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Conversion of Mannose to Fructose by Immobilized Mannose Isomerase from *Pseudomonas cepacia*

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ABSTRACT

The enzyme mannose isomerase (EC 5.3.1.7) catalyzes the isomerization of D-mannose and D-fructose. The conversion of mannose to fructose is the first step in the principal pathway of mannose dissimilation by *Pseudomonas cepacia*. This enzyme is induced during growth on medium containing mannose to levels three- to four-fold higher than observed during growth on glucose or citrate.

Mannose isomerase was purified from extracts of mannose-grown *P. cepacia* and was efficiently immobilized onto a porous, noncompressible, ceramic support. The performance of the immobilized enzyme, compared with the soluble enzyme, was evaluated under a variety of operating conditions to examine its potential for use in a process for the production of fructose syrups containing a higher proportion of fructose than is currently possible using glucose isomerase.

Index Entries: Mannose isomerase; fructose; immobilization; *Pseudomonas cepacia*.

INTRODUCTION

Mannose isomerase (EC 5.3.1.7) catalyzes the interconversion of mannose and fructose. This enzyme has been described from a variety of bacteria including *Pseudomonas* (*1*), *Rhizobium*, (*2*), *Streptomyces* (*3*), and *Mycobacterium* (*4*), and in each case, the enzyme is found intracellularly. The reaction that is catalyzed finds its equilibrium at about 65% fructose and 35% mannose. Therefore, this reaction is quite interesting in view of the huge world-wide demand for fructose for use in high fructose sweet-

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eners. The enzyme currently used for fructose production is glucose (xylose) isomerase, which catalyzes a reaction with an equilibrium of about 50% glucose and 50% fructose. Certainly, glucose is an abundant and inexpensive substrate; however, since the resultant syrup is used in foods as a substitute for sucrose, it must be brought up to approximately 55% fructose to meet sweetness demands. The steps required to increase the fructose concentration in the glucose/fructose syrups add significantly to the processing costs. The use of an enzyme, such as mannose isomerase, that provides a product higher in fructose concentration could eliminate the need for such separation and concentration steps.

Although mannose is not currently manufactured in large quantities, it is very abundant in a crude form from waste liquors of wood pulping operations. The mannose that could be derived from such sources has been estimated to be about 400,000 tons/yr (*5,6*), and methods have been proposed and tested for its recovery from the spent liquor (*6,7*). The method for conversion of wood pulp mannose to fructose must also be economical and would require a relatively inexpensive source of the enzyme and the development of an efficient process for its use. Mannose isomerase from *Pseudomonas cepacia* appears to be the most appropriate enzyme for such a process based on the amount of enzyme produced, its stability, substrate specificity, and the development of simplified methods to purify the enzyme sufficiently for immobilization.

MATERIALS AND METHODS

Strains and Culture Conditions

For the production of mannose isomerase, *Pseudomonas cepacia* was grown in an inorganic salts medium consisting of 50 m*M* phosphate buffer (16 m*M* Na_2HPO_4 and 34 m*M* KH_2PO_4), pH 6.5, 1 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, 0.01 m*M* $FeSO_4$, and 0.2% (w/v) ammonium sulfate. One percent D-mannose was used as the sole source of carbon although 0.5% D-mannose could be used with only slightly lower enzyme yields. The bacteria were grown at 37°C typically for 20 to 24 h.

Preparation of Enzyme Samples

For the preparation of crude cell extracts, cells were collected from the fermentation broth by centrifugation, washed with 20 m*M* phosphate buffer, pH 6.8, recentrifuged, and then suspended in a volume of buffer equivalent to about 1/100th of the original fermentation volume. The washed cells were sonicated, and unbroken cells and cell debris were removed by centrifugation. The resulting crude enzyme extract could be used for immobilization, but it was found preferable to remove nucleic acids by precipitation using streptomycin sulfate (125 mg/mL of extract). It was also found preferable to add the streptomycin sulfate to the soni-

cated extract before removal of cell debris, then centrifuging to remove both cell debris and precipitated nucleic acids. Further purification of the enzyme was accomplished by successive ammonium sulfate precipitations of protein from the extract.

