Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19 No. 1, 70-75 (1991)

Continuous Production of Gluconic Acid and Sorbitol from Glucose and Fructose using Permeabilized cells of *Zymomonas mobilis*

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투과화된 *Zymomonas mobilis* 균체를 이용한 Glucose와 Fructose로부터 Gluconic Acid와 Sorbitol의 생산

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Abstract — Continuous and simultaneous production of gluconic acid and sorbitol from glucose and fructose was carried out by using glucose-fructose oxidoreductase and gluconolactonase of *Zymomonas mobilis*. In order to utilize the enzymes without purification, *Zymomonas mobilis* was permeabilized with toluene. Optimum conditions for permeabilization and reaction kinetics of permeabilized *Zymomonas mobilis* were studied. In batch operation with the permeabilized cells immobilized in alginate beads, about 90% conversion was obtained within 35 h reaction. Continuous production of gluconic acid and sorbitol using the immobilized permeabilized cells was carried out. Optimum conditions for continuous operation with the immobilized cells were; pH 6.2 and temperature 40℃. Maximum productivities for gluconic acid and sorbitol were about 14.5 g/l/h and 14.8 g/l/h respectively at the dilution rate of $0.075\ h^{-1}$ when 300 g/l each of substrates was fed.

Enzymatic production of organic chemicals for the foods and pharmaceuticals has many advantages over chemical process: reaction conditions are mild, by-product formation is minimized due to high specificity for substrate and pollution problem is not serious. When compared with fermentation process, enzyme based process is more favorable because capital investment is relatively small and productivity is higher. Therefore, much effort has been made to replace chemical or fermentation process with enzymatic one.

Sorbitol is, at present, produced chemically by high pressure hydrogenation of dextrose syrup at the concentration of 70%. Hydrogenation is catalyzed by Ni^{2+} ion under the conditions of 40~50 atm and 140~150℃. This process involves generation and storage of H_2 produced by the electrolysis of water. Furthermore, sorbitol syrup produced must be passed through several ion exchange columns to reduce the residual Ni^{2+} ion to below 5 ppm (1). Gluconic acid is presently produced by fermentation process using *Aspergillus niger* or *Gluconobacter suboxydans* (2, 3). In this case, the operation with pure oxygen and high aeration rate is required to meet higher oxygen demand.

Recently, it was reported that glucose-fructose oxidoreductase of *Zymomonas mobilis* simultaneously produces gluconic acid and sorbitol in equimolar concentrations from glucose and fructose in combination with gluconolactonase (1, 4). The oxidoreductase has been proposed to contain a NADP cofactor

Key words: Gluconic acid, sorbitol, *Zymomonas mobilis*, permeabilization
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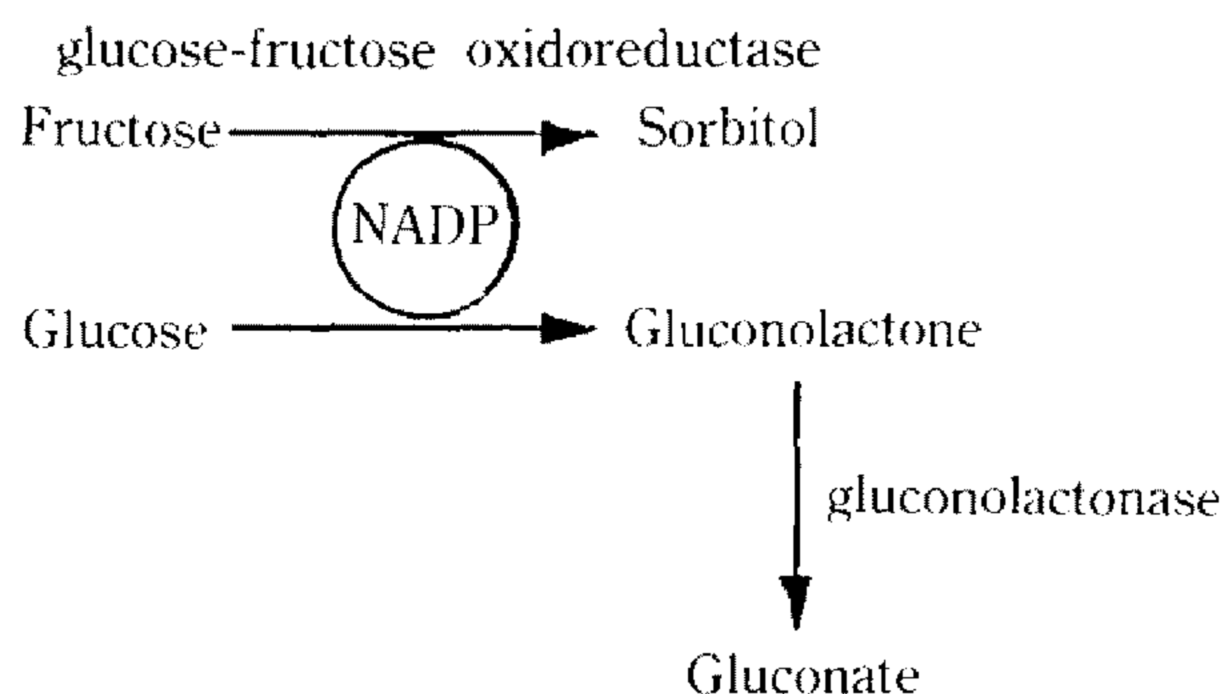

Fig. 1. A schematic diagram of enzyme system for simultaneous production of gluconic acid and sorbitol.

that is tightly bound to the active site of the enzyme (5). A schematic diagram of the enzyme system used is shown in Fig. 1. To utilize the enzymes without purification, *Zymomonas mobilis* was permeabilized with organic solvents, and the permeabilized cells were used for simultaneous production of gluconic acid and sorbitol (1). However, studies on the reaction kinetics of the permeabilized cells and the process development for the continuous production have not yet been carried out in detail.

