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NOTE

Effect of Cross-Linking Agents on L-Sorbose Production by Immobilized *Gluconobacter suboxydans* Cells

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Abstract Biological oxidation of D-sorbitol to L-sorbose using permeated and immobilized cells of *Gluconobacter suboxydans* was carried out to investigate the optimum reaction condition. The stabilization effect of cross-linking agents such as glutaraldehyde, tannic acid, and polyethyleneimine to prevent the leakage of enzymes from beads containing permeated and immobilized cells of *G. suboxydans* was examined by the production of L-sorbose from the mixture of D-sorbitol and gluconic acid. The protein concentration effused from immobilized beads treated with only glutaraldehyde was 5.2 μg/ml after 20 h. The beads of *G. suboxydans* immobilized with alginate and cross-linked with 0.3% glutaraldehyde was the most useful for the oxidation of D-sorbitol to L-sorbose.

Key words: Cross-linking agents, glutaraldehyde, polyethyleneimine, D-sorbitol, L-sorbose

Gluconobacter suboxydans, formerly called *Acetobacter suboxydans*, is a strict aerobe that characteristically accomplishes partial oxidation of glycol and sugar alcohols [6]. The products of this limited oxidation are accumulated rapidly and quantitatively in the growth medium, and many products such as L-sorbose, gluconate and keto-gluconates have commercial value [12]. In particular, L-sorbose was used as an important precursor in the Reichstein synthesis of L-ascorbic acid [7]. This strain contains three kinds of D-sorbitol dehydrogenases (SDH): NAD-dependent D-sorbitol dehydrogenase, NADP-dependent D-sorbitol dehydrogenase, and NAD(P)-independent D-sorbitol dehydrogenase. One of these enzymes, the NAD(P)-independent D-sorbitol dehydrogenase coupled with an electron transport system is the main enzyme in D-sorbitol oxidation, thus proper aeration is considered as one of the important factors affecting L-sorbose production. That permeation with toluene affects an increase in the amount of SDH, which takes part practically in the oxidation of D-sorbitol to L-sorbose. Immobilization of *G. suboxydans* has been reported in several studies [5, 8, 10] to circumvent problems such as decreased activity and product separation inherent either in fermentation or reuse of resting cells. Recently, the technique of chemical cross-linking has been used to enhance the stability of proteins and enzymes [3, 11]. Thus, there are ample examples to illustrate that chemical cross-linking, either inter- or intramolecular, provides a means of preparing stable enzymes and proteins. In fact, immobilization of permeated *G. suboxydans* with sodium alginate was insufficient to prevent the leakage of enzymes from each bead. In this study, we investigated the effect of permeation agents and cross-linking agents on the oxidative conversion of D-sorbitol to L-sorbose and the stability of immobilized beads containing permeated cells.

The *G. suboxydans* KCTC 2111 used in this study was cultured in a 5-l Fermentor (KRC, Korea) using the medium, SYP, consisted of D-sorbitol of 50 g/l, Bacto-peptone of 10 g/l, and Yeast extract of 5 g/l. Approximately 15 g of *G. suboxydans* cells taken from the late exponential phase of growth were harvested by centrifugation, washed twice with a cold 100 mM acetate buffer (pH 5.5) and resuspended in the same buffer to give a final volume of 50 ml. The solution of 90% toluene was added to give a final concentration of 10%. After vigorous stirring for 10 min at 4°C, the cell suspension was centrifuged, washed twice, and resuspended using the same buffer of 50 ml. In order to increase the enzyme stability for long-term operation, we treated permeated cells with cross-linking agents such as glutaraldehyde, tannic acid, and polyethylenimine for 10 min at room temperature before or after immobilization. The 50 ml of permeation

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of *G. suboxydans* cell suspension was mixed with 100 ml of 3% sodium alginate solution for 15 h to give a final concentration of 2%. The mixture was dropped into a 2% $CaCl_2$ solution using a syringe pump through a hypodermic needle (gauge No. 22). The bead size was controlled by air pressure injected vertically by a hypodermic needle. The beads were hardened in 0.2% $CaCl_2$ solution for 30 min and washed twice with a 100 mM acetate buffer fortified with 0.2% $CaCl_2$ at 4°C. The reaction was performed in 0.1 M acetate buffer (pH 5.6) at 30°C. The reactor for immobilized cells was equipped with sintered glass in the bottom for aeration (5 vvm) and its temperature (30°C) was controlled by a water circulator. The protein excreted from the beads was determined by the BCA method [2]. The concentration of L-sorbose and D-sorbitol was analyzed using an HPLC (Beckman system gold) equipped with 156 Refractive Index Detector (Beckman system gold), an Analog Interface Module 406 (Beckman system gold), and an HPLC column heater (Bio-Rad). HPLC columns used were: Beckman column 300 mm × 6.5 mm internal diameter (ID) and guard column 20 mm × 4.6 mm ID. The eluent was water (Baker, HPLC grade) and the flow rate was 0.3 ml/min at 90°C.

