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Continuous production of L-serine by immobilized growing *Corynebacterium glycinophilum* cells

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Summary. Auxotrophic mutant cells of *Corynebacterium glycinophilum* with high L-serine production activity were immobilized by entrapment with various gel materials, such as synthetic prepolymers and natural polysaccharides. The entrapped cells were used for estimation of L-serine productivity in a medium supplemented with glycine as a precursor. Based on the above criteria, including cell growth in gels and cell leakage from gels, calcium alginate was the most suitable gel material. Continuous L-serine fermentation with calcium alginate-entrapped growing cells was successfully achieved in an air-bubbled reactor for at least 13 days.

Introduction

Application of immobilized growing microbial cells has been currently highlighted from the viewpoints of long-term utilization of biocatalysts and continuous operation of stabilized systems (Fukui and Tanaka 1982). Immobilized growing microbial cells possess not only the catalytic faculty of complex reactions but also the ability to self-proliferate and self-regenerate the catalytic systems. Slowinski and Charm (1973) reported the first example of a laboratory application of immobilized growing cells for production of L-glutamate de novo from glucose by *Corynebacterium glutamicum*. Recently, we have demonstrated the efficiency of immobilized growing microbial cells for the production of various substances, such as vitamin B_{12} (Yongsmith et al. 1982), antibiotics (Ogaki et al. 1986; Takashima et al. 1987), a peptide (Okada et al. 1987a) and an enzyme (Okada et al. 1987b). In particular, we have successfully achieved continuous production of oxytetracycline by *Streptomyces rimosus* (Ogaki et al. 1986) and the α-mating factor by *Saccharomyces cerevisiae* (Okada et al. 1987a).

L-Serine is a valuable amino acid used as an additive in cosmetics and a component of an intravenous nutrient solution. At present, practical production of L-serine, which is hard to obtain by direct fermentation from carbohydrates, is mainly carried out by fermentative conversion of glycine. Recently, some mutants of methylotrophic bacteria were employed for batch production of L-serine from glycine and methanol in a resting state (Watanabe et al. 1987; Yamada et al. 1986). It has also been reported that *Klebsiella aerogenes* harbouring a hybrid plasmid that coded for L-serine hydroxymethyltransferase (SHMT) of *Escherichia coli* was used for the production of L-serine from glycine and formaldehyde under semi-anaerobic conditions (Hamilton et al. 1985). However, continuous production of L-serine from glycine has not been established hitherto, although a few strains of methylotrophic bacteria and recombinant cells with high producing ability are available as useful biocatalysts.

An L-serine auxotroph of *Corynebacterium glycinophilum* that has high activity of SHMT and low activity of L-serine dehydratase to degrade L-serine has been found to accumulate a large amount of L-serine in a glucose-salt medium supplemented with glycine (Kubota 1985). L-Serine synthesis from glycine in the *C. glycinophilum* mutant is catalyzed by SHMT and the glycine-cleaving enzyme system [EC 2.1.2.10] coupled with tetrahydrofolate and nicotinamido adenine dinu-

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cleotide (NAD^+) as cofactors, 2 mol of glycine and 1 mol of NAD^+ being consumed in the formation of 1 mol of L-serine, CO_2, NH_3 and NADH (Kubota and Nakazawa 1985).

The mutant strain is now employed for commercial production of L-serine using batch fermentation. In order to improve the L-serine production system, it is necessary to construct a continuous system with immobilized growing cells with these complex catalytic systems. This paper describes immobilization of the auxotrophic cells of *C. glycinophilum* and continuous fermentation of the amino acid by the entrapped growing cells.

Materials and methods

Synthetic resin prepolymers. Photo-crosslinkable resin prepolymer (ENT-4000) (Sonomoto et al. 1979; Tanaka et al. 1977) was obtained from Kansai Paint Co., Tokyo, Japan. Water-miscible urethane prepolymer (PU-6) (Sonomoto et al. 1980) was provided by Toyo Tire & Rubber Co., Osaka, Japan. Photosensitive resin prepolymer (PVA-SbQ) is a derivative of polyvinyl alcohol (PVA; polymerization degree, 1800; saponification degree, 100%) introduced by styrylpyridinium groups (SbQ; 1.2 mol% to PVA) as photosensitive functional sites (Ichimura 1984).

