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Fructose production by immobilized *Arthrobacter* cells

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SUMMARY

Immobilized *Arthrobacter* cells (NRRL-B-3728) were used for continuous isomerization of glucose to fructose in a bioreactor system. The system utilized stationary phase (55 h) cells (2.2×10^9 CFU/ml saline) immobilized onto K-carrageenan (3% w/v) beads [cells were heated at 65°C for 10 min to inactivate endogenous proteolytic enzymes]. Immobilized-cell preparations were hardened using three different glutaraldehyde systems. Glutaraldehyde (0.2 M) treated-immobilized cells (pH 7.0, 5°C for 30 min) exhibited good gel strength and high glucose isomerase activities. Maximal bioreactor isomerization of 44% was achieved when a buffered feedstock containing 40% glucose was fed into the column (60°C) at a flow rate of 0.2 ml/min. The biological half-life of glucose isomerase activities in this system was 400 h. Scanning electron microscopy revealed large numbers of cells distributed within the beads. A thin layer surrounding the beads following glutaraldehyde treatment was mainly due to cross-linking reactions between cell proteins and glutaraldehyde. This layer prevented leaking of cells during continuous isomerization reaction.

INTRODUCTION

Enzymatic isomerization of glucose to fructose received considerable attention due to the high sweetening index of fructose [22]. Glucose polymers such as starch are hydrolyzed to glucose and then used for this isomerization [8].

Many microorganisms (bacteria and actinomycetes) are capable of producing glucose isomerase [6,8,9,19]. Various techniques of immobilizing either partially purified glucose isomerase (GI) or cells were reported [11,14,16,17,23,25]. Several brands of immobilized GI products are also commercially available and these include: Sweetzyme (*Bacillus coagulans*) of Novo Industri A/S, Maxazyme GI-Immob (*Actinoplanes missouriensis*) of Gist Brocades N.V., Taka-Sweet (*Streptomyces olivaceous;* no longer available), Taka-Sweet FM (*Flavobacterium arborescens*) of Miles laboratories Inc., Ketozyme (*Actinoplanes missouriensis*) of Universal Oil Products, Inc. and Optisweet 22 (*Streptomyces rubiginosus*) of Kali-chemie [27]. The methodology

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used for manufacturing of each preparation is quite different. The first three products are derived from whole or homogenized cells and each immobilization process is coupled with a particle formation technique [4,5,12], while the others are derived from purified soluble GI which is covalently bound to chemically modified matrices such as alumina oxides [24]. However, new effective immobilization methods are necessary to improve efficiency and economics of the process [3]. This communication describes the use of such a process using *Arthrobacter* cells for continuous isomerization of glucose to fructose in a bioreactor system. Factors affecting the immobilization of the organism for optimal bioreactor operation were also examined.

MATERIALS AND METHODS

Arthrobacter spp. NRRL-B-3728 was allowed to grow in medium [26] containing (g/l): glucose, 30; peptone, 10; yeast extract, 1.0; $(NH_4)_2HPO_4$ 10.6; KH_2PO_4, 3.6; and $MgSO_4 \cdot 7H_2O$, 0.37 and desired pH. Glucose and magnesium sulfate were separately sterilized (121°C, 15 min) and then added to the sterile medium. The same culture media of desired pH and glucose level were incubated aerobically in a shaker water bath. Growth was monitored by plating suitable dilutions on surface of Tryptic Soy Agar plates (TSA, Difco Laboratories, Detroit MI) and incubating for 48 h at 30°C.

Cell immobilization

Two systems were used: bead type for the bioreactor system and cylindrical type for physical testing. *Arthrobacter* cells (30.0 g, wet weight) obtained from stationary phase (55 h) were washed and suspended in 30 ml sterile saline (2.2×10^9 cells/ml) preheated to 50°C. Sterile colloidal K-carrageenan (6.0 g/134 ml saline) kept at 60°C was added to the cell-suspension and mixed well. The mixture was then converted to 5 mm beads by pipetting it (dropwise at a constant rate) into one liter sterile, cold (4°C) ammonium phosphate buffer (0.04 M, pH 7.0) containing 0.3 M KCl. After equilibration, the beads were hardened using 0.2 M glutaraldehyde and packed in the jacketed glass column (3.3 × 37 cm) of the bioreactor system.

