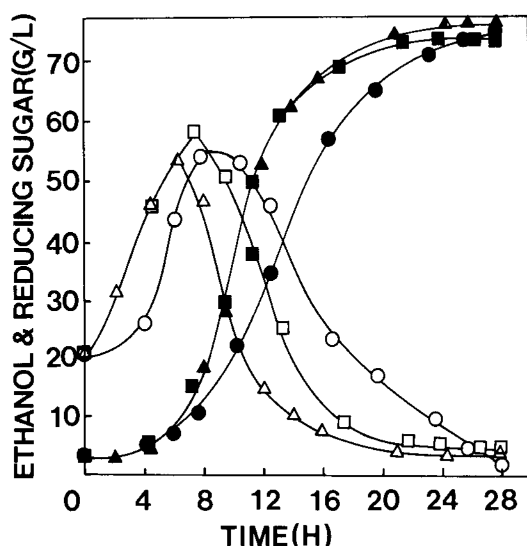

Fig. 1. Effect of temperature on SSF process using inulinase and Z. *mobilis*. Closed symbols; ethanol, open symbols, reducing sugars. ●—●, 30°C; ▲—▲, 35°C; and ■—■, 37.5°C.

Table 3
Effect of Enzyme Dosage on Hydrolysis and SSF Process Using JA Juice (154.2 g/L) at 35°C

Enzyme dosage, %, v/w	Hydrolysis yield,[a] %	Final ethanol concentration, g/L	Ethanol yield, %	Ethanol productivity,[a] g/L/h
0.10	39.8	62.3	79.1	2.6
0.15	42.4	73.0	93.9	3.0
0.20	52.0	75.6	95.9	3.5
0.25	57.7	76.0	96.4	3.7
0.30	58.6	76.1	96.6	3.7

[a]Hydrolysis yield and ethanol productivity were calculated on the basis of hydrolysis and fermentation for 29 and 20 h, respectively.

The effect of enzyme dosage on hydrolysis and SSF process with JA juice (154.2 g/L) at 35°C are also examined (Table 3). The enzyme concentration was varied in the range from 0.1 to 0.3% (v/w) at pH 5.0. The degree of hydrolysis increased as inulinase concentration became higher. However, the hydrolysis progressed very slowly at this temperature (35°C) and attained only 58.6% in 29 h with 0.3% (v/w) of inulinase. It should be because of the product inhibition by the fructose and glucose produced. In the SSF process, the higher ethanol yield of more than 95% of theoreti-

Table 4
Comparison of Various SSF Processes in Batch Fermentation

System	Final ethanol concentration, g/L	Ethanol yield, %	Ethanol productivity,[a] g/L/h
Free enzyme-cell system	76.6	97.2	3.7
Separately immobilized system	74.0	93.9	3.6
Coimmobilized system	72.0	91.4	3.5

[a]Ethanol productivity was calculated on the basis of fermentation for 20 h.

cal yield could be obtained in the enzyme concentrations above 0.2% (v/w). The fermentation was nearly completed in 24 h, in most cases, except with the low enzyme dosages of 0.1 and 0.15% (v/w). Considering the ethanol yield and productivity, the optimal conditions for SSF process were selected as the temperature of 35°C and inulinase concentration of 0.25% in the present study.

Comparison of Batch SSF Processes

Various SSF processes, using free enzyme and cells, immobilized enzyme and cells, and coimmobilized enzyme and cells, were compared batchwise at 35°C and pH 5.0 with 154.2 g/L JA juice supplemented with 0.3% $CaCl_2$ as an alginate gel stabilizer. For SSF using a separately immobilized system, the inulinase was immobilized on chitin (434.2 U/5 g of chitin) and the *Z. mobilis* cells were immobilized in sodium alginate beads. For the SSF using a coimmobilized system, the inulinase immobilized on chitin was entrapped in sodium alginate gel beads with *Z. mobilis* cells. As shown in Table 4, ethanol yield and productivity were slightly higher in free enzyme-cells system than others. The decrease in the fermentation rate, resulting in lower productivity in the batch SSF, was likely owing to the rate-limiting of hydrolysis reaction caused by mass transfer limitation between the biocatalysts and substrates (*24,25*). If the recovery of biocatalysts and operational facilities of continuous fermentation are taken into consideration, it is plausible that the coimmobilization system has many advantages over the other systems.

Continuous SSF Using Coimmobilized System

The continuous SSF process by coimmobilized inulinase and *Z. mobilis* cells was carried out in a packed-bed column reactor at 35°C and pH 5.0 (Fig. 2). The reactor was packed with 65 mL of sodium alginate beads containing 92 g cells and 4245 U of inulinase activity/L of beads, which were

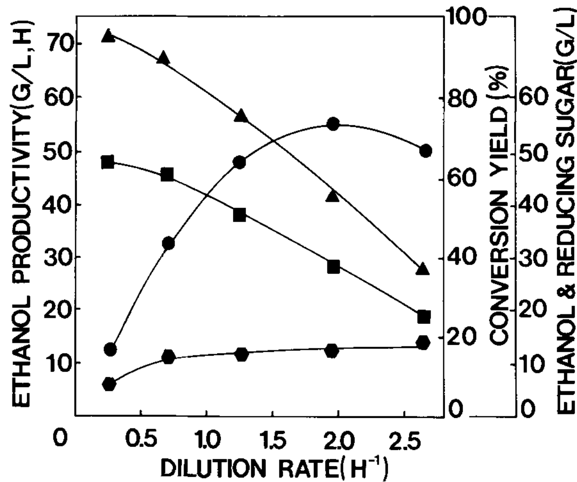

Fig. 2. Continuous SSF using coimmobilized system at various dilution rates. ▲—▲, conversion yield; ■—■, ethanol; ●—●, productivity; and ⬢—⬢, reducing sugar.

equivalent to 68 g cells and 3136 U of enzyme activity/L of total reactor volume. The 10% JA juice, containing 0.3% $CaCl_2$ as gel stabilizer, was fed into the column.