The main objective of this work is to characterize the reaction kinetics of the permeabilized *Zymomonas mobilis* and to develop a continuous process for the simultaneous production of gluconic acid and sorbitol using the permeabilized cells immobilized in alginate beads.

Materials and Methods

Microorganism and culture condition

Zymomonas mobilis ATCC 31821 was used. The medium composition for cultivation was as follows: 10% glucose, 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.5% yeast extract. No phosphate salts were added to the final growth medium in order to minimize the level of phosphorylated intermediates in the cell. The pH was adjusted to 5.0 and temperature was controlled at 30℃.

Cell permeabilization

Culture broth was harvested at the late exponential growth phase and separated using a centrifuge. Centrifugated cells were suspended in 0.1 M citrate buffer, pH 6.2. To the cell suspension was added toluene and the mixture was shaken with vortex for predetermined time. The solvent-treated cells were separated by centrifugation at 2000 g and washed twice with 0.85% saline solution.

Immobilization of permeabilized cells

0.6 g (dry weight) of permedabilized cells were suspended in 10 m*l* of 0.85% saline solution and mixed with equal volume of 4% sodium alginate solution. The cell suspension was dropped into 0.1 M $CaCl_2$ solution through hyperdermic needle using syringe pump (Sage Instrument, USA). Alginate beads formed were stirred for 30 minutes. Average size of beads was about 2 mm in diameter.

Batch operation

Free or immobilized cells were incubated with substrates solution in a 200 m*l* reactor with magnetic stirring under controlled temperature and pH. Unless otherwise noted, two substrates (glucose and fructose) were added in equimolar concentration. The pH of reaction mixture was controlled with a titrator (Metrohm, Switzerland). The biomass concentration in batch operation with the free or the immobilized cells was about 9.0 g (dry cell weight) per liter of reaction mixture. Aliquots were taken at intervals to determine the concentration of substrates and products.

Continuous operation

The permeabilized cells immobilized in alginate beads were incubated in the same reactor as used in batch operation. Stock solution containing two substrates in equimolar concentration was fed at predetermined flow rate and reaction mixture was withdrawn at the same flow rate as that of feeding using a peristaltic pump. The biomass concentration in continuous operation was the same as that in batch operation.

Analytical method

Concentrations of glucose, fructose, gluconic acid and sorbitol were determined by HPLC (Waters Assoc., Milford, MA, USA). The column was Sugar-PAC I (300×6.5 mm, Waters Assoc.) and column temperature was maintained at 90℃. Water contai-

ning 50 mg/l calcium EDTA was used as a mobile phase and flow rate was 0.6 ml/min. Column eluent was detected with Refractive Index (Waters Assoc.).

Results and Discussion

Optimum conditions for permeabilization

Microorganism could be permeabilized in order to use enzymes without purification by various methods: treatment with organic solvents (6-8), or detergents (9, 10), osmotic shock (11, 12) and enzymatic treatment (13). In this work, *Zymomonas mobilis* was permeabilized with organic solvents such as toluene, acetone, ethyl acetate and ethylether. Among organic solvents tested, toluene was found to be most effective for permeabilization. In order to search the optimum conditions for permeabilization with toluene, the effects of toluene concentration and treatment time on permeabilization were investigated. As shown in Fig. 2, permeabilization was most effective when *Z. mobilis* was treated with 10% toluene for 3 min. The extent of permeabilization was estimated by measuring the products formed in batch operation using the solvent-treated cells.

Kinetic characterization of permeabilized cells

Reaction kinetics with both the permeabilized and the non-permeabilized cells was studied. Production profiles for gluconic acid and sorbitol with the permeabilized cells are shown in Fig. 3(A). Gluconic acid and sorbitol were simultaneously produced in equimolar concentrations, and consumption profiles for glucose and fructose were almost identical as expected. It was found that molar concentrations of substrates consumed were almost the same as those of products formed. Production of gluconic acid and sorbitol with the non-permeabilized cells was also conducted. As shown in Fig. 3(B), the production profiles for gluconic acid and sorbitol were different, and concentrations of products formed were much lower compared with those using the permeabilized cells.

The permeabilized cells were incubated with reaction mixture containing cofactors and metal ions such as NADP, ATP, ADP, NAD, $MgSO_4$ and K_2HPO_4, and production of gluconic acid and sorbitol was observed. As shown in Fig. 4, production profiles for gluconic acid and sorbitol were similar to those with the non-permeabilized cells. Consumption profiles for glucose and fructose were also si-

