KOREAN J. FOOD SCI. TECHNOL.
Vol. 11, No. 3 (1979).

Studies on Whole Cell Immobilized Glucose Isomerase

I. Preparation and Properties of Whole Cell Immobilized Glucose Isomerase

Byung Yoon Ahn[1] and Si Myung Byun
Department of Biological Science and Engineering
The Korea Advanced Institute of Science, Seoul
(Received July 2, 1979)

포도당 이성화 효소의 세포 고정화에 관한 연구

Ⅰ. 세포 고정화 효소의 제조와 성질

안 병 윤[1] · 변 시 명
한국과학원 생물공학과
(1979년 7월 2일)

Abstract

With cells of *Streptomyces spp* K-45 isolated from soil, the immobilization of glucose isomerase by a series of treatments ; heat, carefully manipulated drying, extrusion with a thickening agent, and glutaraldehyde-induced crosslinking, was presented. This was aimed to obtain a mechanically stable form of whole cell containing glucose isomerase. The resulted pellet form had a good mechanical strength, compared with a commercial product, and showed 26 % of the activity recovery. The specific activity was 48.1 units per g of the dry material.

The immobilized glucose isomerase generally showed properties similar to those of the soluble enzyme ; optimal pH at 7.5~9.0, optimal temperature at 80~85°C, activation energy of 10.9 kcal/mole, and K_m for glucose of 10.9 M. The immobilized enzyme was very thermostable and pH stable.

Introduction

Glucose isomerase (hereafter called GI) have been immobilized by the various methods, entrapment of whole cell containing GI[1~4], covalent binding[5,6], entrapment of soluble enzyme[7~11], adsorption[12~15], cell aggregation[16], and heat treatment of cells[17~21]. Among them, the immobilization of whole cell containing GI rather than GI itself, has advantages especially in the industrial considerations. Isolation and the succeeding purification of the intracellular enzyme are tedious and difficult, regarding low overall yields and increased cost of the enzyme[1].

The problem encountered, however, is that whole cell immobilized product is physically unstable and dispersed in the aqueous solution by repeated usage. Although commercial pellet forms of the whole cell immobilized enzyme, which are physically stable, are available in the market, the technology is not open

[1] Present Address; Animal Products Technology Laboratory, The Korea Institute of Science and Technology, Seoul, Korea
현주소 : 한국 과학 기술 연구소 축산 가공 연구실

due to the company's "know-how" and hence the domestic manufacturers of isomerized sugar utilize the product of NOVO company[22].

In the present communication, we aimed to obtain a physically stable form of whole cell immobilized GI. Using a *Streptomyces* strain screened form soil, the enzyme was immobilized by a simple whole cell immobilization method and the subsequent reaction kinetics were studied.

Materials and Methods

Materials

Among the about 200 strains of *Streptomyces spp* and 60 strains of *Bacillus spp* isolated from soil, 2 strains showed 2.5~3.5 units of GI activity per ml of culture broth, while *Streptomyces albus* ATCC 21132 showed 0.5~1.0 units. By the morphological characteristics of colonies, we identified them tentatively as *Streptomyces spp* K-34 and K-45 respectively. The K-45 strain was used throughout the work.

Glutaraldehyde, L-cysteine and carbazole were from Aldrich Chem. Co., Inc. (Milwaukee, Wisconsin), and Glucostat from Worthington Biochem. Corp. (Freehold, New Jersey). All other chemicals used were of reagent grade.

Cultivation of Microorganism

Streptmyces spp K-45 was cultured in 14 *l* fermentor (New Bruinswick Sci., New Jersey) with a medium containing 1 % peptone, 1 % yeast extract, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.03 % $CoCl_2 \cdot 6H_2O$, and 0.3 % KH_2PO_4 (initial pH 7.2). The culture condition was 1.5 VVM of aeration and 400 RPM of agitation at 30°C for 40 hr. Cells were collected by the centrifugation of the culture broth at 5,000 × g for 20 min (Sorvall RC-5 refrigerated centrifuge), washed twice with 0.05 M phosphate buffer, pH 7.2 and resuspended in the same buffer.