The maximum ethanol productivity of 55.1 g/L/h was obtained at a dilution rate, $D=1.96$/h with 55% conversion yield. The productivity was decreased to 32.7 g/L/h with an increased conversion yield of 90%. On the basis of liquid phase mean residence time, the maximum ethanol productivity reached 104 g/L/h, which is higher than previously reported for the yeast *Kluyveromyces marxianus* (*10*).

In order to assess the operational stability of the continuous SSF, using coimmobilized inulinase and *Z. mobilis* cells, the reactor containing 65 mL of alginate gel was operated at a fixed dilution rate ($D=0.26$/h) using 100 g/L JA juice supplemented with 0.3% $CaCl_2$. Figure 3 shows the ethanol concentration monitored from the exit line. Relatively high conversion to ethanol (average 48.6 g/L) could be maintained stably longer than 14 d without causing serious problems, such as disruption of beads or pressure drop of the reactor.

CONCLUSIONS

The direct conversion of inulin into ethanol by inulinase and *Z. mobilis* could be achieved in various one-step batch and continuous SSF processes. Among these, the continuous SSF process using coimmobilized inulinase and *Z. mobilis* was particularly interesting in terms of ethanol productivity and operational stability.

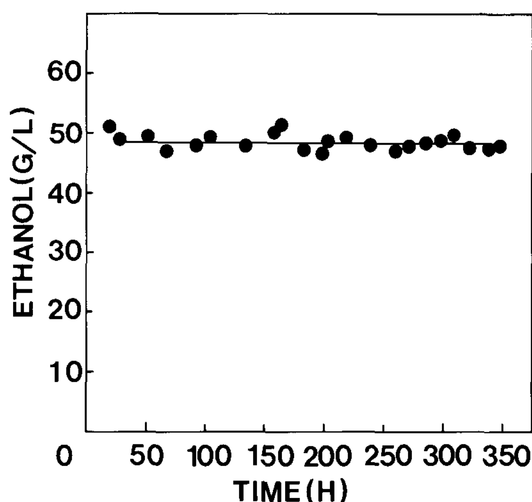

Fig. 3. Operational stability of continuous SSF using coimmobilized inulinase and *Z. mobilis.*

REFERENCES

1. Fleming, S. E. and GrootWassink, J. W. D. (1979), *CRC Crit. Rev. Food Sci. Nutr.* **12,** 1.
2. Sachs, R. M., Low, C. B., Vasavada, A., Sully, M. J., Williams, L. A., and Ziobro, G. (1981), *Calif. Agric.* **35,** 4.
3. Snyder, H. E. and Phaff, H. J. (1981), *J. Biol. Chem.* **237,** 4.
4. Duvnjak, Z., Kosaric, N., Kliza, S., and Hayes, D. (1982), *Biotechnol. Bioeng.* **24,** 2297.
5. Williams, L. A. and Ziobro, G. (1982), *Biotechnol. Lett.* **4,** 45.
6. Kim, K, and Hamdy, M. K. (1986), *Biotechnol. Bioeng.* **28,** 138.
7. Toran-Diaz, I., Jain, V. K., Allais, J.-J., and Baratti, J. (1985), *Biotechnol. Lett.* **7,** 527.
8. Guiraud, J. P., Daurelles, J., and Galzy, P. (1981), *Biotechnol. Bioeng.* **23,** 1461.
9. Margaritis, A. and Bajpai, P. (1982), *Biotechnol. Bioeng.* **24,** 1473.
10. Margaritis, A. and Bajpai, P. (1982), *Biotechnol. Bioeng.* **24,** 1483.
11. Ryu, Y. W., Kim, C. H., and Kim, S. I. (1983), *Kor. J. Agric. Chem. Soc.* **26,** 119.
12. Ryu, Y. W., Kim, C. H., and Kim, S. I. (1984), *Kor. J. Appl. Microbiol. Bioeng.* **12,** 51.
13. Rogers, P. L., Lee, K. J., and Tribe, D. E. (1980), *Process Biochem.* **15,** 7.
14. Rogers, P. L., Lee, K. J., and Tribe, D. E. (1979), *Biotechnol. Lett* **1,** 165.
15. Lee, G. M., Kim, C. H., Lee, K. J., Abidin, Z., Han, M. H., and Rhee, S. K. (1986), *J. Ferment. Technol.* **64,** 293.
16. Hsu, S. C. and Lockwood, J. L. (1975), *Appl. Microbiol.* **29,** 422.
17. Weinen, J. (1978), *J. Inst. Brew.* **88,** 222.
18. Miller, G. L. (1959), *Anal. Chem.* **31,** 426.

19. *Sigma diagnostics,* Procedure No. 510. Sigma Chemical Co.
20. Byun, S. M. and Nahm, B. H. (1978), *J. Food Sci.* **43,** 1871.
21. Rhee, S. K., Lee, G. M., Kim, C. H., Abidin, Z., and Han, M. H. (1986), *Biotechnol. Bioeng. Symp. No. 17,* 481.
22. Zittan, L. (1981), *Starch* **33,** 373.
23. Swings, L. and De Ley, J. (1977), *Bacteriol. Rev.* **41,** 1.
24. Kim, C. H., Lee, G. M., Han, M. H., and Rhee, S. K. (1987), *Kor. J. Appl. Microbiol. Bioeng.* **15,** 55.
25. Hagerdal, B. H. (1984), *Biotechnol. Bioeng.* **26,** 771.