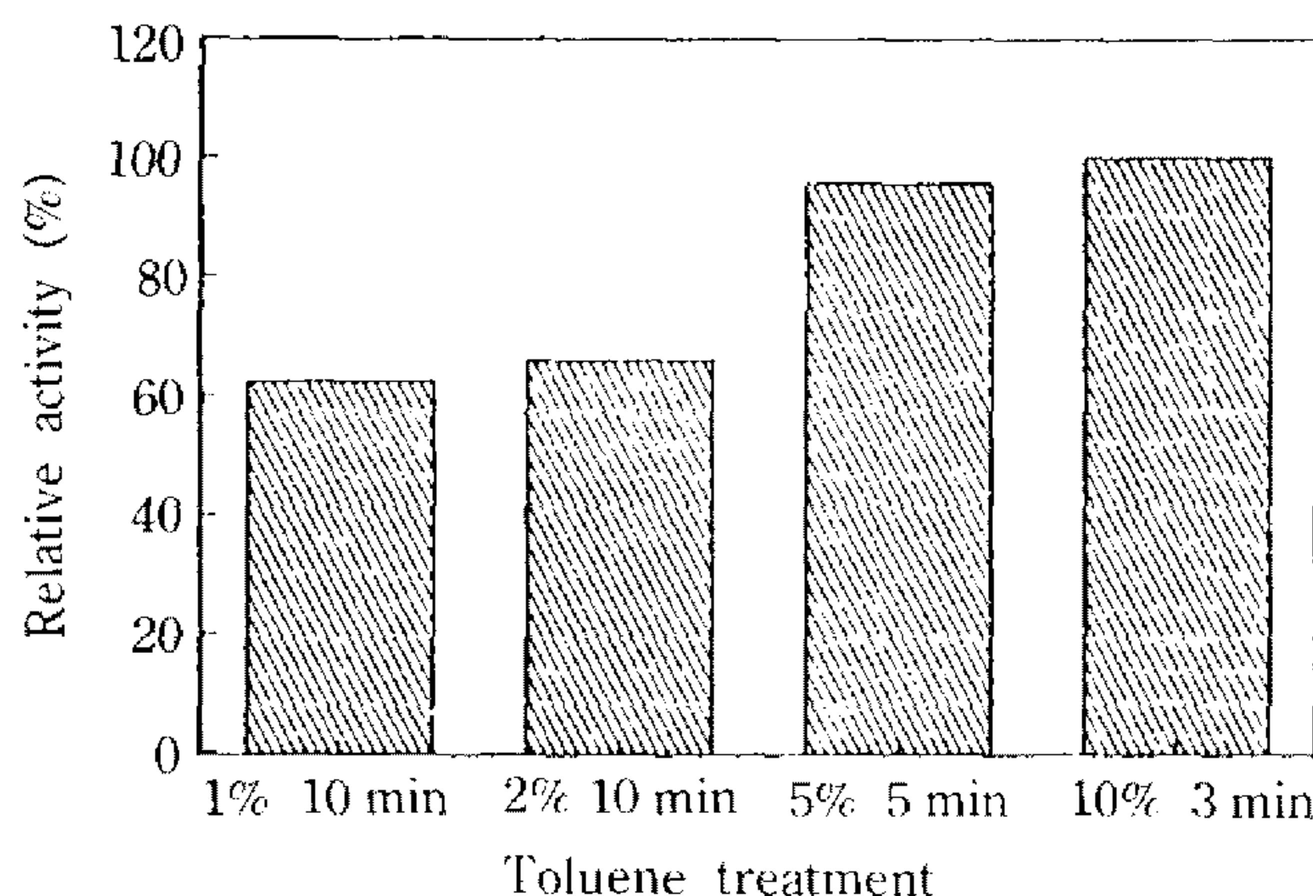

Fig. 2. Effect of treatment methods with toluene on permeabilization of *Zymomonas mobilis*.

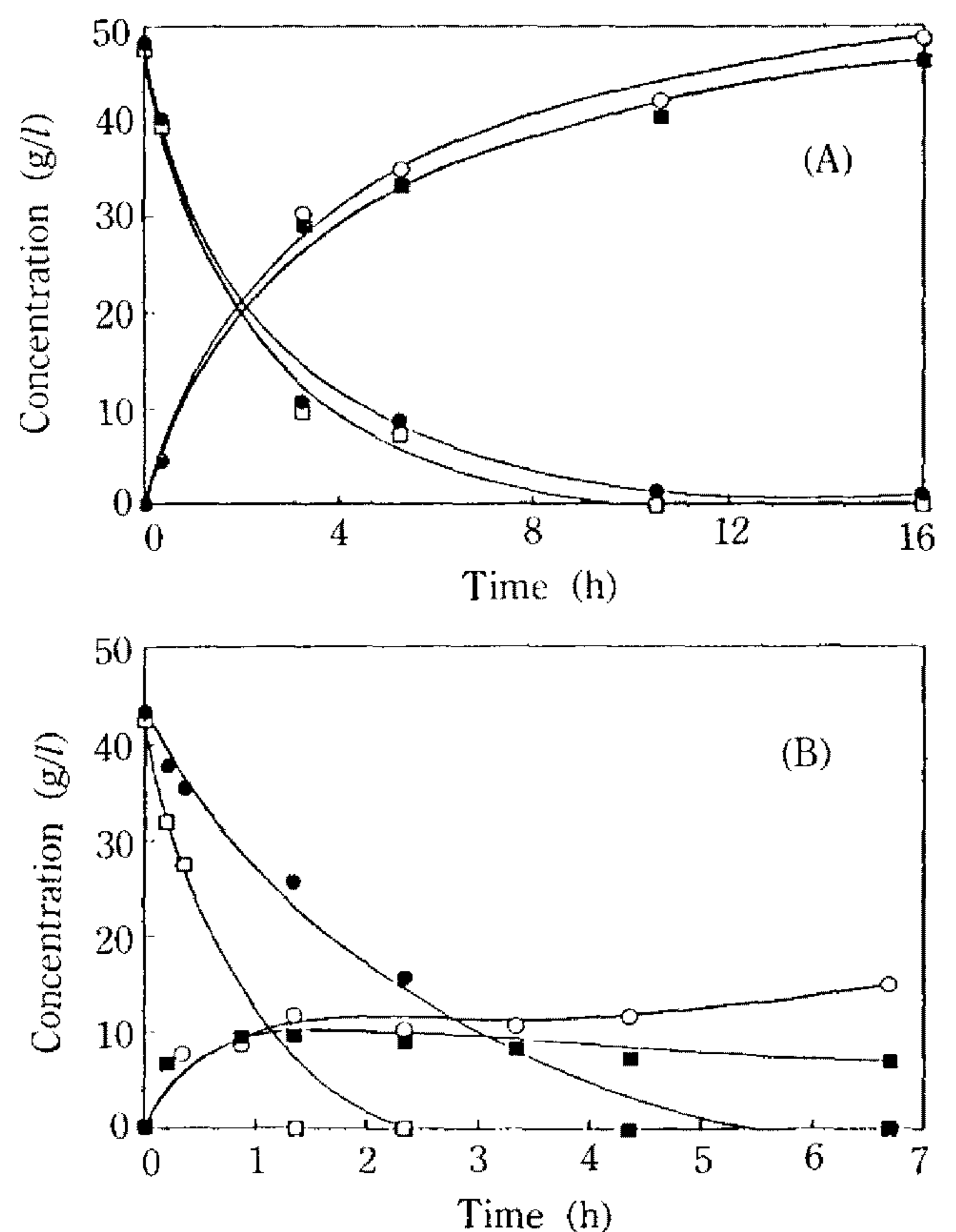

Fig. 3. Production of gluconic acid and sorbitol in batch process with the permeabilized (A), and the non-permeabilized (B) cells of *Z. mobilis*.
Reaction conditions were; pH 6.2 and temperature 40℃. Symbols are; glucose (□), fructose (●), gluconic acid (○), sorbitol (■).