Enzyme assay

Cells of K-45 strain were sonicated for 5 min at 15 KC with Artek-300 Sonic Disembrator and centrifuged at 10,000 × g for 30 min. The clear supernatant was used as a soluble enzyme after dialysis against water at 4°C.

The incubation mixture of the enzyme contained 1 ml of 0.1 M phosphate buffer (pH 7.2), 0.2 ml of 0.05 M $MgSO_4 \cdot 7H_2O$, 0.2 ml of 1 M D-glucose, 0.4 ml of distilled H_2O, and 0.2 ml of the enzyme solution in the final volume of 2.0 ml. Before the enzyme solution was added, the mixture was preincubated at 65°C for 10 min and whole reaction mixture was incubated for 1 hr. After incubation was completed, the reaction was stopped by adding 2.0 ml of 0.05 N perchloric acid solution. The concentration of fructose produced was determined by the cysteine-carbazole method[23] and the glucose determined with Glucostat (glucose oxidase-peroxidase system) for the backward reaction[24].

For the immobilized enzymes, the above assay system was scaled up 5 folds with 20~50 mg of the immobilized enzyme. The reaction mixture was stirred well during the reaction with a submerged magnetic stirrer. One unit of glucose isomerase activity was defined as the amount of enzyme which catalyzes the production of 1 μmole of fructose for 1 min under the condition of assay.

Immobilization

Heat treatment: To inactivate thermally unstable enzymes except GI and fix the enzyme to cell membrane, the resuspended cell (320 g) in 2.5 *l* of 0.05 M phosphate buffer, pH 7.2 was heated slowly to 65°C with vigorous agitation and maintained at 65°C for 15 min. The resulted cell was collected by the centrifugation and was washed twice with distilled H_2O.

Dehydration: The heat-treated cells were dehydrated either by lyophillization or anhydrous acetone to 10 % of its original weight. Since it was found that the water content of cell slurry was very important for the good agglomerization of the cell aggregates in the following extrusion step, 320 g of the heated cell was lyphillized for 24 hr to yield about 30 g of partially dehydrated cells.

Extrusion: To the partially dehydrated cell, 0.5 % (w/w) of soluble starch powder was added as a thickening agent and the mixture was mixed well. This slurry was extruded through a syringe with 0.8 mm of the needle diameter onto the glass plate. The fiber preparation was allowed to be dried for 10 hr at room temperature. The dried fiber was cut into small pellet forms (0.8 × 3.0 mm of size).

Crooslinking: The pellets were treated with various concentrations of glutaraldehyde in 0.05 M phosphate buffer, pH 7.2 for different times. The mixture was swirled gently with a glass rod during the reaction. The immobilized cell pellets were washed completely with water to remove the extra reagent and dried at room temperature for 12 hr. Final pellet form was stored at 4°C until used.

Kinetics

Kinetic parameters of the immobilized GI were determined by the method of Byun *et al.*[25] under the assay conditions described previously. Analyses of sugars were carried out by the cysteine-carbazole method for the forward reaction and by the Glucostat method for the backward reaction, respectively. As comparison, the soluble enzyme, which was prepared by the method of Takasaki[26], was also used in this study.

Results and Discussion

Whole cell immobilization

The GI from *Streptomyces spp* K-45 was an intracellular enzyme and remarkably stable in high temperature. No decrease in the enzyme activity was observed by the heat treatment at 65°C for 15 min. This heat stability of the enzyme was also found in other *Streptomyces* species and that the strain of the K-45 required xylose or xylan as an inducer for production of GI as was reported[4,7,17,20,26]. Fig. 1 shows that maximum activity of the enzyme per ml of culture broth was reached at 48 hr of incubation and the enzyme activity paralleled cell growth, hereafter.

Whole cell immobilization method is recommended for the intracellular enzyme[1], since it is not economical to extract, purify and immobilize the enzyme. Hence commercial products of whole cell immobilized GI are available and yet the technology remained in secret.