Initially, in order to confirm the order of mixing of cross-linking agent, we used glutaraldehyde, which is well known as a cross-linking agent [1]. The choice of concentration of a cross-linking agent was determined by preliminary experiments. The optimum concentration of the cross-linking agent on bioconversion efficiency was 0.3% (v/v) (data not shown). As shown in Table 1, activity of the beads treated with 0.3% (v/v) glutaraldehyde solution before immobilization was more effective in the throughout working period than that of immobilized *G. suboxydans* which was post-treated with glutaraldehyde under the same conditions.

Tanaka *et al.* [9] reported that addition of polycationic polymers, such as polyethyeneimine and tannic acid, to the immobilization medium was effective in increasing the stability of fumarase activity in the immobilized cells. To confirm the most effective cross-linking agent affecting the biological oxidation of D-sorbitol to L-sorbose, we also used tannic acid and polyethyleneimine to treat beads,

Table 1. Effect of treatment order with 0.3% (v/v) glutaraldehyde on relative oxidative activity of SDH of immobilized *G. suboxydans* cells.

Treatment order	Relative activity (%)[a]	
	0 h	170 h
No treatment	100.0	19.6
Before bead formation	95.0	38.4
After bead formation	8.9	0.5

[a]Oxidative activity of immobilized *G. suboxydans* cells without treatment of glutaraldehyde was taken as 100%.

Table 2. Effect of various cross-linking agents on the oxidation of D-sorbitol to L-sorbose by immobilized *G. suboxydans* cells.

Cross-linking agent	Conversion (%)[a]
Glutaraldehyde	66.0
Polyethyleneimine	9.3
Tannic acid	74.0

[a]Conversion was estimated from the oxidation of D-sorbitol to L-sorbose after 20 h of reaction. Substrate was composed of D-sorbitol (50 g/l) and gluconic acid (50 g/l). Cell concentration used was 80 mg/ml.

respectively. They were used in the same concentration as glutaraldehyde. As shown in Table 2, the oxidative activity of polyethyleneimine-treated beads was very poor and conversion by beads treated with glutaraldehyde and tannic acid was 66% and 74%, respectively.

However, the oxidative activity decreased rapidly in the case of tannic acid-treated beads (data not shown). This results suggested that glutaraldehyde was the most useful cross-linking agent among the agents used in this work for oxidation of D-sorbitol to L-sorbose by the immobilized cells of *G. suboxydans*. The low or rapidly decreased activity of beads treated with polyethyleneimine and tannic acid was thought to be caused by the release of periplasmic SDH from *G. suboxydans* cells. In order to elucidate the SDH release from the beads, the protein concentrations effused from each bead were investigated (Fig. 1). In the case of only polyethyleneimine-treated beads, the effusing protein concentration from beads was higher than any other case. Therefore, glutaraldehyde was better in the prevention of protein efflux from immobilized *G. suboxydans* cells than polyethyleneimine. It should be noted that the inhibition effect of protein leakage by double treatment of glutaraldehyde and polyethyleneimine was superior to that of other cases.

On the contrary, the oxidative activity of SDH of double-treated beads was almost 0% (Fig. 2D). These

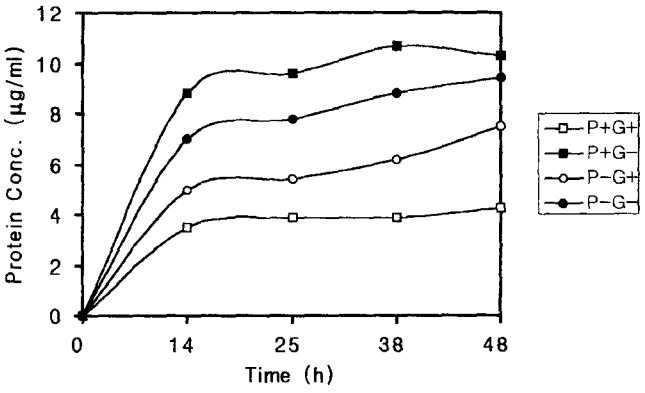

Fig. 1. The effect of cross-linking agents on protein release from immobilized *G. suboxydans* cells; P, polyethyleneimine; G, glutaraldehyde; -, not treated; +, treated.

