Bacitracin Production by Whole Cells Immobilized in Polyacrylamide Gel

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Received for publication 1 October 1978

Whole cells of *Bacillus* sp., bacitracin-producing bacteria, were immobilized in polyacrylamide gel prepared by using 5% total acrylamide (95% acrylamide monomer and 5% *N,N'*-methylenebis acrylamide). Bacitracin production by the immobilized whole cells was examined by using various fermentation media. In starch-bouillon medium, the initial activity of immobilized whole cells for bacitracin production was 20 to 25% that of an equivalent amount of washed cells. With successive utilizations in 1% peptone as reaction medium, activity of the immobilized whole cells increased gradually and reached a steady-state maximum having a value of 80 to 90% of the activity obtained initially with washed cells, whereas the washed cells lost most activity when utilized successively. What appeared to be growth of the bacteria in the gel was observed by electron microscopy. Therefore, the activation of immobilized whole cells during successive utilizations apparently resulted from the growth of whole cells in the gel, especially at the gel surface.

In the past few years, much interest has been expressed in the use of microorganisms immobilized in solid supports (1-3, 9-11, 13). In general, intracellular enzymes in immobilized microorganisms have been used for the production of bioactive materials. A possible advantage of whole microorganisms compared with purified enzymes includes the ability to catalyze a series of linked reactions, some of which may require cofactors.

On the other hand, some work has been done using living immobilized whole cells for fermentation products (2, 9, 12). We have reported continuous hydrogen production by immobilized whole cells of *Clostridium butyricum* (8) and α -amylase production by immobilized *Bacillus subtilis* (T. Kokubu, I. Karube, and S. Suzuki, Eur J. Appl. Microbiol., in press). Furthermore, microbial electrodes using immobilized whole cells have been developed for the determination of biological oxygen demand (BOD) (6) and a vitamin (T. Matsunaga et al., Anal. Chim. Acta, in press). In these cases, bacteria are active for a long time in the gel. Recently, preliminary work on penicillin G production by immobilized whole cells of *Penicillium chrysogenum* has been reported (Morikawa et al., Biotechnol. Bioeng., in press). These immobilized whole cells have a potential for application in a new type of

fermentation. Since the fermentation can be performed with a column system when immobilized whole cells are used, the fermentation process can be controlled easily.

The peptide antibiotic bacitracin is synthesized by the protein thiotemplate mechanism present in *Bacillus* species (7). Recently, cell-free synthesis of bacitracin has been also studied in some detail (7). Therefore, we selected bacitracin for the production of secondary metabolite by immobilized whole cells.

In this paper, bacitracin-producing bacteria, *Bacillus* sp., were immobilized in polyacrylamide gel, and the production of bacitracin was carried out from a simple nutrient. Furthermore, the growth of the bacteria in the gel was examined by electron microscopy.

MATERIALS AND METHODS

Materials. Acrylamide and *N,N'*-methylenebisacrylamide (BIS) were obtained from Wako Pure Chemicals Inc., and bacitracin (75 U/mg) was obtained from Kyowa Hakko Co. Meat extract and peptone were purchased from Kyokuto Pharmaceutical Co. Other reagents were commercially available analytical reagents or laboratory grade materials. Deionized water was used in all procedures.

Culture of microorganism. *Bacillus* sp. (KY 4515) used was maintained on glucose-bouillon (GB) agar. The GB was also used for the seed medium. At the end of the incubation period (11 h), 8 ml of seed medium was added to 72 ml of starch-bouillon (SB)

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medium containing 1% soluble starch, 1% peptone, 0.5% meat extract, 0.25% NaCl, and 1×10^{-5} M MnSO_4 (pH 7.2). Fermentation was performed under aerobic conditions (reciprocal type shaker, 150 strokes per min) at 30°C. The whole cells were harvested 13 h after inoculation, at which time the bacitracin concentration in the broth was usually 350 to 450 U/ml, and the maximum rate of bacitracin production was about 40 U/ml per h.