We felt it necessary to prepare mechanicllly stable forms of whole cell immobilized GI, since preliminary prepared products were mechanically unstable and dispersed easily in aqueous solution by repeated use Much efforts were done to solve this problem and we treated the cells of K-45 with a series of treatments; (a) heated the cell at 65°C for 15 min, (b) dehydrated to 10 % of the original weight, (c) added 0.5 % soluble starch and extruded with a syringe of 0.8 mm needle diameter, (d) dried and cut into pellets, and (e) crosslinked the pellets with 5 % glutaraldehyde for 3 hr. As shown in Fig. 2, final product showed excellent mechanical strength and this was not dispersed into suspension in water by repeated use (5 times). The overall recovery of the enzyme activity

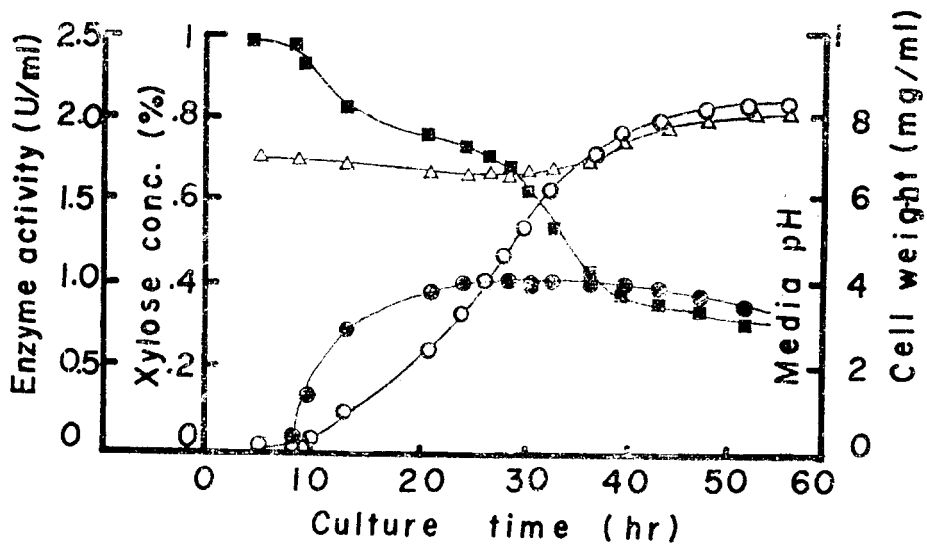

Fig. 1. Time course of cell growth and glucose isomerase production
Method for incubation of *Streptomyces spp* K-45 is described in the text(●-●, cell growth; ○-○, glucose isomerase activity; △-△, pH; ■-■, residual xylose concentration).

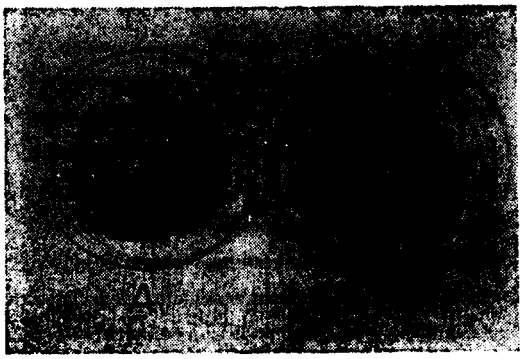

Fig. 2. Final preparations of whole cell immobilized glucose isomerase

A: Commerical product of NOVO Sweetzyme

B: Final preparation of whole cell immobilized glucose ismerase of *Streptomyces spp* K-45

on the immobilized steps was 26 % and the final preparation showed the specific activity of 48.1 units per g of dry material as summarized in Table 1.

Table 1. Results of whole cell immobilization

Enzyme preparation	Total amount (*l* or g)	Enzyme activity (unit/ml or g)	Total activity (unit)	Activity recovery (%)
Initial culture broth	10 *l*	1.13 (unit/ml)	11,285	100
Heat treated suspension	2.5 *l*	4.51 (unit/ml)	11,285	100
Immobilized enzyme	61 g	48.1 (unit/g)	2,934	26.1

Much studies were carried out on the immobilization of GI; entrapment of whole cell in polymers such as cellulose acetate, collagen, or polyacrylamide[1~4], cell aggregation of heat treatment[10~21], crosslinking with glutaraldehyde[1,4]. In this experiment, we modified the above methods and improved to prepare the pellet form by dehydration for good agglomerization of cells, and by extrusion, followed by crosslinking of the resulted pellets to obtain the mechanical strength.