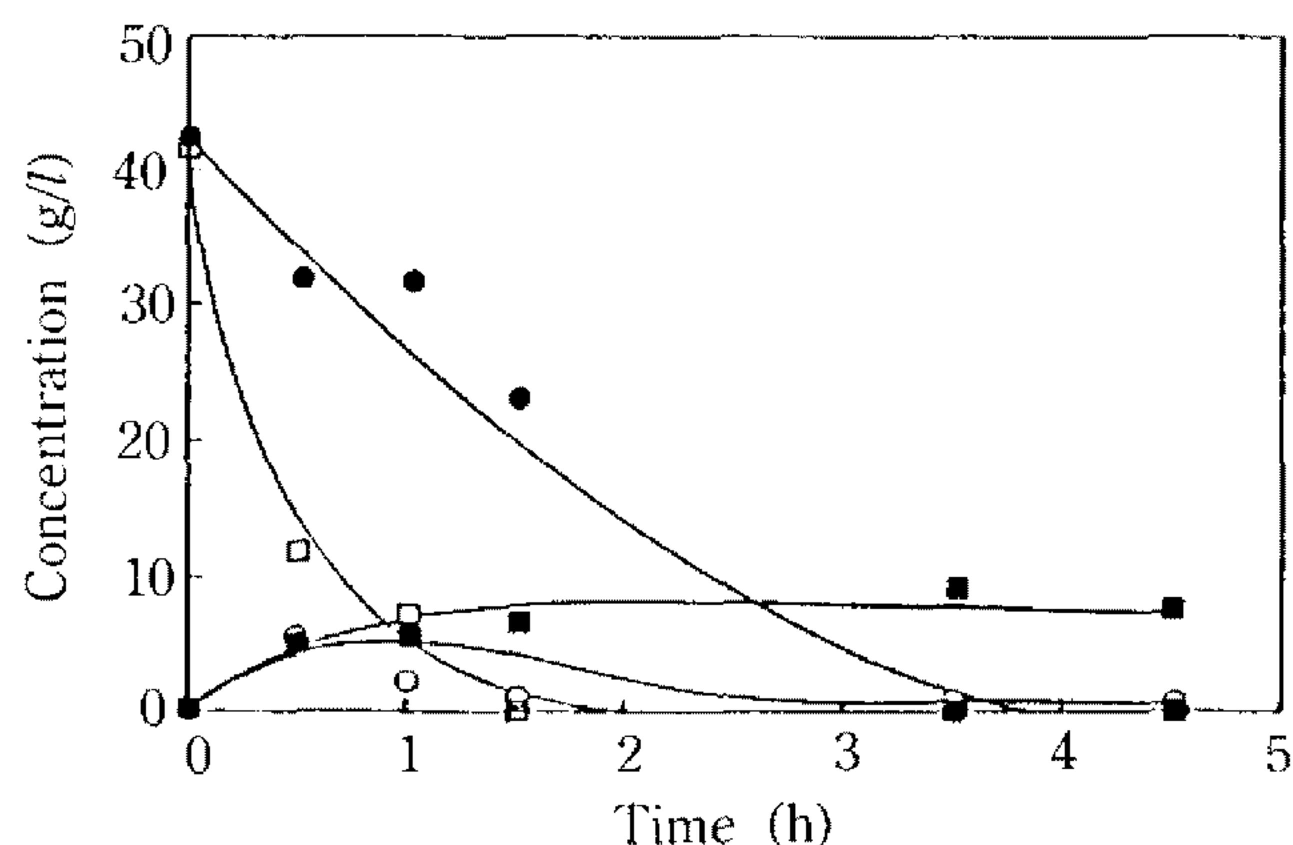

Fig. 4. Effect of cofactors addition on production of gluconic acid and sorbitol with the permeabilized cells of *Z. mobilis*.
Reaction conditions were; pH 6.2 and temperature 40℃. Symbols are; glucose (□), fructose (●), gluconic acid (○), sorbitol (■). Cofactors added; 0.005 M ATP, 0.005 M ADP, 0.0005 M $NADP^+$, 0.0005 M NAD^+, 0.0075 M $MgSO_4$, 0.05 M K_2HPO_4.