For the cylindrical type, K-carrageenan gel-cell mixture was poured into a plastic cylinder consisting of 8 small ones (1.0 × 1.9 cm each) stacked vertically and taped in place. The packed cylinder was kept for 0.5 h at 4°C, and the tapes surrounding each of the small cylinders were removed and the gel cylinder sliced using thin thread to obtain small gel cylinders. The small cylinders were then equilibrated for 0.5 h in cold ammonium phosphate buffer (0.04 M, pH 7.0) containing 0.3 M KCl and used for gel strength determination. This was conducted by ascertaining the force (Newtons) required to destroy the gel as measured with an Instron Universal Testing Instrument (Model 122, Instron Corp., Canton, MA) equipped with a disc plate plunger 6.0 cm in diameter.

Assay for glucose isomerase and proteolytic enzymes

The *Arthrobacter* cells, harvested from specified growth phase, were washed twice with sterile saline and resuspended in deionized water. For GI, one ml cell suspension was added to 4.0 ml reaction mixture containing 0.2 M glucose, 0.4 M $MgSO_4 \cdot 7H_2O$ in tris (hydroxymethyl aminomethane) buffer (pH 8.0, 0.05 M) and incubated for 30 min (70°C) in a shaker water bath. Five ml of 10% aqueous trichloroacetic acid (Baker Chemical Co., Phillipsburg, NJ) solution was then added to stop the reaction. Fructose formed was determined [7] and reported as μmol fructose/mg protein/30 min incubation. Assay of GI in immobilized cell preparations was conducted by adding 10.0 ml reaction mixture to 5.0 g immobilized *Arthrobacter* cells and fructose determined using the same procedure except for stopping the reaction by removal of the beads. Specific activities were reported as μmol fructose/g beads/30 min. For proteolytic enzymes, 10.0 g wet cells were macerated in a cold mortar using an equal weight of powdered quartz sand and distilled water, followed by centrifugation for 20 min at 34 800 × *g*. The macerated cells were extracted twice, supernatants combined and protein was determined using the modified Lowry method reported by Hebert et al [10]. Proteolysis was as-

sayed following the method of Kunitz [15]. Specific activities in the supernatants were reported as ΔO.D. (280 nm)/mg protein/l h incubation.

Effect of hardening treatments on GI and gel strength of immobilized-cells

Three treatments (A, B, and C) were used to examine the role of different glutaraldehyde levels (0.1–0.5 M) on both the strength of the immobilized cell preparations and their GI activites. In A, the glutaraldehyde was in aqueous solution and reaction performed at 30°C for 2.0 h in shaker water bath; in B and C, the glutaraldehyde was in ammonium phosphate buffer (pH 7.0, 0.5 M) and reaction was conducted at 10°C for 60 min and 5°C for 30 min, respectively. In all cases the immobilized cells were added to the glutaraldehyde solutions containing 0.3 M KCl at a ratio of 3:5 (w/v) to prevent gel distortion. After each treatment, excess glutaraldhyde was removed by washing with ammonium phosphate buffer (0.04 M, pH 7.0) containing 0.3 M KCl. Samples were examined for both gel strength and GI activities.

Continuous isomerization of glucose

Following a 0.2 M buffered glutaraldehyde treatment for 30 min at 5°C, immobilized-cell beads were packed into the column. Feedstock (FS) containing the desired glucose level, 0.02 M Mg^{2+} and 0.3 M KCl in 0.05 M tris buffer of specified pH kept at 60°C (or otherwise stated) was fed to the column by continuously pumping the FS upward using a peristaltic pump at a known flow rate. The effluent from the top of the column was also recycled, when necessary, and fructose levels determined at intervals. The half-life of the column (defined as the time [in h] required for the bioreactor to lose 50% of its initial GI activities) was determined at 10 h intervals.

