Journal of Solid-Phase Biochemistry, Vol. 2, No. 3, 1977

PREPARATION AND PERFORMANCE OF CELLULOSE BEAD-ENTRAPPED WHOLE CELL GLUCOSE ISOMERASE[1]

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Accepted January 16, 1978

A novel simple and mild technique to entrap glucose isomerase active *A. missouriensis* cells within α-cellulose beads has been developed. Regenerated α-cellulose beads were shown by SEM to be highly loaded with microbial cells. Kinetics, high operational stability, reactor performance, and low cost of carrier showed the entrapped cell preparation to be suitable for continuous isomerization of 45% glucose solution.

INTRODUCTION

Immobilization of glucose isomerase (GI) for production of high-fructose syrup has been studied intensively for the past few years (1–3). Immobilization techniques employed include adsorption, entrapment, covalent bonding, and/or cross-linking. Both purified and whole cell glucose isomerase have been immobilized. As carrier, microbial cells (4–6), DEAE Sephadex (7), porous glass (8, 9), DEAE-cellulose (10, 11), collagen (12), cellulose (13, 14, 16), cellulose triacetate (17), phenol formaldehyde resins (18), control pore alumina (19), gelatin (20), and so on, have been suggested. Most research has involved *Bacillus* and *Streptomyces* species enzymes. We have succesfully developed a simple and mild technique to entrap whole cell *Actinoplanes missouriensis* GI in cellulose fiber (15, 16). The activity recovery of immobilized glucose isomerase was 40–60%, and a half-life of 42 days in continuous plug flow reactor operation could be demonstrated for glutaraldehyde-treated fiber entrapped enzyme.

[1]This paper was presented at the 4th Enzyme Engineering Conference, Bad Neuenahr, Germany. Sponsored by the Engineering Foundation, New York, New York.

Further advantages in reactor kinetics and performance in practical operation could be realized with an immobilized cell preparation in bead form. This article describes preparation of α-cellulose in bead form with entrapped whole cell *A. missourienses* glucose isomerase and characterization of the immobilized enzyme. This is believed to be the first time an enzyme has been immobilized within regenerated pure cellulose beads.

MATERIALS AND METHODS

Materials

α-Cellulose (DP 880) was obtained from Rauma-Repola Inc., Finland. Dimethylformamide (Merck A.G.) was purified by vacuum distillation, and *N*-ethylpyridinium chloride was prepared from equimolar quantities of pyridine and ethylchloride at 77°C. Whole cell glucose isomerase (EC 5.3.1.5) Maxazyme GI, *Actinoplanes missouriensis*, activity 1000 μmol/min·g, 16.7 μkat/g was a gift from Gist-Brocades N.V.

Glucose Isomerase Activity Assay

Whole cell glucose isomerase was incubated for 10 min at 65°C in 1 ml of pH 6.8 0.25 M maleate buffer containing 0.1 M $MgSO_4 \cdot 7H_2O$ and 0.13 M KCl. After adding 1 ml of 10% glucose in the same buffer containing in addition 0.002 M $CoCl_2 \cdot 6H_2O$, the reaction was carried out for 20 min with magnetic stirring. The reaction was stopped with 10 ml of 0.1 M perchloric acid. Fructose produced was then determined by the cystein-carbazole method (21). For blank, glucose was added after perchloric acid. Activity is expressed as micromoles per minute per gram of dry weight (105°C, 2 h) of enzyme cellulose beads under these conditions.

Preparation of Cellulose Bead-Entrapped Whole Cell Glucose Isomerase

Three grams of α-cellulose (DP 880) was dissolved in a melt of 150 g of *N*-ethylpyridinium chloride (NEPC) and 75 g of dimethylformamide (DMF) according to the method of Linko et al. (15). After dissolution, temperature was lowered to 30°C, followed by immediate addition of 9 g of dry (5.6% H_2O) cells and removal of air by vacuum suction. Whole cell GI cellulose was then transferred to a stainless steel cylinder of 4-cm dimaeter fitted with perforated Teflon disks with orifices of ϕ 0.8 mm. The contents were forced through the holes by slight application of pressure into water at 23°C. The dropping distance was 3–5 m. Whole cell GI cellulose emer-

ged out as streams, breaking during dropping into short pieces that formed into beads before reaching the water's surface.