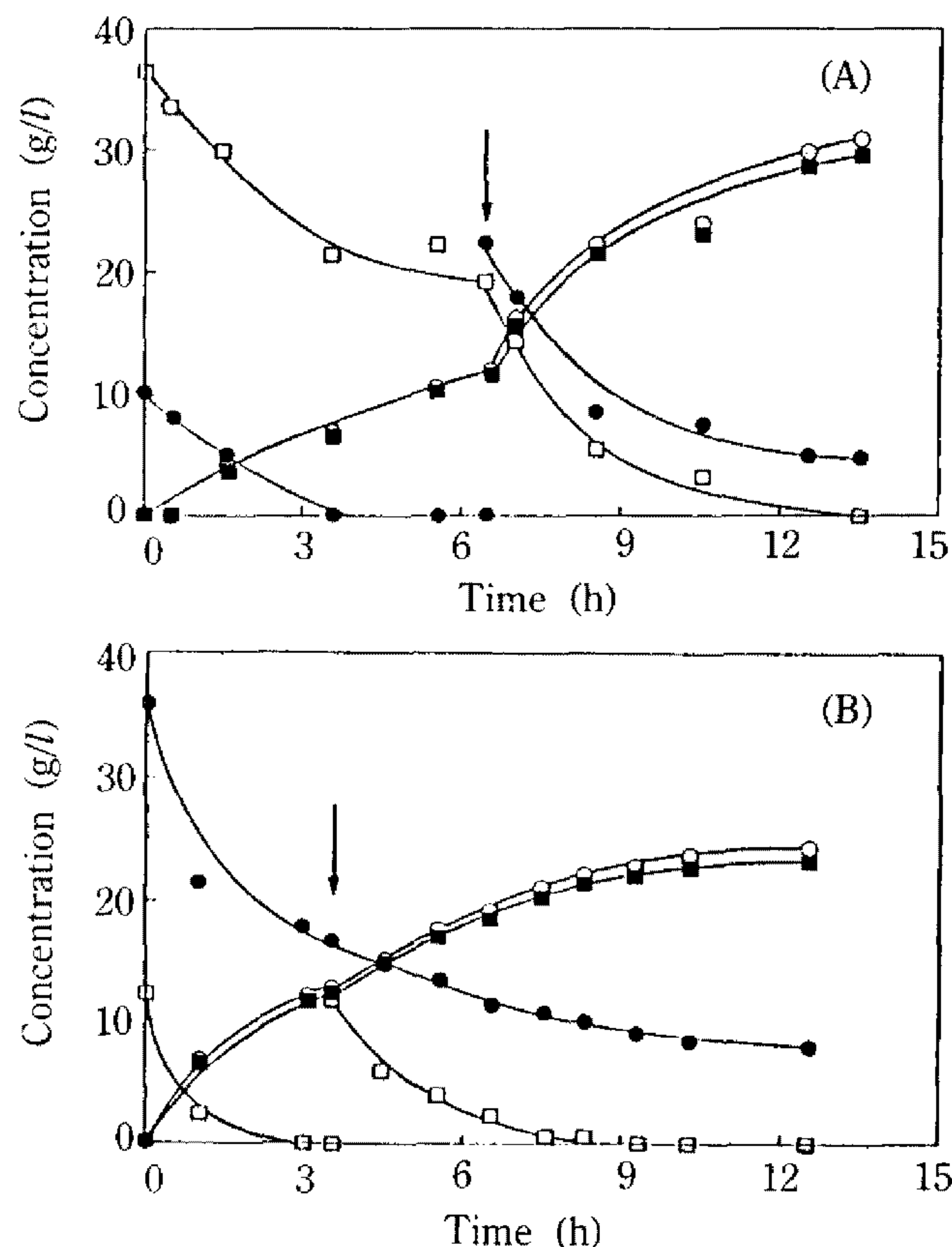

Fig. 5. Time courses of production and consumption at different molar ratios of glucose to fructose.
Reaction conditions were; pH 6.2 and temperature 40℃. Frucose (A) or glucose (B) was supplemented at the arrow indicated. Symbols are; glucose (□), fructose (●), gluconic acid (○), sorbitol (■).

milar to those obtained with the non-permeabilized cells. Glucose-fructose oxidoreductase is known to contain a NADP cofactor that is tightly bound to the active site. Thus, it is likely that when *Z. mobilis* was permeabilized with toluene, soluble cofactors and high energy compounds leaked out of the cells, and thereby resulted in shutdown of the enzyme reactions for sugar metabolism except for glucose-fructose oxidoreductase and gluconolactonase.

In order to verify the involvement of a NADP (H) cofactor in the reaction of an oxidoreductase inside the permeabilized *Z. mobilis*, the permeabilized cells were incubated with different molar ratio of glucose to fructose. When glucose concentration was three times higher than fructose, the production of gluconic acid and sorbitol ceased as fructose was completely consumed [Fig. 5(A)]. Further production of gluconic acid and sorbitol was observed when fructose was supplemented upon its depletion. Similar result was obtained when fructose concentration was higher than glucose [Fig. 5(B)]. These observations seem to be coincident with the fact that a NADP (H) cofactor is spontaneously regenerated by the enzymatic catalysis itself at the active site of the enzyme, and that glucose-fructose oxidoreductase of *Z. mobilis* requires equimolar concentrations of glucose and fructose for simultaneous production of gluconic acid and sorbitol with spontaneous regeneration of the cofactor.

Batch kinetics with free permeabilized cells

Batch production of gluconic acid and sorbitol was conducted with the free permeabilized cells at different concentrations of substrates. Operational conditions were the same as described for purified glucose-fructose oxidoredutase; temperature 40 ℃, pH 6.2. The concentration of the free cells was about 9.0 g (dry cell weight) per liter of reaction mixture in each experiment. As shown in Fig. 6, conversion yield reached up to 95% within 12 h at 300 g/*l* each of substrates. Maximum productivities for gluconic acid and sorbitol increased with increasing concentration of substrates and its values were about 23.8 g/*l*/h and 24.0 g/*l*/h respectively at 300 g/*l* each of substrates. Productivity of