Immobilization of microorganism. In a typical procedure, the cells were collected by centrifugation ($10,000 \times g$ for 30 min) and washed twice in saline and resuspended in saline (bacterial concentration was 200 mg [wet weight] per ml). To 25 ml of this suspension, 20 ml of 25% acrylamide monomer solution (dissolved in saline, 95% acrylamide, and 5% BIS) and 52 ml of saline was added. The system was blanketed with nitrogen, and the polymerization was initiated with 2.0 ml of 5% ammonium persulfate (APS) and 1.0 ml of 5% N,N,N',N' -tetramethylethylenediamine (TEMED) and allowed to proceed for 20 min in an ice bath. Immobilized whole cells were cut into small blocks (8 to 27 mm³) with a knife blade, and they were washed thoroughly with saline.

Bacitracin production by immobilized whole cells. In a typical procedure, the bacitracin production by immobilized whole cells was performed in a batch system as follows: 15 g of immobilized whole cells (contained 0.75 g of cells, wet weight) was added to 25 ml of a fermentation medium in a 500-ml Sakaguchi flask, and the medium was incubated in a reciprocal type shaker (150 strokes per min) for 4 h at 30°C.

For repeated batch production of bacitracin by immobilized whole cells, they were washed thoroughly with saline after incubation, suspended in a new medium, and incubated again for 4 h.

Assay of bacitracin. Bacitracin produced by the immobilized whole cells was assayed by the paper-disk method, using *Micrococcus luteus* (ATCC 10240) as an assay microorganism (5). Bacitracin (75 U/mg) was used as a standard.

Electron microscopy. Samples for electron microscopy examination were fixed with 2.5% (wt/vol) glutaraldehyde in phosphate buffer (pH 7.0). The wet gels were placed in a series of water—acetone, acetone, and isoamylacetate solutions. Thin sections were prepared with a knife blade, coated with gold, and examined in a JEM scanning electron microscope (100C-ASID4D) operated at 40 KV.

RESULTS

Immobilization of whole cells. Washed cells were resuspended in the SB medium and incubated for 4 h at 30°C. Bacitracin was produced by washed cells and the rate of production was usually 13 to 18 U/ml per h (cell concentration was about 20 mg [wet weight] per ml). Whole cells of *Bacillus* sp. were immobilized in polyacrylamide gel. Table 1 shows the various conditions for immobilization of whole cells and bacitracin produced. Bacitracin produced by immobilized whole cells is indicated as relative to the original washed cells. The activity of bacitracin

production was highest when whole cells were immobilized in the gel prepared with 5% total acrylamide (95% acrylamide monomer and 5% BIS) (Table 1). On the other hand, no effect of BIS content on production of bacitracin was observed. However, the best productivity of bacitracin by immobilized whole cells was only 20 to 25% of that by washed cells. These results suggest that the lower rate of bacitracin production is mainly caused by the inactivation of enzymes in whole cells with polymerization reagents (especially acrylamide and APS as shown in Table 1) and may be partly due to hindered diffusion of the substrates and/or product through the gel.

Effect of air on bacitracin production. The effect of air (oxygen) on the bacitracin production by immobilized whole cells was examined. The incubation of immobilized whole cells was carried out under ordinary conditions and under nitrogen. Bacitracin produced under anaerobic condition was only 30% that under aerobic condition (Table 2).

Effect of media on bacitracin production. Preliminary experiments indicated that the activity of bacitracin production by immobilized whole cells was remarkably reduced at the second reaction when a fermentation medium was used. Therefore, the effect of media on bacitracin

TABLE 1. Bacitracin production by whole cells entrapped in various acrylamide gels

Acrylamide ^a	BIS ^b	APS	TEMED	Bacitracin produced ^c
4	5	0.2	0.025	24
6	5	0.2	0.025	15
8	5	0.2	0.025	8
10	5	0.2	0.025	5
5	5	0.2	0.025	17
5	10	0.2	0.025	16
5	15	0.2	0.025	17
5	20	0.2	0.025	18
5	5	0.1	0.05	24
5	5	0.1	0.10	20
5	5	0.2	0.025	17
5	5	0.2	0.05	13
5	5	0.4	0.025	8
5	5	0.4	0.05	6

^a Values indicate, in percent, total weight of acrylamide per total volume used in immobilization procedure.

^b Values indicate, in percent, BIS weight per total weight of acrylamide monomer.