Chemicals. κ-Carrageenan was the gift from Tanabe Seiyaku, Osaka, Japan. Antifoamer, KM-70, was purchased from Shinetsu Chemicals, Tokyo, Japan. Sodium alginate (500 cps) was the product of Nacalai Tesque, Kyoto, Japan. All other chemicals were also obtained from commercial sources.

Organism and cultivation. A mutant of *C. glycinophilum* (AJ 3411), an L-leucine auxotroph of the parent strain (ATCC 21341) (Kubota 1985), was grown at 30° C for 24 h with shaking (220 rpm) in a 500-ml flask containing 50 ml of the following medium (pH 6.8): glucose, 5 g; peptone, 10 g; yeast extract, 10 g; NaCl, 5 g, in 1 l deionized water.

Immobilization of cells. Cultured cell suspension (2 ml) was aseptically immobilized with ENT-4000, PU-6, sodium alginate or κ-carrageenan in a similar manner to that described previously (Sonomoto et al. 1981). Beads made from calcium alginate or κ-carrageenan were ca. 4 mm diameter. The PVA-SbQ-entrapped cells were prepared as described previously (Nakajima et al. 1986).

Medium for L-serine production. For L-serine production the following medium (pH 6.7) was used: glycine, 30 g; L-leucine, 0.3 g; glucose, 100 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; DL-methionine, 1 g; α-biotin, 0.2 mg; thiamin. HCl 1 mg; nicotinamide, 2.5 mg; folic acid, 1 mg; casamino acids, 0.21 g; $CaCO_3$, 50 g, in 1 l deionized water. In the case of calcium alginate- and κ-carrageenan-entrapped cells, 50 m*M* $CaCl_2$ was added to the production medium.

Batch production of L-serine. The immobilized cells or 2 ml precultured cell suspension were cultivated in a 500-ml shaking flask containing 50 ml production medium at 30° C with shaking (220 rpm).

Continuous production of L-serine. A schematic diagram of the air-bubbled reactor used for continuous production is illustrated in Fig. 1. The calcium alginate-entrapped cells (volume, 70 ml) were used for continuous production of L-serine. The entrapped cells and concentrated production medium supplemented with $CaCl_2$ and antifoamer (final concentrations, 50 m*M* and 0.05%, respectively) were transferred to the reactor. Since the reactor volume was adjusted to 200 ml with sterile deionized water, the final concentrations of the medium components in the reactor correspond to those of the production medium described above. Sterile air was introduced from the bottom of the reactor through an air sparger at a rate of 1 l/min. Fresh production medium without $CaCO_3$ was supplied continuously to the reactor at a flow rate of 50 ml/day using a peristaltic pump.

Analytical methods. L-Serine produced and residual glycine were determined by a bioassay method using *Leuconostoc mesenteroides* ATCC 8042 as the test microorganism (Kubota 1985). Cell masses of the free cells and cells leaked from gels were expressed by the absorbance of culture broth at 562 nm after dissolving $CaCO_3$ in the medium with HCl.

Results and discussion

Selection of cell-entrapping gel materials

When L-leucine auxotrophic cells of *C. glycinophilum* were cultivated in production medium, L-serine was produced along with growth of the bacterium (Fig. 2). Although this mutant strain has less L-serine dehydratase activity compared to the parent strain, L-serine was gradually degraded after maximum production of L-serine together with the almost complete consumption of glucose, as reported previously (Kubota 1985).

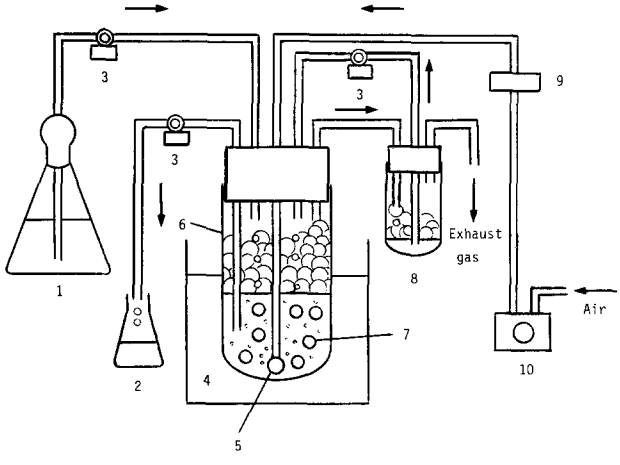

Fig. 1. Schematic diagram of a system for continuous production of L-serine: *1,* medium reservoir; *2,* product reservoir; *3,* peristaltic pump; *4,* water bath; *5,* air sparger; *6,* reactor; *7,* immobilized cells; *8,* column for trapping foam; *9,* air filter; *10,* air pump