The enzyme-containing beads were further treated with 2.5% glutaraldehyde in 0.1 M phosphate buffer of pH 7.0 at 23°C for 1 h to "fix" the cell wall and to cross-link enzyme molecules. The treated beads were washed thoroughly with water and vacuum-dried at 35°C to about 21% dry weight.

Apparent Michaelis–Menten Constants K_{mf} and K_{mb}

Apparent K_m for forward and backward reactions for free and entrapped cell GI was measured using a batch process at 65°C, pH 7.5, 0.003 M Mg^{2+} and 0.0003 M Co^{2+}. The initial rate of forward reaction at various initial substrate concentrations was determined by the cysteine-carbazole method for fructose (20). For backward reaction, the glucose formed was determined by the glucose oxidase peroxidase method (22).

Pressure Drop across the Column

Glucose solution (45%, 60°C) was pumped through the packed column reactor by Multifix pump type R 102 using various linear flow rates. Pressure drop across the column was measured both by mercury manometer and a pressure gauge.

Scanning Electron Microscopy

Japan Electron Optics Laboratory Company JSM-U3 scanning electron microscope (SEM) operated at an acceleration voltage of 15 kV was employed. Bead samples (21% dry weight) were dehydrated by an ethanol–water mixture of gradually increasing ethanol concentration (10, 40, 70, and 100%), followed by amyl acetate–ethanol mixtures with increasing amyl acetate concentration (10, 40, 70, and 100%) and finally by critical point drying. Dehydrated samples were then coated with a thin layer of carbon and gold.

Continuous Isomerization

Continuous isomerization of 45% glucose solution (pH 7.5, 0.003 M Mg^{2+}, 0.0003 M Co^{2+}, 0.1% methyl-*p*-hydroxybenzoate, and 0.01% propyl-*p*-hydroxybenzoate) was carried out up to 40 days in two parallel jacketed glass columns (A, 20 × 89 mm and B, 15 × 156 mm) with a G3 glass filter at the bottom. Before packing of column reactors, whole cell

GI cellulose beads (bulk density ~0.67 g/ml) were soaked in 45% glucose solution for 30 min, and air was removed by vacuum at 60°C. Equal total activities (713 μmol/min) were used in both columns. Column temperature was maintained at 60°C. The reactor system was equipped with a preheating coil and air remover.

RESULTS AND DISCUSSION

Preparation of Whole Cell GI Cellulose Beads

Cellulose and cellulose derivatives have been widely used in chromatography and in immobilization of enzymes, mostly as fiber or powder for absorption and covalent binding of purified enzymes. To improve operational characteristics, cellulose in bead form has been prepared. Known methods for the preparation of cellulose beads, such as viscose and cuprammonium processes (23–25), are too severe for entrapment of biologically active cells or enzymes. Other methods have involved cellulose derivatives as raw material, requiring hydrolysis after bead formation (14) and activation of cellulose beads before immobilization. The present method of dissolving cellulose in a melt of *N*-ethylpyridinium chloride–dimethylformamide mixture (15, 16) is simple and mild, and thus suitable for entrapment of biologically active whole cells.

Bead size distribution was affected by applied nitrogen pressure, viscosity of cellulose whole cell suspension, and the dropping distance to water. Nitrogen pressure of 0.1 kp/cm^2, suspension viscosity of 1400 cP, and dropping disatance of 3.5–5.0 m were found to be suitable to obtain beads 1–2 mm in diameter. After washing three times with 4 liters of water, neither solvent nor salt was detected.

Cross-linking with 2.5% glutaraldehyde in 0.1 M phosphate buffer of pH 7 for 1 h at 23°C did not decrease enzyme activity. Vacuum drying of GI cellulose beads (dry weight 6%) to dry weight of about 21% had no effect on total activity. Drying reduced bead size from about 1–2 mm to 0.7–1.4 mm.