Other assays procedures

Glucose was determined using the glucose oxidase method [1], and total reducing sugar by the Bernfield procedure [2].

Scanning electron microscopy (SEM)

The SEM was used to examine the surface and interior of the gel beads before and after cell immobilization and hardening treatments. Whole beads and/or bead sections were fixed by immersion in 2.5% glutaraldehyde sodium phosphate buffer (0.13 M, pH 7.3) for 1 h. The beads were then washed in three changes of buffer and fixed in 2% osmium tetroxide for 1 h, washed again, and dehydrated using an ascending series of ethanol levels (50, 70, 80, 90 and 100%, v/v) for 15 min at each step. Beads were dried (30 min) at critical point in a Samdre 780A critical point dryer (Tousimis Research Corporation, Rockville, MD) using liquid carbon dioxide. Specimens were mounted on aluminum stubs, coated with 25 mm of a gold-platinum mixture (8:2 ratio, w/w) in a Hummer Sputter Coater (Alexandria, VA) and examined using Phillips 505 SEM at 15.1 kV.

Statistical analysis

Results obtained were subjected to one-way analysis of variance [21].

RESULTS

Growth and GI synthesis

In early log phase, the relative specific activity of GI was 75.7% of maximal and persisted to early stationary phase. Maximal specific GI activities of 21.7 μmol fructose/mg protein/30 min (considered herein as equal to 100% relative specific activities) were noted (Fig. 1) as growth reached late stationary phase (55 h). The pH of the medium was not altered during growth, whereas residual glucose level in medium after 60 h incubation was 59.0% of the initial value.

Characterization of proteolytic activities

The crude enzyme extract of *Arthrobacter* cells exhibited pH optima at 8.0 and 6.0 (Table 1). When the crude enzyme extract was heated at 65°C, a sharp decline in the relative proteolytic activities

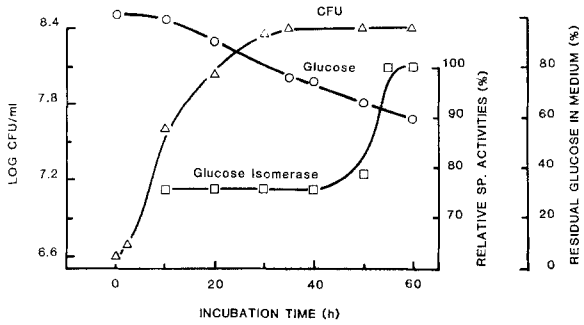

Fig. 1. Time-course study of growth and glucose isomerase synthesis by *Arthrobacter* spp during 60 h incubation at 30°C in a medium containing 3% glucose (pH 7.0). Maximal specific enzyme activity of 21.7 μmol fructose/mg protein/30 min was considered to be 100%.

(measured at pH 8.0) was observed, reaching 10% of original value after 10 min heating, and complete inactivation after 30 min. On the other hand, relative specific activities of GI decreased by 15 and 45% of original after heating (65°C) for 10 and 30 min, respectively. It is of interest to note that viable cell number after 10 min heating at 65°C in tris buffer (0.05 M, pH 7.0) containing 0.02 M Mg^{2+} was not affected, but 50 min heating at 65°C killed the entire population. However, 52% of the GI activities were detected in the heat inactivated cells, indicating the thermal stability of GI.

Table 1

Effect of pH on specific activities of proteolytic enzymes in crude extracts of *Arthrobacter* cells. Results are average of three experiments

Buffer used	pH value	Sp. activities[a] × 10^{-2} ± SD
Ammonium phosphate	5.5	1.0 ± 0.2
	6.0	2.4 ± 0.2
	6.5	1.0 ± 0.3
Tris	7.0	0.8 ± 0.2
	7.5	1.0 ± 0.1
	8.0	5.8 ± 0.2
	8.5	4.0 ± 0.2
	9.0	2.4 ± 0.3

[a] Δ O.D. (280 nm)/mg protein/1 h.