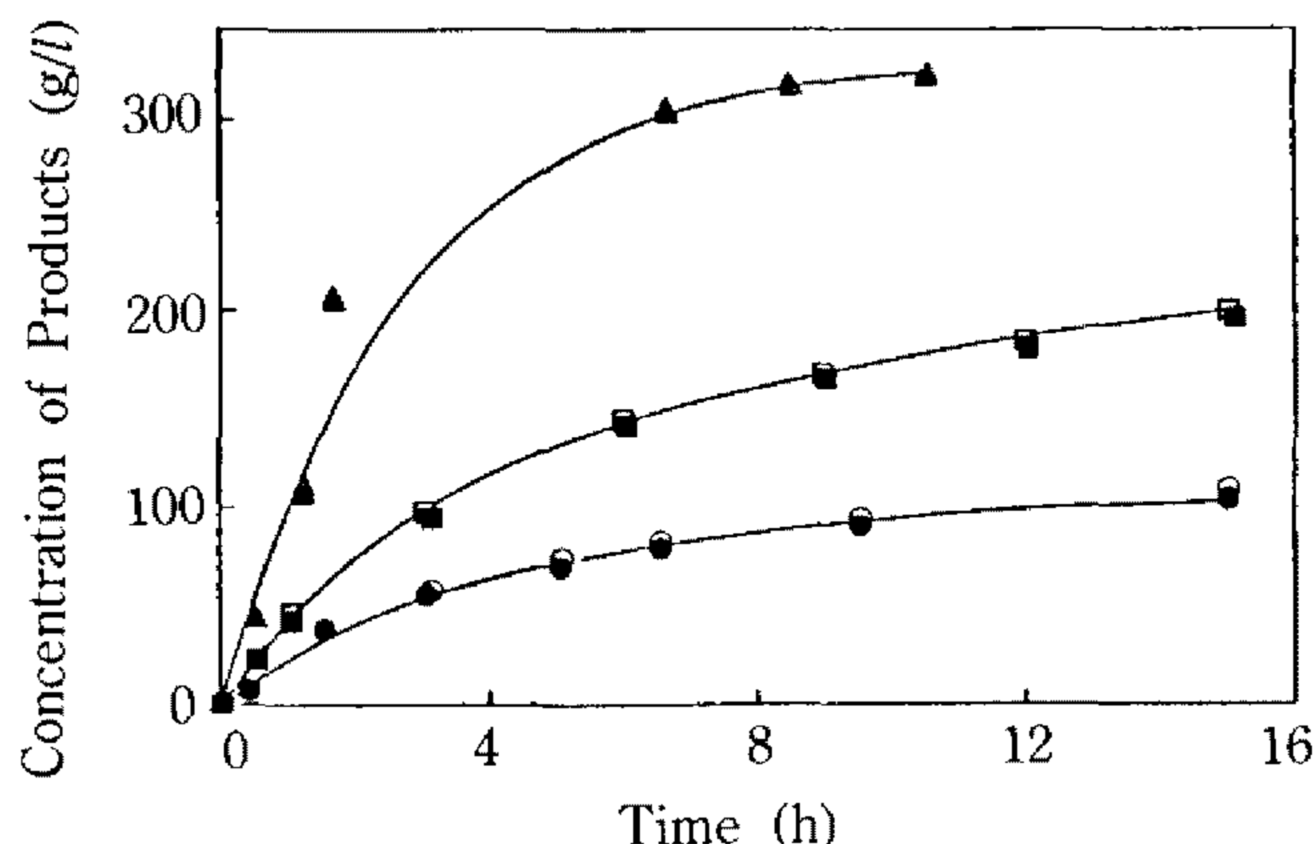

Fig. 6. Production of gluconic acid and sorbitol with the permeabilized free cells at different concentrations of substrates.
Reaction conditions were; pH 6.2 and temperature 40℃. Symbols are; 100 g/l each of substrates (gluconic acid (○), sorbitol (●)), 200 g/l each of substrates (gluconic acid (□), sorbitol (■)); 300 g/l each of substrates (gluconic acid (▲)).