Apparent Michaelis–Menten Constant K_m for Forward (K_{mf}) and Backward (K_{mb}) Reactions

Lineweaver–Burk plots of initial reaction rate data for both whole cell and entrapped cell GI for forward and backward reactions are shown in Figs. 1 and 2. A value of 0.83 M was obtained for K_{mf} and of 1.02 for K_{mb} for free and immobilized enzymes, indicating no effect of entrapment on enzyme–substrate complex formation and no significant external diffusion

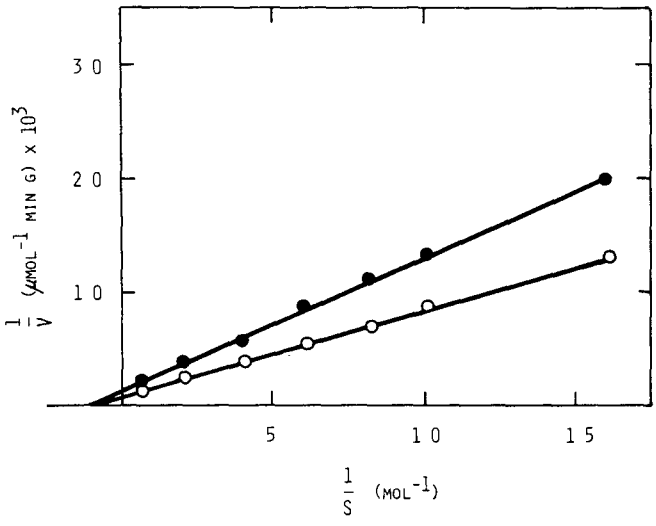

FIG. 1. Lineweaver–Burk plots of free (○) and immobilized (●) glucose isomerase for forward reaction glucose to fructose.

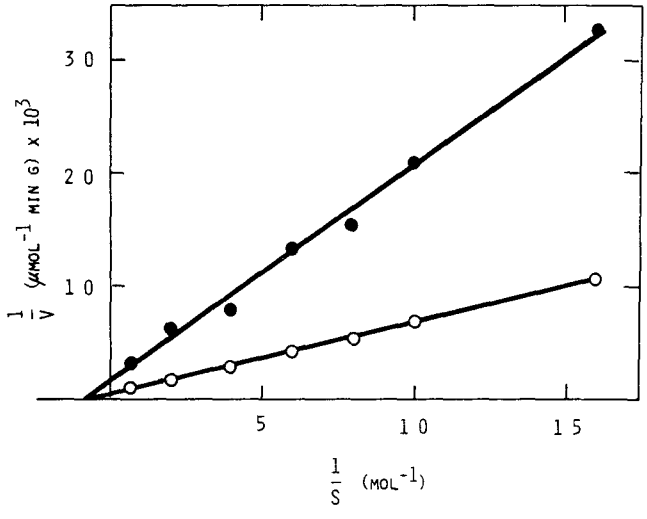

FIG. 2. Lineweaver–Burk plots of free (○) and immobilized (●) glucose isomerase for backward reaction fructose to glucose.

effect at employed reaction conditions. A K_{mf} of 0.83 has also been reported for cellulose fiber-entrapped GI (16). Widely varying K_m values have been reported for glucose isomerase, depending on enzyme source and reaction conditions (see Table 1). In general higher values were reported for K_{mb} than for K_{mf}.

Scanning Electron Microscopy

Scanning electron micrographs obtained from partially dried whole cell GI cellulose beads (Fig. 3) showed *Actinoplanes* cells entrapped within beads in large quantities. Vacuum drying resulted in shrinkage of cellulose beads, and fine cracking of bead surface structure may be easily seen. This did not, however, have an adverse effect on activity. Gradual dehydration and critical point drying of beads preserved the structure well.