It was found that the water content of the cell materials was very important for the extrusion process which determined the hardness and mechanical strength of immobilized whole cell matrix. Two methods, lyophillization and drying with acetone, were used and both methods resulted to form good pellets. Although the acetone treatment was a simple to use, a fluctuation of activity decrease to 70~98 % resulted in accordance to the delicate difference in the treating procedures and hence we used the method of lyophillization.

The partially dehydrated cell slurry was extruded through the needle of syringe and cut into small pellet forms after drying. Three main objectives were taken into consideration in the preparation of the immobilized enzyme, which were the activity recovery, good mechanical strength resulting in physical stability during the operation, and minimum mass transfer. Keeping a good mechical strength, proper additives were considered for the improvement of mass transfer of the extruded pellet. Among many candidates, some thickening agents (gelatin, agar, alginate, gums, and pectin), filter aid (celite), some biochemicals having functional amino groups or other functional groups (AE-cellulose, cyanuric acid, and 4,4′-diamine 2,2′-biphenylsulfonic acid), and some proteins (albumin and casein) were examined. Results showed no positive effects except the soluble starch. Soluble starch, therefore, was used as a thickening agent in the extrusion process.

It was also very important to determine the concentration and treating time of glutaraldehyde with respect to the deactivation of the enzyme during crosslink and mechanical strength of pellet of cells. As was suggested[27], glutaraldehyde seemed to react with amino groups in cellular membrane and enzymes. To determine the relationship between the concentration of glutaraldehyde and the enzyme activity, therefore, the whole cell suspension was treated in various concentrations of the reagent for 3 hr while gently swirling. The cells were washed with water to remove the unreacted reagent and the enzyme activity was determined with part of preparation. The rest of cell suspension was sonicated to extract the enzyme which was not crosslinked and centrifuged to separate the supernatant and the disrupted cell debris. The enzyme activities of both were determined to know the degree of crosslinking. As shown in Fig. 3, almost 75 % of enzyme activity was inactivated by treatment of 0.4 % glutaraldehyde and remained constant. The preparation which was obtained by treatment with

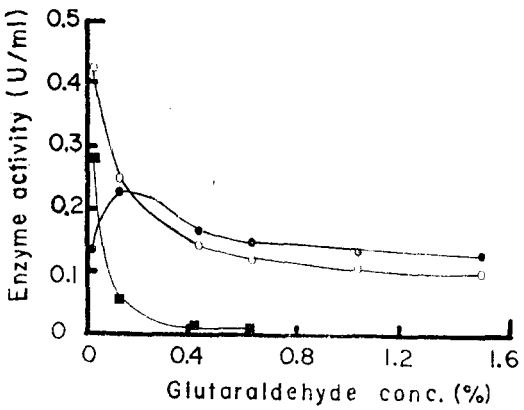

Fig. 3. Effect of glutaraldehyde concentration on crosslink of enzyme to membrane
The whole cells were treated with various concentrations of glutaraldehyde in 0.05 M phosphate buffer, pH 7.2 at 25°C for 3hr. The unreacted glutaraldehyde was removed by centrifugation and washing with water. Total activity of the treated cell was determined(○-○). The treated cell was sonicated for 5 min at 15 KC to extract the enzyme which was not bound to cell membrane. The soluble portion(■-■) and cell debris (●-●) were separated by centrifugation and the enzyme activities of each portion were determined.

0.6 % glutaraldehyde for 1 hr or 0.1 % for 3 hr showed the highest activity.

To determine the effect of treating time of glutaraldehyde on immobilization, the whole cell was treated

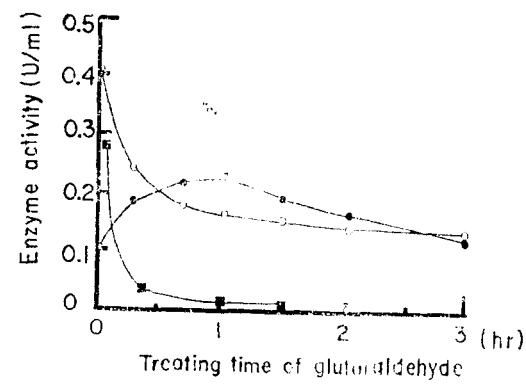

Fig. 4. Effect of treating time of glutaraldehyde on crosslink of enzyme to membrane.
The same procedure was carried out as described in Fig. 3 except using different period of time with 1 % of glutaraldehyde. Total enzyme activity of whole cell (○-○); the enzyme activity of soluble portion(■-■); after sonication the enzyme activity of cell debris after sonication (●-●).