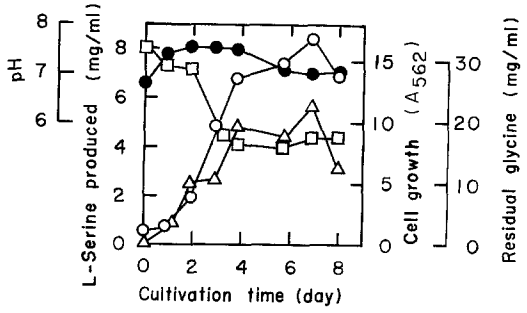

Fig. 2. Time course of L-serine production by free cells of *Corynebacterium glycinophilum* in a batch system. Cultivation was performed as described in Materials and methods: ○, L-serine produced; △, cell growth; □, residual glycine; ●, pH

Production of L-serine by the cells entrapped with different gel materials was examined (Table 1). The PU-6, PVA-SbQ, and κ-carrageenan provided as much L-serine accumulation as the free cells. On the other hand, the ENT-4000- and calcium alginate-entrapped cells showed moderate L-serine production. However, leakage of cells from the former gels was marked, while the latter gave less cell leakage (Table 1). For constructing continuous production systems with immobilized growing cells, one should consider the cell-holding ability of gels on account of its importance to productivity and ease of operation of the systems (Ogaki et al. 1986). The ratios of L-serine produced to cell leakage indicate that the cells inside calcium alginate gels produced L-serine more efficiently than the others (Table 1). From microscopic observations, small amounts of the cells were found to occupy the cavities of the PU-6 and PVA-SbQ gels after cultivation of the immobilized cells. κ-Carrageenan gels were liable to be damaged under the cultivation conditions employed. The calcium alginate-entrapped cells grew well and remained near the surface of the beads at a very high density after cultivation.

Figure 3 illustrates a typical time course of L-serine production and cell leakage by the entrapped and free cells in a batch system. Calcium alginate-entrapped cells were slightly inferior to their free counterparts with regard to the L-serine production rate. However, cell leakage from the calcium alginate beads was less than one-third of that of the free system even after 12 days of cultivation.

From the results obtained here, calcium alginate was selected as the gel material for subsequent experiments.

Repeated use of entrapped C. glycinophilum cells

The calcium alginate-entrapped cells of *C. glycinophilum* were repeatedly used to examine the stability of L-serine productivity, durability of the beads and cell leakage from the gels. The entrapped and free cells were cultivated for 12 days for the first batch and then every 7 days from the second to the fifth batch in fresh production medium. The cell-entrapping gel beads could be employed in the five successive batches of cultivation without any loss of gels. L-Serine was successfully produced by the entrapped cells at least during the five times of repeated use (Fig. 4). Sirirote et al. (1988) have recently reported repeated production of L-serine from methanol and glycine with an immobilized resting methylotroph. However, repeated use was not feasible without reactivation of the calcium alginate-entrapped cells in

Table 1. L-Serine production by immobilized growing *Corynebacterium glycinophilum* cells

Gels	L-Serine produced (mg/ml)	Cell leakage (A_{562})	L-Serine / Cell leakage
None	6.96	15.3	0.45
PVA-SbQ	6.59	13.8	0.48
PU-6	7.32	18.0	0.41
ENT-4000	4.68	7.02	0.67
κ-Carrageenan	6.11	13.6	0.45
Calcium alginate	5.49	4.13	1.33

Gel abbreviations and cultivation of immobilized and free cells in 50 ml production medium for 6 days are as described in Materials and methods