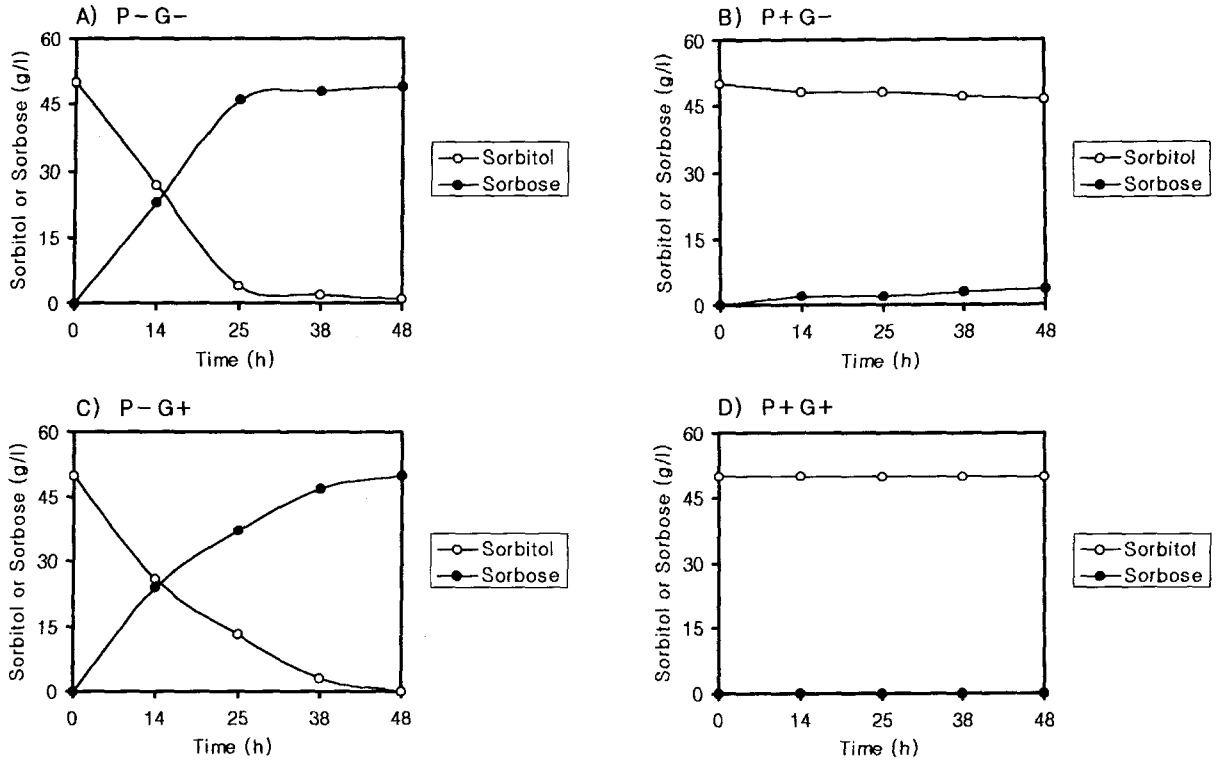

Fig. 2. The effect of various cross-linking agents on the oxidation of D-sorbitol to L-sorbose by immobilized *G. suboxydans* cells; P, polyethyleneimine; G, glutaraldehyde; -, not treated; +, treated. Substrate was a mixture of D-sorbitol (50 g/l) and gluconic acid (50 g/l).

results suggest that the polythyleneimine affect the leakage of the enzyme and also inhibit the enzyme activity. Thus, considering the activity and stability of SDH, *G. suboxydans* beads treated with only glutaraldehyde were the most useful for the oxidation of D-sorbitol to L-sorbose (Fig. 2C). The beads without any treatment presented a slightly higher oxidative activity (Fig. 2A), but a higher protein concentration effused from the beads. The half-life of enzyme activity of original beads was shorter than that of glutaraldehyde-treated beads in long-term operation [4].

In conclusion, considering the effect of order of treatment, type, and concentration of cross-linking agents on the release of immobilized periplasmic SDH enzyme of *G. suboxydans*, we believe that treatment with 0.3% glutaraldehyde before bead formation produces a higher and more stable oxidative activity of SDH of immobilized *G. suboxydans* cells.

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