Hardening of immobilized beads by glutaraldehyde

In treatment A (Table 2), complete inactivation of the GI was evident at 0.1 M glutaraldehyde, and

Table 2

Effect of different glutaraldehyde levels and treatments on gel strength and GI activities of immobilized-cell preparation. Results are average of three experiments

Treatment used	Glutaraldehyde (M conc.)	Newton force applied (±SD)	Sp. activities[a] ±SD
A	0.0	93.2 ± 1.8	84.0 ± 2.1
(H_2O, 2 h, 30°C)	0.1	104.8 ± 2.5	0.0 ± 0.0
	0.2	130.8 ± 3.5	0.0 ± 0.0
	0.3	144.9 ± 3.3	0.0 ± 0.0
	0.5	157.3 ± 5.4	0.0 ± 0.0
B	0.0	93.1 ± 1.5	84.0 ± 1.8
(Buffer[b], 1 h, 10°C)	0.1	110.1 ± 2.8	65.9 ± 1.8
	0.3	111.1 ± 3.1	58.5 ± 2.0
	0.5	111.1 ± 2.7	51.1 ± 1.7
C	0.0	93.0 ± 1.6	84.1 ± 1.6
(Buffer[b], 30 min, 5°C)	0.1	100.0 ± 2.5	74.9 ± 1.4
	0.2	106.0 ± 3.4	71.5 ± 1.3
	0.3	106.1 ± 2.8	69.0 ± 2.3
	0.5	106.0 ± 2.9	64.4 ± 1.9

[a] μmol fructose/g beads/30 min.
[b] Ammonium phosphate buffer (pH 7.0, 0.5 M).

the applied forces required to destroy the gel rapidly increased at higher levels of glutaraldehyde. On the other hand, the C treatment resulted in higher residual GI activities and lower forces required to destroy the gel as compared to B. The use of 2.0 M glutaraldehyde in C treatment was therefore selected.

Continuous isomerization of glucose

Both glucose level and flow rate of feedstock influenced this reaction (Fig. 2A). Maximal equilibrium of 44% conversion was achieved using flow rates of 1.0 ml/min for FS containing 3.6% glucose and 0.2 ml/min for FS containing 40% glucose. The results also indicated that a flow rate of 0.2 ml/min

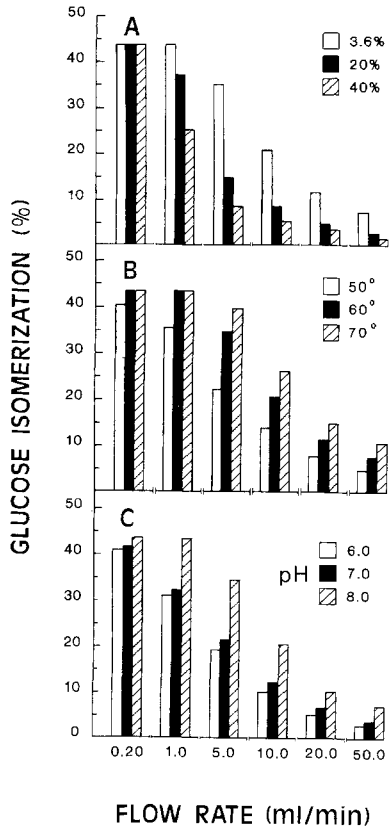

Fig. 2. Histograms showing factors affecting the continuous isomerization of glucose in bioreactor when feed stocks at specified flow rate were used. (A) represents glucose level in Tris buffer (0.05 M, pH 8.0) containing 0.3 M KCl and 0.02 M Mg^{2+}; (B) different temperatures (50, 60, or 70°C) in same tris buffer containing 3.6% glucose; (C) varied pH of same tris buffer with 3.6% glucose.