with 1 % glutaraldehyde for various times. As was described in Fig. 3, the soluble and immobilized fraction were tested for activities. As shown in Fig. 4, most of the enzyme was bound to cell membrane within 1 hr and was not extracted into supernatant by sonication, while 70 % of the enzyme activity was extracted from the untreated cells (0 time).

Although the highest activity was obtained by the treatment with 0.6 % glutaraldehyde for 1 hr from Fig. 3 and 4, this treatment did not result to form the acceptable mechanical strength of final pellets, which may determine the operational strength and packing properties. Therefore, we treated the extuded pellets with 5 % glutaraldehyde for 3 hr and this treatment gave very excellent mechanical strength of the final products.

Fig. 5 shows the final preparations of whole cell immobilized GI treated with different concentrations of glutaraldehyde. Final pellet forms of the whole cell immobilized GI showed a good mechanical strength and operational stability compared with a commercial product. This immobilized enzyme, however, showed a drawback of diffusional limitation and this result will be presented in the second part of this study elsewhere with the operational stability[29].

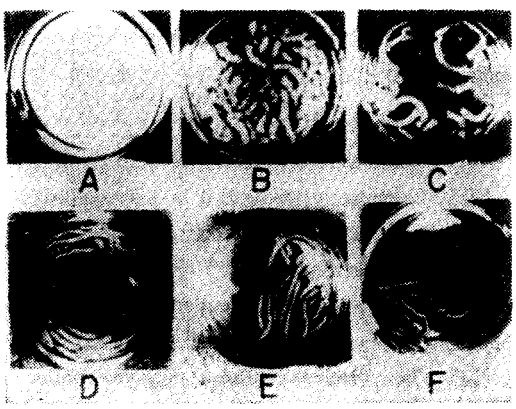

Fig. 5. Final preparations of whole cell-immobilized glucose isomerase treated with various concentrations of glutaraldehyde.
A: No glutaraldehyde treatment
B: Treated with 0.1% glutaraldehyde
C: Treated with 0.5 % glutaraldehyde
D: Treated with 1 % glutaraldehyde
E: Treated with 3 % glutaraldehyde
F: Treated with 5 % glutaraldehyde

Since this work was primarily concerned on the mechanical strength of the final products, further work should be carried out to improve the mass transfer. One possibility seems that improvement of the extruder, which extrudes it from the compressed reservoir into vacuum chamber, will pupped the fiber of cell aggregates to form fine porosity of the fiber.

Characteristics of the immobilized enzyme.

Using the GI preparation of the K-45 which was whole cell immobilized, some kinetic parameters were determined. Comparative studies were also carried out with the soluble enzyme. As results, there were no great differences between both preparations as shown in Table 2.

Glucose isomerase from the K-45 was observed to be a D-xylose isomerase which showed better affinity to xylose and acted more preferentially on xylose than glucose[7,28]. Other kinetic parameters were agreed closely with the values reported for the free and cell bound enzymes of *Streptomyces*[6,7,17,20].

The heat stability of the whole cell immobilized and soluble enzyms was examined by incubating test tubes containing each enzyme solution respectively at temperatures from 50 to 90°C for 24 hr at pH 7.2.