^c SB medium was used. Values indicate production rate of immobilized whole cells, expressed as a percentage of the production rate of an equivalent amount of washed cells incubated under similar conditions.

cin productivity was examined. Bacitracin productivity in a medium containing meat extract or peptone was higher than that in a medium containing carbohydrate (Table 3). Furthermore, the productivity was retained at the second reaction of immobilized whole cells. This tendency was also observed in the case of bacitracin production by washed cells. Therefore, 0.5% meat extract and 1% peptone were selected as media for repeated batch production of bacitracin. Repeated batch production of bacitracin was attempted by using immobilized whole cells and washed cells (Fig. 1 and 2). Bacitracin produced by washed cells decreased gradually after several utilizations, but bacitracin produced by the immobilized whole cells increased with successive utilizations. The bacitracin produced in saline appeared to result from synthesis from whole-cell nutrient pools.

Since bacitracin production in the 1% peptone medium was higher than that in the 0.5% meat extract medium, the 1% peptone medium (containing 0.25% saline and 1×10^{-5} M MnSO_4) was employed for further work.

Bacitracin production from amino acids and ATP. Bacitracin production by immobilized whole cells was attempted from asparagine, aspartic acid, histidine, phenylalanine, isoleucine, ornithine, lysine, glutamic acid, leucine,

TABLE 2. Effect of air on production of bacitracin^a

Cells	Bacitracin (U/ml)	
	in air	in N ₂
Immobilized	19	7.5
Washed	91	34

^a Reaction time was 5 h; SB was used as medium.

TABLE 3. Effect of media on production of bacitracin by immobilized and washed cells

Cells	Medium ^a	Bacitracin produced (U/ml)	
		Reaction 1	Reaction 2 ^b
Immobilized whole cells	SB	12	4.1
	GB	7.2	2.0
	Peptone	19	16
	Meat extract	19	31
Washed cells	SB	54	12
	GB	34	4.2
	Peptone	82	65
	Meat extract	64	68

^a Peptone and meat extract media contained 0.25% NaCl and 1×10^{-5} M MnSO_4 .

^b Second reaction was performed after 12 h of storage at 4°C.

FIG. 1. Repeated batch production of bacitracin by washed cells and immobilized cells, 0.5% meat extract was used for reaction medium. Symbols: ●, Washed cells; ○, immobilized cells; ×, immobilized cells (0.85% saline was used as a reaction medium).

FIG. 2. Repeated batch production of bacitracin; 1% peptone was used for reaction medium. Symbols: ●, Washed cells; ○, immobilized cells.

cysteine, and ATP (Fig. 3). No activation of bacitracin production was observed under these conditions. The amount of bacitracin produced from amino acids and ATP was lower than that from 1% peptone.

Effect of bacteria content on bacitracin production. Figure 4 shows bacitracin produced by the gel containing various amounts of the bacteria. The rate of bacitracin production increased with increasing the amount of whole cells in the gel. A lag in bacitracin production was observed in the case of the gel containing the smallest amount of bacteria. However, the activity of each gel came to the same level with successive utilizations.

Microscopic observation on immobilized cells. Electron microscopic observation of the

FIG. 3. Effect of substrates on bacitracin production by immobilized whole cells. Symbols: $\circ$, 1% peptone; $\times$, amino acids (2.5 mM each) and ATP (2mM).

FIG. 4. Effect of cell contents in the gel on bacitracin production. Immobilization of whole cells was carried out under the standard conditions, except for cell contents in the gel employed; 1% peptone was used for the reaction medium. Symbols: $\circ$, 10%; $\bullet$, 5%; $\times$, 0.5%.

entrapped cells in the polyacrylamide gel was performed. Figure 5 shows the electron micrographs of whole cells immediately after immobilization and also after 56 h of use for bacitracin production. The apparent growth of cells in the gel (especially at the surface layer) was observed after successive utilizations (total, 56 h of reaction time), whereas the amount of cells evidently had decreased in the center of the gel.

DISCUSSION

Washed cells of *Bacillus* sp. produced bacitracin as described by Cornell and Snoke (4). However, bacitracin productivity decreased with successive utilizations of washed cells. Furthermore,