was required to reach equilibrium for all feedstocks, regardless of glucose level present. At flow rates of 50 ml/min or higher (data not shown), the isomerization reaction was low and almost the same due to short contact time between substrate and GI in the bioreactor. Fig. 2B shows that the percent isomerization in the effluent was 44% at both 60 and 70°C and flow rate of 1.0 ml/min, whereas at 50°C the reaction did not reach equilibrium even at a flow rate of 0.2 ml/min. Before achieving equilibrium, the percent isomerization at 70°C was always the highest. At flow rates of 50 ml/min or higher (data not shown), the results were almost the same due to the short contact time between the substrate and the enzyme. The data for effect of tris buffer pH at 60°C (Fig. 2C) indicate that maximal isomerization occurred at pH 8.0 and reached equilibrium at flow rate of 1.0 ml/min, whereas isomerization was 41.5 and 42.7% at FS flow rate of 0.2 ml/min using feedstocks of pH 6.0 and 7.0, respectively. The column exhibited a constant isomerization of 44% for 160 h, 39% for the following 120 h, and 34% for another 70 h. Thereafter, a gradual decline was noted, achieving 22% after 400 h (half-life) and reaching 15% after 420 h, when a feedstock (pH 8.0, 60°C) containing 40% glucose and 0.02 M Mg^{2+} was used.

Scanning electron microscope (SEM)

The SEM of the outer (Fig. 3A) and interior (Fig. 3B) surfaces of K-carrageenan gel beads containing *Arthrobacter* cells showed large numbers of cells. Surface cells were not visible following the glutaraldehyde hardening treatment (Fig. 3C). This may be due to the high cross-linking interaction between cell proteins and glutaraldehyde which also prevented the washout of cells from the surface of beads.

DISCUSSION

The data revealed that the test organism should be allowed to grow for 55 h in medium containing 3% glucose at pH 7.0 and 30°C to maximize the enzyme production. It was also noticed that 0.04 M