Table 2. Kinetic parameters of the whole cell-immobilized glucose isomerase of *Streptomyces spp* K-45

	Soluble enzyme	Whole cell immobilized enzyme
Optimum pH	7.8~8.0	7.5~9.0
Optimum temperature(°C)	80~85	80~85
E_a(kcal/mole)	14.9	10.6
K_{mf}(M)	0.133	0.227
V_{mf}(μmole/min)	0.712	0.255
K_{mb}(M)	0.309	0.465
V_{mb}(μmole/min)	0.371	0.476
K_{eq}	1.207	1.097
K_m for xylose(M)	0.059	—
V_m for xylose(μmole/min)	3.238	—

E_a: activation energy
K_{mf}: Michaelis constant for forward reaction
V_{mf}: maximum forward reaction rate
K_{mb}: Michaelis constant for backward reaction
V_{mb}: maximum backward reaction rate
K_{eq}: equilibrium constant
V_m: maximum reaction rate

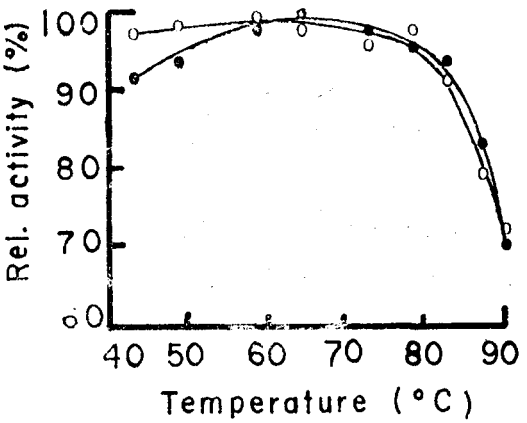

Fig. 6. Effect of temperature on the stability of enzymes
Immobilized enzyme(●-●) and soluble enzyme (○-○) were incubated for 24 hr in 0.05 M phosphate buffer (pH 7.2). Residual activity was determined at the enzyme assay condition described in the text.

Residual activities were determined at the assay condition (temperature 65°C). As shown in Fig. 6, both enzyme preparations gave good heat stability to 80°C.

The pH effect on the stability of the enzymes was also examined by incubating them at pHs from 4 to 10 for 12 hr at 70°C and the residual activities were determined at the assay condition (pH 7.2). As results, the enzymes were the most stable at pH 7.0 and

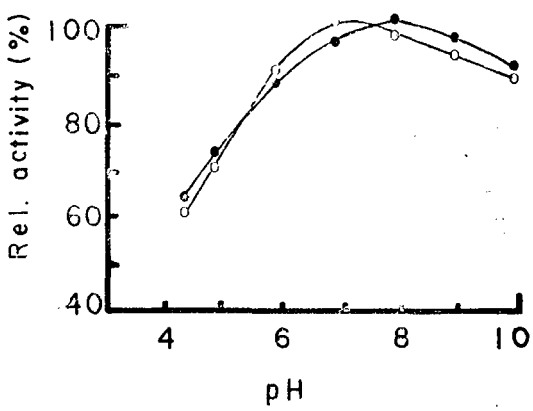

Fig. 7. Effect of pH on the stability of enzymes
Immobilized enzyme (●-●) and soluble enzyme (○-○) were incubated at 70°C for 12 hr in 0.05 M phosphate buffer of different pH. Residual activity was determined at the enzyme assay condition described in the text.

the immobilized one showed slightly broader pH stability than the soluble one (Fig. 7).

In this study we obtained a mechanically stable form of whole cell immobilized GI which showed good pH and heat stabilities and operational stability[29]. The preparation, however, showed little lower activity recovery to compare with the preparations by other methods[2,9,15]. Further studies should be continued to solve this drawback.

요 약

비교적 높은 역가의 포도당 이성화 효소를 생산하는 방사선균을 토양에서 선별하여 이성화 효소의 세포 고정화를 행하였다. 특히 최종 제품(pellet form)의 물리적 견고성을 얻기 위하여 세포를 65℃로 15분간 열처리하고 선택적 건조를 행하여 얻은 세포 slurry를 가용성 전분과 섞은후 사출시켜 pellet form으로 만들었다. 5% glutaraldehyde를 가교제로서 pellet 균과를 3시간 처리함으로 효소의 세포 고정화를 이룩하였다. 최종 제품은 물리적 견고성이 양호하였고 효소의 회수율은 26 %였으며 비활성도는 건물 g당 48.1 단위였다.

세포 고정화시킨 이성화 효소는 가용성 효소와 매우 유사한 효소학적 성질을 보여 주었다. 즉 최적 pH ; 7.5~9.0, 최적 온도 ; 80~85℃, 활성화 에너지 ; 10.9 kcal/mole, 포도당에 대한 K_m 값 ; 10.9 M이었다. 고정화 효소는 열안정과 pH 안정성이 양호함을 보여주었다.