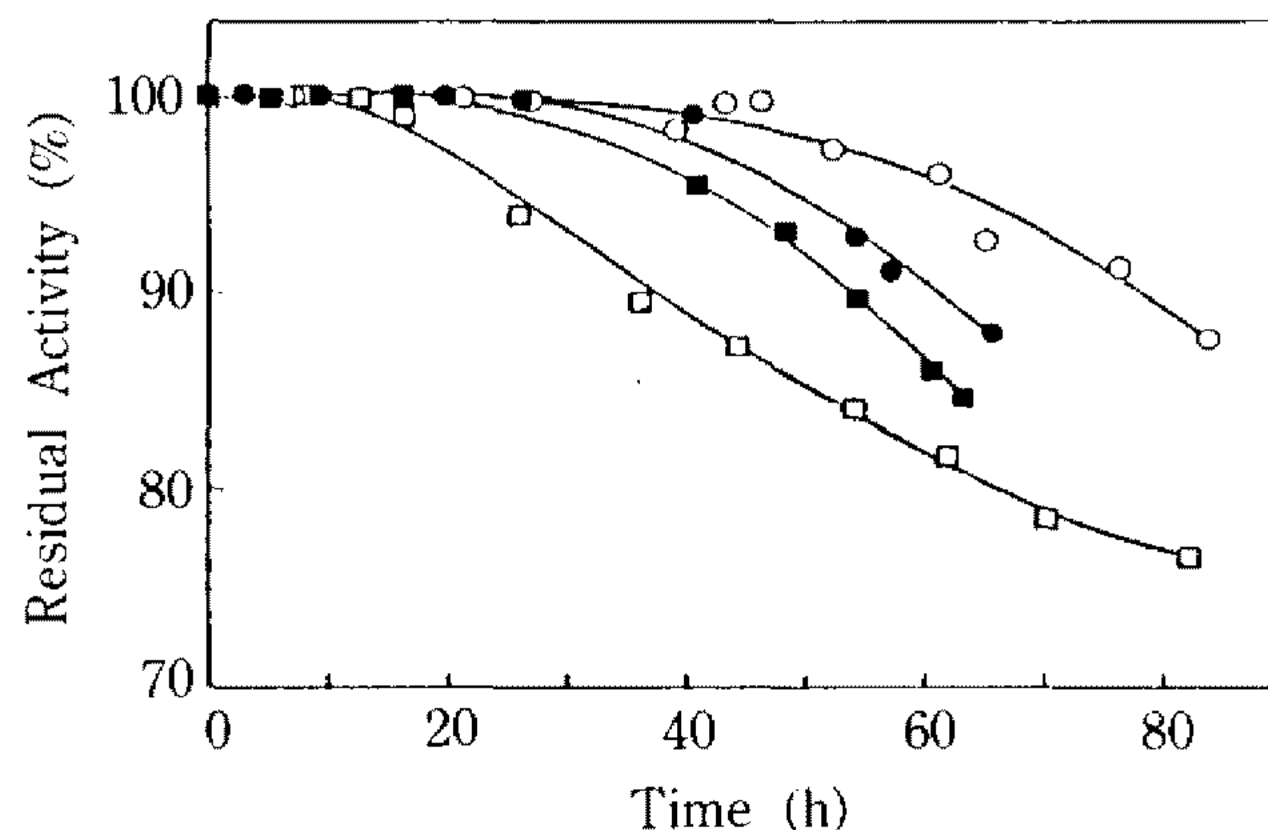

Fig. 7. Time courses of residual activity of immobilized cells in continuous operation at different pH.
Symbols are; pH 6.2 (○), pH 6.6 (●), pH 7.0 (■), pH 7.4 (□).

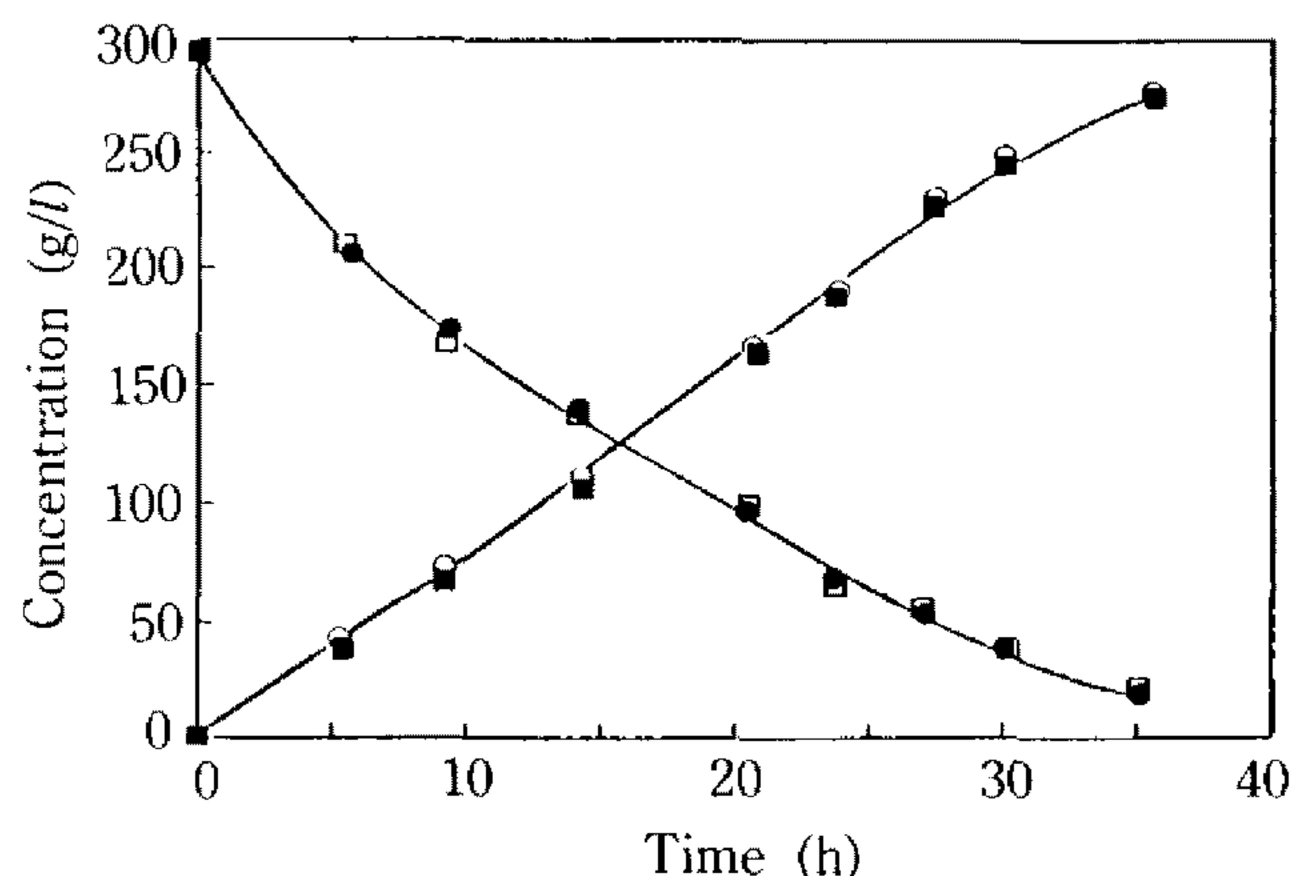

Fig. 8. Time courses of production of gluconic acid and sorbitol in batch process with the immobilized cells.
Reaction conditions were; pH 6.2 and temperature 40℃. Concentration of substrates was 300 g/l each. Symbols are; glucose (□), fructose (●), gluconic acid (○), sorbitol (■).

gluconic acid was almost identical with that of sorbitol. High productivities for gluconic acid and sorbitol at elevated concentration of substrates indicate that this is a highly promising process for production of gluconic acid and sorbitol in place of the conventional methods.

Optimum conditions for the immobilized cells

The permeabilized cells were immobilized in alginate beads and optimum operating conditions were investigated. Optimal temperature was about 40~45℃ for both free and immobilized cells.

Maximum activity of the immobilized cells was obtained at pH 7.4. Since the pH for optimum activity for the immobilized cells was different from that for the free cells, optimum pH for long term operation was investigated with the immobilized cells in continuous operation (Fig. 7). The operational stability decreased more rapidly with increasing pH and half-life at pH 6.2 was about 9 days. From the practical standpoint, optimum pH for the immobilized cells was selected to be 6.2 in this work.