Pressure Drop across Column

The degree of pressure drop across the column is important in industrial-scale continuous operations. In order to obtain the maximum degree of conversion with a given enzyme preparation, pressure drop should be minimized. Effect of linear flow rate on pressure drop was investigated to determine the suitability for industrial applications. It could be observed that pressure drop and linear flow rate (0–1.6 cm/sec) were directly proportional, as shown in Fig. 4. Pressure drop across the bed at a linear flow rate of 1 cm/sec was of the same order of magnitude as that reported for diisocyanate-treated cellulose beads by Chen and Tsao (14). These authors also observed a marked hysteresis phenomenon both with microcrystalline and porous DEAE-cellulose particles. The hysteresis and

TABLE 1. **K_m Values for Glucose Isomerase**

Microorganism	K_{mf}(M)	K_{mb}(M)	Reference
For soluble enzyme			
S. albus	0.14	0.23	1
L. brevis	0.92		1
B. coagulans	0.09		1
For immobilized enzyme			
Streptomyces sp.	0.580	0.936	26
S. venezuelae	0.25	0.383	12
Streptomyces sp.	0.21	0.40	9
S. phaeochromogenas	0.238	0.49	27

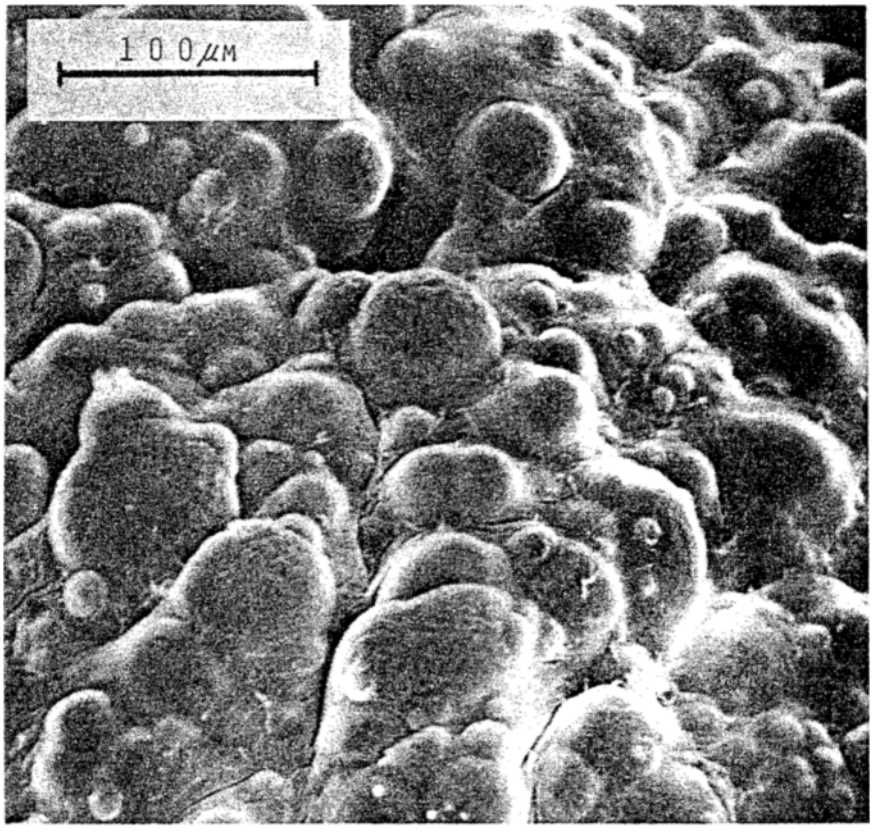

FIG. 3. Scanning electron micrograph of *A. missouriensis* cells entrapped within α-cellulose beads.

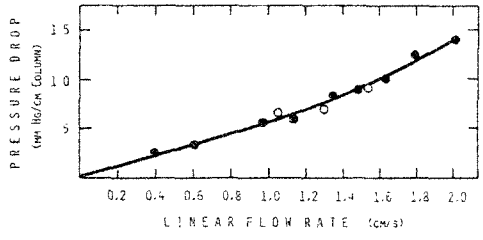

FIG. 4. Pressure drop in column of immobilized glucose isomerase at various linear flow rates. Linear flow rate increased (●), decreased (○).