References

1. Vieth, W. R., Wang, S. S. and Saini, R.: *Biotech. Bioeng.*, **15**, 565 (1973)
2. Kolarik, M. J., Chen, B. J., Emery, Jr., A. H. and Lim, H. C.: in "*Immobilized Enzyme in Food and Microbial Process*", Edited by Olson, A. S. and Cooney, C. L., p. 71, Plenum Press, New York and London (1974)
3. Moskowitz, G. T.: *U. S. Patent*, 3,843,442 (1974)
4. Saini, R. and Vieth, W. R.: *J. Appl. Chem. Biotechnol.*, **25**, 115 (1975)
5. Havewala, N. B. and Pitcher, Jr., W. H.: in "*Enzyme Engineering*" Edited by Pye, E. K. and Wingard, Jr., L. B., vol. 2, p. 315, Plenum Press, New York (1974)
6. Lee, Y. Y., Fratzke, A. R., Wun, K. and Tsao, G. T.: *Biotech. Bioeng.*, **18**, 389 (1976)
7. Strandberg, G. W. and Smiley, K. L.: *Appl. Microbiol.*, **21**, 588 (1971)
8. Giovenco, S., Morisi, F. and Pansolli, P.: *FEBS Lett.*, **36**, 57 (1973)
9. Kent, C. A. and Emery, A. N.: *J. Appl. Chem. Biotechnol.*, **24**, 663 (1974)
10. Kasumi, T., Kawashima, K. and Tsumura, N.: *J. Ferment. Technol.*, **52**, 321 (1974)
11. Linko, Y. Y. and Pohjola, L.: *FEBS Lett.*, **62**, 77 (1976)
12. Messing, R. A. and Filbert, A. M.: *J. Agri. Food Chem.*, **23**, 920 (1975)
13. Messing, R. A. and Horseheads, N. Y.: *U. S. Patent*, 3,868,304 (1975)
14. Park, Y. K. and Toma, M.: *J. Food Sci.*, **40**, 1112 (1975)
15. Huitron, C. and Limon-Lason, J.: *Biotech. Bioeng.*, **20**, 1377 (1978)
16. Lee, C. K. and Long, M. E.: *U. S. Patent*, 3,821,086 (1974)
17. Takasaki, Y., Kosugi, Y. and Kanbayashi, A.: in "*Fermentation Advances*" Edited by Perman, D., p. 561, Academic Press, New York (1969)
18. Takasaki, Y. and Kanbayashi, A.: *Japanese Patent*, 19,086 (1972)
19. Hamilton, B. K., Colton, C. K. and Cooney, C. L.: in "*Immobilized Enzymes in Food and Microbial Process*" Edited by Olson, A. C. and Cooney, C. L., p. 85, Plenum Press, New York and London (1974)
20. Park, Y. K., Chisaki, C., and Moretti, R. H.: *J. Food Sci.*, **41**, 1383 (1976)
21. Kim, S. M.: *M. S. Thesis of Korea Adv. Inst. of Sci.*, (1978)
22. Personal communication to Miwon Corp., Lucky Corp. and Sunil Company (1978)
23. Dische, Z. and Browenfreund, E.: *J. Biol. Chem.*, **192**, 583 (1953)
24. Worthington Biochem. Corp.: "*Glucostat for the Enzymatic Determination of Glucose*" New Jersey, U. S. A. (1972)

25. Byun, S. M. and Wold, F.: *J. Korean Food Sci. Technol.*, 8, 253 (1976)
26. Takasaki, Y.: *Agr. Biol. Chem.*, **30**, 1247 (1966)
27. Richards, F. M. and Knowles, J. R.: *J. Mol. Biol.*, **37**, 231 (1968)
28. Takasaki, Y., Kosugi, Y. and Kanbayashi, A.: *Agr. Biol. Chem.*, **33**, 1527 (1969)
29. Ahn, B. Y. and Byun, S. M.: Submitted to *J. Korean Food Sci. Technol.*, **11**, (4), (1979)