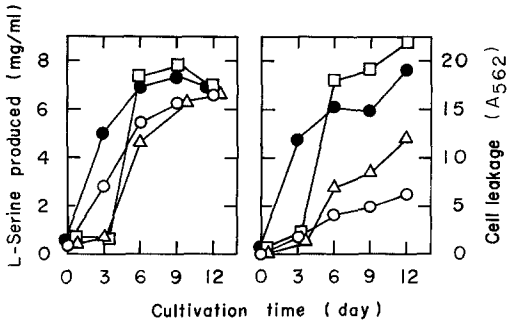

Fig. 3. Time course of L-serine production by immobilized *C. glycinophilum* cells. Gel abbreviations and cell cultivation are as described in Materials and methods: ○, calcium alginate-entrapped cells; △, ENT-4000-entrapped cells; □, PU-6-entrapped cells; ●, free cells

appropriate media. The productivity of L-serine greatly decreased to less than one-sixth of the initial amount after five repeated batch reactions even with reactivation. Although the free cells of *C. glycinophilum* also showed high L-serine formation, a marked fluctuation in productivity was observed after the third batch cultivation, at which the cell mass of free cells was almost constant in the system (Fig. 4). As shown in Fig. 4, leakage of the cells from calcium alginate gels scarcely increased during repeated cultivation. From the third to the fifth batch, L-serine was produced at the maximum level (6–7 mg/ml) in the early phase of immobilized cell cultivation, during which cell leakage was very small. Thereafter, the leaked cells increased with cultivation time ($A_{562} \geqq 10$ after 7 days). This result indicates that most of the L-serine produced might be derived from cells that proliferated well inside the gels and not from the leaked cells ($A_{562} \geqq 10$).

Thus, calcium alginate seems to be a suitable gel material for entrapping cells to provide continuous L-serine production with immobilized growing *C. glycinophilum* cells.

Continuous production of L-serine

Continuous production of L-serine by the calcium alginate-entrapped cells was performed in production medium containing 0.05% of an antifoamer. As shown in Fig. 5, after the immobilized cells were cultivated in the reactor for 4 days, continuous fermentation was started (flow rate, 50 ml/day; working volume, 200 ml). The system was stirred by sparging air at a rate of 1 l/min and automatically controlled at pH 7.6. L-Serine productivity was maintained within a range of 4.0 to

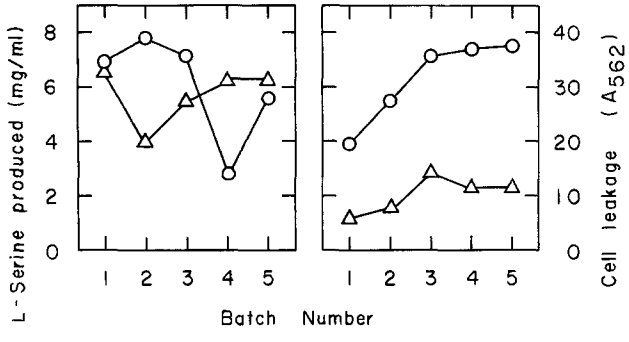

Fig. 4. L-Serine production in repeated use of free and immobilized *C. glycinophilum* cells. Cells were cultivated for 12 days for the first batch and then every 7 days for four successive batches: ○, free cells; △, calcium alginate-entrapped cells

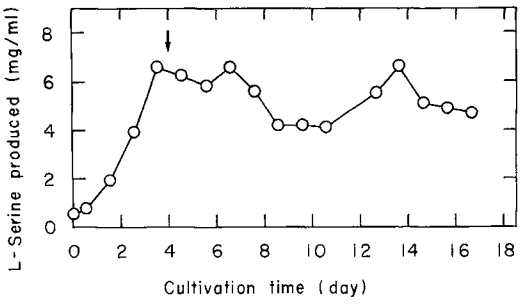

Fig. 5. Continuous production of L-serine by calcium alginate-entrapped growing *C. glycinophilum* cells. Detailed operational conditions are described in Materials and methods: *arrow*, start of continuous cultivation

6.0 mg/ml (1.0–1.5 mg · ml^{-1} per day) for at least 13 days under the conditions employed (Fig. 5). During continuous fermentation, leakage of cells from the alginate beads was kept at the same level as observed in batch cultivation. These results suggest that a sufficient amount of physiologically active cells trapped in the gel matrices results in stabilization of the continuous production system of L-serine. Highly aerated conditions were found to be necessary for high productivity of L-serine in continuous fermentation and also in the batch cultivation (data not shown). Therefore, a foam-trapping column was incorporated in the reactor, as shown in Fig. 1, because of vigorous foaming of the cultured broth even in the presence of antifoamer.

The results obtained here indicate that L-serine can be produced continuously and efficiently by immobilized growing *C. glycinophilum* cells. Such a continuous bioreactor system might be applicable to the production of other useful substances requiring highly aerobic conditions.

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