the collection of washed cells from reaction medium was difficult. Therefore, *Bacillus* sp. was immobilized in polyacrylamide gel. The gel prepared with 5% total acrylamide was suitable for immobilization of *Bacillus* sp. These conditions are very similar to those used previously (Mori-kawa et al., in press). Because air was required for the production of bacitracin by immobilized whole cells, whole cells in the gel had respiratory activity. As shown in electron micrographs, growth of the bacteria was observed at the surface layer of the gel, and the bacteria could not be observed in the central area of the gel. The diffusion of oxygen through the gel matrix may be a limiting factor of bacitracin production. In this study, leakage of the bacteria from the gel is not desirable for bacitracin production. The growth of leaked bacteria was observed when a fermentation medium (SB) was employed for bacitracin production. Therefore, diluted peptone was chosen as the nutrient for the bacteria, and the growth of the leaked and contaminating bacteria could not be observed during the incubation time. The activity of bacitracin production increased with increasing utilization cycles of immobilized whole cells and reached a steady-state maximum. Furthermore, the apparent half-life of bacitracin production was estimated to be at least 1 week. On the other hand, the activation of bacitracin production was not observed when amino acids and ATP were used as substrates. Furthermore, the rate of activation increased as the amount of bacteria in the gel increased. These results suggested that the activation of bacitracin production was caused by the growth of bacteria in the gel. Therefore, the gel was examined by electron microscopy. The growth of the bacteria was observed at the inner layer (near the surface) of the gel, which was used for repeated batch production of bacitracin. The growth of the bacteria during incubation provides an obvious explanation for the increase in the activity of bacitracin production with successive utilizations. A steady state of bacitracin production could mean that the interstitial space of the gel was filled with active bacteria, or may be controlled by the diffusion rate of substrates and/or product through the gel. However, the detailed mechanism of a steady-state production of bacitracin is far from being fully understood.

The rate of bacitracin production by immobilized whole cells was slightly higher than that of common fermentation.

No attempt has been made to use the immobilized whole cells for a long time or to optimize the process for antibiotic production. Furthermore, carriers for immobilization of the bacteria

FIG. 5. Electron micrographs of the gel. Gel immediately after immobilization (a). Outer surface (b), inner layer near surface (c), and center of the gel (d) after 56 h of incubation.

which have good mechanical properties and good diffusibility of substrates must be considered in any further studies.

Further developmental studies in this laboratory are being directed toward applying the immobilized whole cells to a reactor for the continuous production of bacitracin.

LITERATURE CITED

1. Abbott, B. J. 1976. Preparation of pharmaceutical compounds by immobilized enzymes and cells. *Adv. Appl. Microbiol.* **20**:203-257.
2. Abbott, B. J. 1977. Immobilized cells, p. 205-233. In D. Perlman (ed.), *Annual report on fermentation processes*, vol. 1. Academic Press Inc., New York.
3. Chibata, I., and T. Tosa. 1977. Transformations of organic compounds by immobilized microbial cells. *Adv. Appl. Microbiol.* **22**:1-27.
4. Cornell, N., and J. E. Snoke. 1964. Biosynthesis of bacitracin and protein. *Biochim. Biophys. Acta* **91**:533-536.
5. Hoff, D. A., R. E. Bennett, and A. R. Stanley. 1947. A sensitive cylinder plate assay for bacitracin. *Science* **106**:551-552.
6. Karube, I., T. Matsunaga, S. Mitsuda, and S. Suzuki. 1977. Microbial electrode BOD sensors. *Biotechnol. Bioeng.* **19**:1535-1547.
7. Katz, E., and A. L. Demain. 1977. The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* **41**:449-474.
8. Karube, I., T. Matsunaga, S. Tsuru, and S. Suzuki. 1976. Continuous hydrogen production by immobilized whole cells of *Clostridium butyricum*. *Biochim. Biophys. Acta* **444**:338-343.
9. Kierstan, M., and C. Buche. 1977. The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotechnol. Bioeng.* **19**:387-397.
10. Mosbach, K., and P. Larson. 1970. Preparation and application of polymer entrapped enzymes and microorganisms in microbial transformation process with special difference to steroid 11- β hydroxylation and Δ' dehydrogenation. *Biotechnol. Bioeng.* **12**:19-27.
11. Shimizu, S., H. Morioka, Y. Tani, and K. Ogata. 1975. Synthesis of coenzyme A by immobilized microbial cells. *J. Ferment. Technol.* **53**:77-83.
12. Slowinski, W., and S. E. Charm. 1973. Glutamic acid production with gel-entrapped *Corynebacterium glutamicum*. *Biotechnol. Bioeng.* **15**:973-979.
13. Vieth, W. R., S. Wang, and R. Saini. 1973. Immobilization of whole cells in a membraneous form. *Biotechnol. Bioeng.* **15**:565-569.