Batch kinetics with the immobilized cells

The kinetic study was carried out with the immobilized cells at 300 g/l each of substrates in batch operation. As shown in Fig. 8, production rates of gluconic acid and sorbitol decreased when compared with those of free cells. About 90% conversion was obtained within 35 h while free cells showed almost complete conversion within 12 h. Results obtained above strongly indicate that severe diffusional limitation occurred with the immobilized cells. Other immobilization method to reduce the diffusional limitation is in progress in our laboratory.

Continuous production with the immobilized cells

Using the continuous operation system with the immobilized cells, the effect of dilution rate on both conversion yield and productivity was investigated under optimized conditions (Fig. 9). Maximum pro-

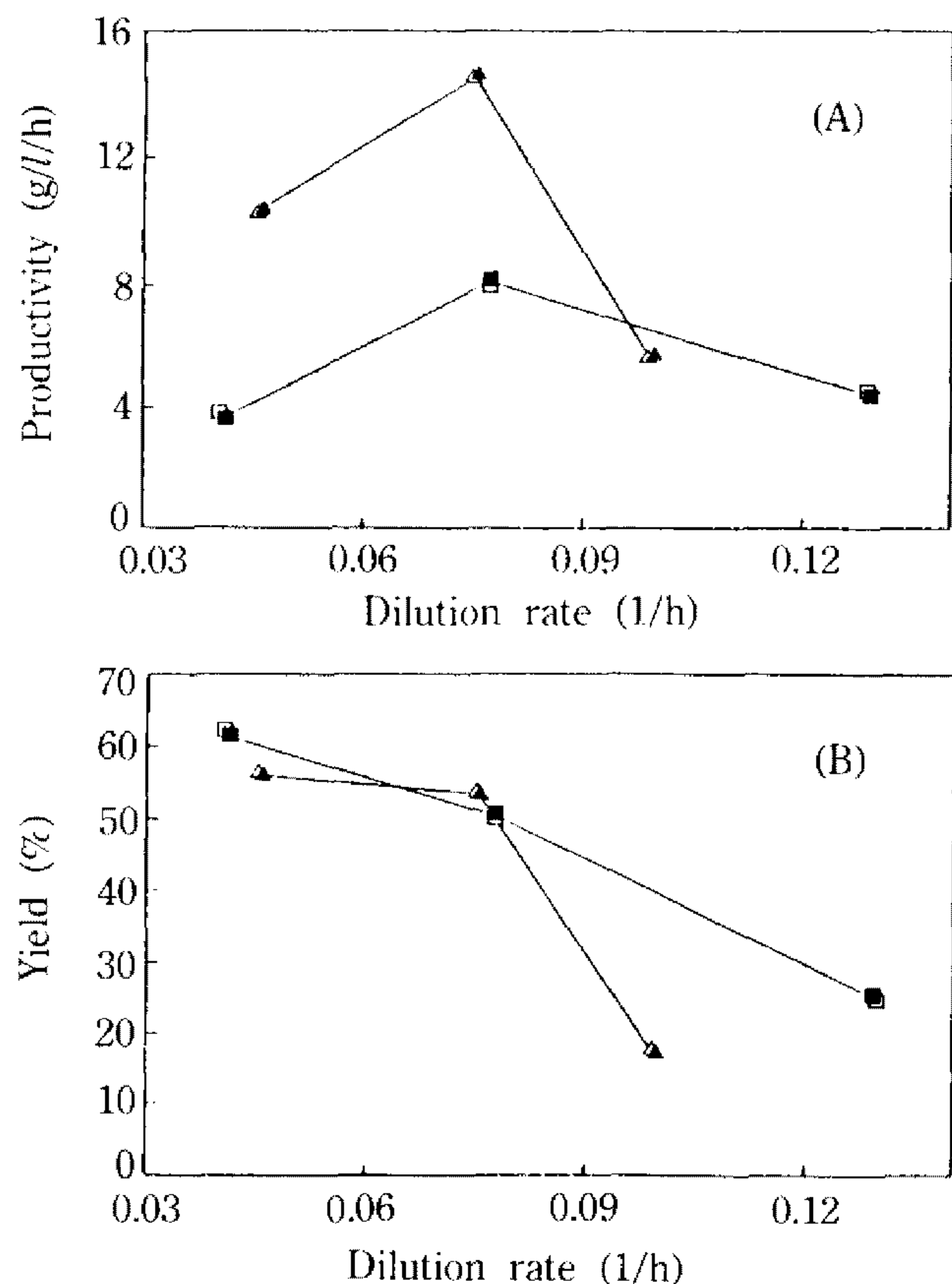

Fig. 9. Effect of dilution rate on productivity (A) and yield (B) in continuous process.
Operational conditions were; pH 6.2 and temperature 40℃. Symbols are; 200 g/l each of substrates (gluconic acid (□), sorbitol (■), 300 g/l each of substrates (gluconic acid (△), sorbitol (▲)).

ductivities for gluconic acid and sorbitol were obtained at the dilution rate of 0.075 h^{-1} when 200 g/l or 300 g/l each of substrates was fed. Maximum productivities were found to be about 14.5 g/l/h for gluconic acid and 14.8 g/l/h for sorbitol respectively at 300 g/l each of substrates. Conversion yields in continuous operation were about 60% even at low dilution rate, and this seems to be due to the fact that affinity of an oxidoreductase for substrates was known to be relatively low (5). It is expected that increase of cell loading in alginate beads could increase the conversion yield and thereby productivity. Further research to increase the conversion yield and productivity is in progress in our laboratory.

The process described in this article is very simple and highly productive for the simultaneous production of gluconic acid and sorbitol. A cost-effective process for separating gluconic acid from sorbitol shall be developed in order for this process to be economically feasible. It is believed that such an innovative effort on enzyme technology can significantly improve the overall process economics in the production of valuable organic compounds.

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(Received January 22, 1991)