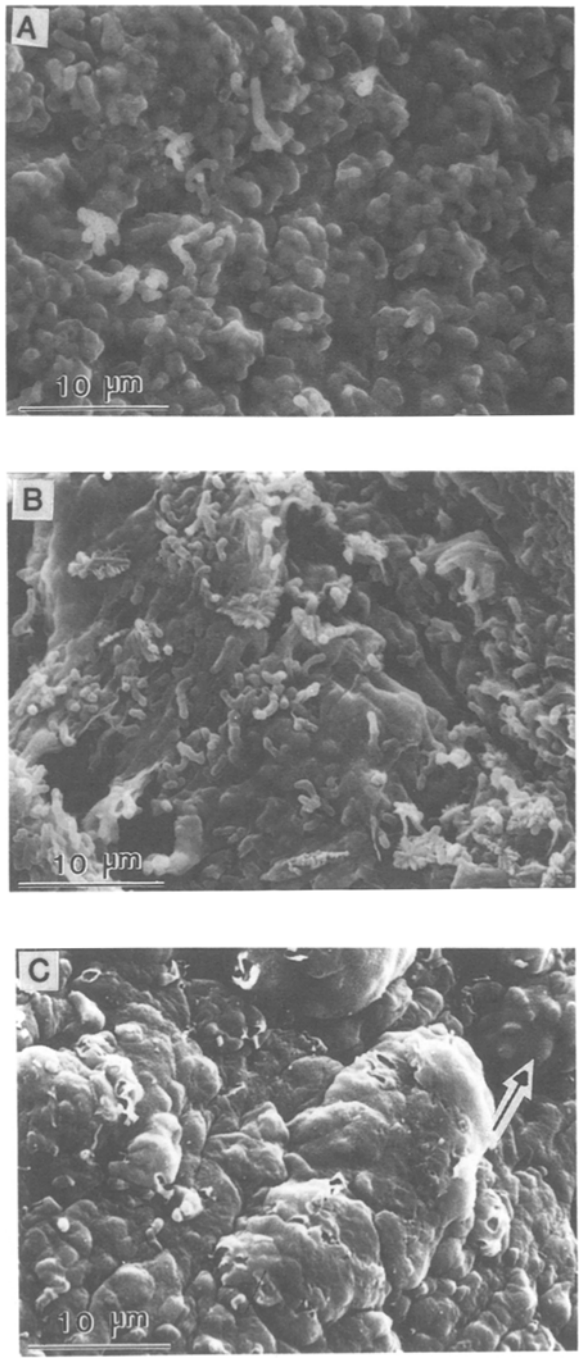

Fig. 3. SEM demonstrating the outer surface (A) and interior (B) of the K-carrageenan beads after cell immobilization and prior to hardening; (C) outer surface containing *Arthrobacter* cells following glutaraldehyde treatment. Arrow shows the surface cells almost covered with a thin film due to the interaction with glutaraldehyde. Magnification, ×2400. Bar = 10.0 μm.

Mg ions is required in reaction mixture to achieve enzyme activation to highest specific activities of GI. The GI activities exhibited optimum pH and temperature of 9.0 and 80°C, respectively. However, pH 8.0 was used to eliminate the dark color formed in the reaction mixture at pH 9.0 and 80°C, which is probably due to interaction between the aldehyde group of sugars and the free amino groups of the basic amino acids in cell protein (Millard reaction). A sharp decline in the enzyme activity due to denaturation was also noted when a temperature over 80°C was applied, therefore 70°C was used.

The cells exhibited at least two proteolytic enzymes, with pH optima at 8.0 and 6.0. These proteolytic activities can destroy the GI and cause a sharp decline in the isomerization reaction during processing operation. Therefore, a heat treatment was applied as described, to inhibit the proteolysis. The proteolytic enzymes were heat labile with 90% of the activities destroyed after 10 min heating at 65°C, whereas 85% of the GI activities remained active (indicating a heat stable enzyme).

The effects of different K-carrageenan concentrations for immobilization of cells were tested for both GI activities and gel strength. Three percent K-carrageenan exhibited adequate GI activities as well as high gel strength (data not shown).

In glutaraldehyde treatment (Table 2), complete inactivation of GI and high increase in gel strength (treatment A) were evident, probably due to the facilitated diffusion of 0.1 M glutaraldehyde in the aqueous solution (pH 2.8 for 2 h at 30°C) into the beads. In C treatment, the activity of GI was highest and the gel strength was lowest due to use of buffered glutaraldehyde (pH 7.0) at lower temperature and shorter reaction time. Therefore, most of the reaction between glutaraldehyde and cell proteins occurred on the external surface of the beads and only slightly on the internal surfaces. This was evidenced by the formation of a very thin brown layer surrounding the beads in treatments B and C, while in A the beads were dark throughout. These results agree with those of Nguyen and Luong [20], who stated that the viscosity of the solute solution and the K-carrageenan gel are inversely related to operating temperatures higher than 25°. Such a

combined effect will increase the diffusion of the glutaraldehyde.

Comparative analysis of data collected from the bioreactor studies (Fig. 2) revealed that the higher the temperature and slower the flow rate the greater the glucose isomerization to fructose. The presence of high levels of glucose in feedstock, longer times, and slower flow rates were required to reach the maximal conversion. However, this conversion was high at 70°C as compared to 60°C using the same flow rates above 1.0 ml/min. The temperature of 60°C was employed to increase bioreactor stability. No physical changes occurred to the beads due to the presence of K ions as a gelation agent in the feedstock.

In conclusion, our test organism showed high GI activities and its immobilized-cell preparations using K-carrageenan exhibited relatively high half life during continuous mode operation. Therefore, it is believed that this type of immobilization can be industrially applied. The design of different reactors for given applications is feasible using the K-carrageenan immobilization method, since it allows production of different sizes and shapes of beads.

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