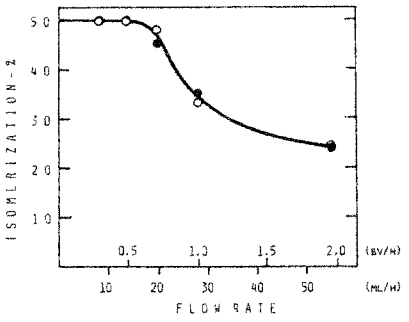

FIG. 5. Effect of flow rate on percent isomerization of 45% glucose with immobilized enzyme in column A (20×89 mm) (○) and column B (15×156 mm) (●).

compression of particles could be eliminted by diisocyanate treatment. No hysteresis was observed in the present work with a gradual linear flow rate decrease, and no compression or deformation of beads could be detected during operation.

Continuous Isomerization

The degree of isomerization was determined using various flow rates at different column diameter/height ratios (A, 1/4.4; B, 1/10.4). Figure 5 indicates that the rate of isomerization was virtually unaffected by nearly doubling the linear flow rate, indicating no significant external diffusion effect. As compared with a number of other preparations, the beads were neither compacted nor swollen, which allowed the reactor to be fully packed from the beginning, an advantage in industrial operations.

Operational stability of the two columns was equal (Fig. 6). A 50% conversion could be maintained for 23 days, followed by a gradual decrease that corresponds to a half-life of 42 days. Color formation during

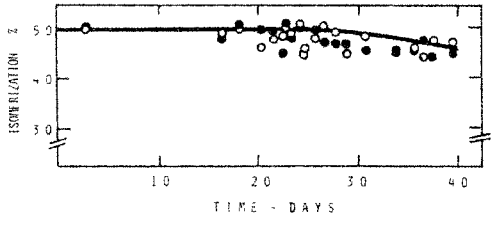

FIG. 6. Continuous isomerization of 45% glucose with immobilized enzyme in column A (○) and column B (●) (pH 7.5, 60°C, flow rate 8.4 ml/h, 0.003 M Mg^{2+}, 0.0003 M Co^{2+}).

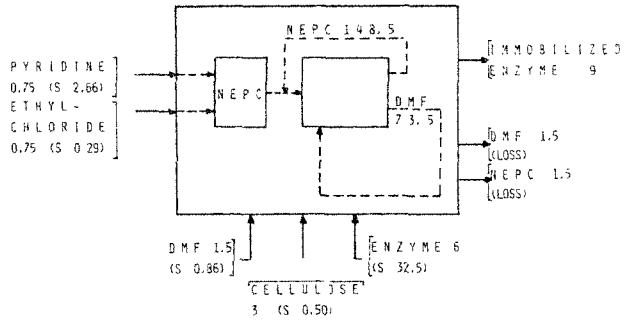

FIG. 7. Material balance (kg) of entrapment of *A. missouriensis* cells in α-cellulose beads.

the process was negligible as indicated by the absorbance of the syrup of about 0.01 at 420 nm.

Material Costs in Entrapment

Chen and Tsao (14) reported that cellulose beads prepared by dissolving cellulose acetate in acetone and dimethylsulfoxide, followed by precipitation from water, and regeneration to cellulose by hydrolysis, cost about \$1.50/lb without enzyme (~\$3.30/kg). Material balance of entrapment with the new technique is shown in Fig. 7. Material costs for regenerated cellulose alone were approximately \$0.3/kg, assuming recycling of NEPC (with 99% recovery) and DMF (with 98% recovery), and for whole cell GI cellulose beads a total of \$4.2/kg. Thus, the cost of immobilized catalyst is influenced mostly by the cost of the enzyme.

ACKNOWLEDGMENT

Financial support from the Academy of Finland and Neste Oy Foundation is gratefully acknowledged.

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