Immobilization of Mannose Isomerase

Mannose isomerase preparations were immobilized onto a macroporous, incompressible, ceramic support (*8*). Approximately 1 g of support (60–80 mesh) was added to 4 mL of the enzyme preparation, pH 6.6, containing typically 16,000 U of enzyme activity and 4.3 mg of protein/mL. The enzyme was incubated with the support at 4°C with intermittent shaking. Enzyme loading onto the support was estimated by measuring enzyme loss from the supernatant using assay procedures described below. Estimates made in this manner were compared with the activity of an enzyme preparation stored under the same conditions without the presence of support in order to discount the loss of enzyme owing to inactivation. Once sufficient enzyme was loaded, the remaining soluble enzyme was removed and the support washed extensively with 100 m*M* Tris buffer, pH 6.8. The support was then packed into a fixed-bed reactor and connected to a peristaltic pump for feed delivery. The reactor was maintained at 4°C overnight, during which time the immobilized enzyme was flushed with over 1000 bed volumes of the same Tris buffer to remove any free or weakly-bound enzyme. The immobilized enzyme was subsequently flushed with the mannose substrate solution for several hours prior to assay.

In some cases in which the immobilized enzyme was to be sacrificed, for example in experiments designed to determine temperature optimum or substrate specificity, a small sample of the immobilized enzyme was added to individual 50 mL flasks containing substrate and gently swirled. At appropriate time intervals, the support was allowed to settle and aliquots of the supernatant were withdrawn for analysis.

Enzyme Assays

Immobilized mannose isomerase activity was typically determined at 40°C using a feedstock containing 0.01*M* D-mannose and 0.2*M* Tris buffer at the appropriate pH. Mannose isomerase activity was determined in extracts primarily by measuring mannose dependent formation of fructose according to the method described previously (*9*). Assay mixtures (0.5 mL) containing 0.2*M* Tris buffer, pH 7, 0.01*M* D-mannose, and appropriately diluted cell extract were incubated at 40°C. After 15 min, 0.5 mL of ethanol containing 0.1% (w/v) resorcinol and 1.0 mL of 10*N* HCl were added. The tubes were incubated at 80°C for 8 min and then placed in ice water to stop the reaction. The absorbance at 484 nm was determined and compared to a standard curve relating absorbance to fructose concentration. An absorbance of 0.1 was equivalent to 150 nmol fructose/ml of

Table 1
Specific Activity of Enzymes Related to Mannose Utilization

Carbon source[a]	Mannose isomerase[b]	Fructokinase[b]
Mannose	126	30
Glucose	57	3
Citrate	69	6

[a]Bacteria were grown in minimal salts medium supplemented with 0.5% of the indicated carbon source.
[b]Enzyme activity expressed as nmol of product formed/min/mg protein.

assay mixture. One unit of activity is equivalent to the production of 1 nmol of fructose/min. The conversion of mannose to fructose (as well as any reactions with other sugar substrates) was also determined using HPLC and known standards.

To determine the pH optimum of the immobilized enzyme, the percent conversion of mannose to fructose was measured after a set period of time with the reactor in a batch recycle mode of operation. Before each test of the immobilized enzyme, the reactor was operated under standard conditions of pH 7 at 40°C to test for any inactivation of the enzyme. With each change of an operating variable, the immobilized enzyme was allowed to equilibrate by passing a minimum of 50 bed volumes of feed through the column before the product concentrations were measured.

For measurement of substrate specificity, individual sugars or a mixed sugar substrate was used as the feed for the immobilized enzyme in a batch recycle mode of operation. Samples were removed at various times and analyzed by HPLC, as previously described (*10*).

RESULTS

Pseudomonas cepacia grew well on a minimal salts medium supplemented with a variety of carbon sources including glucose, mannose, or citrate. Mannose isomerase activity was determined for cultures grown on each of these substrates, and it was shown that growth on mannose induces the highest level of mannose isomerase activity (Table 1). This is consistent with the proposed pathway for mannose utilization by *P. cepacia* (Fig. 1) in which mannose isomerase is responsible for the conversion of mannose to fructose, which is subsequently phosphorylated and ultimately metabolized via the Entner-Doudoroff or pentose phosphate pathways. It was shown previously (*11*) that mannose isomerase levels could be elevated still further when mutants deficient in fructokinase activity are grown on, or in the presence of, D-mannose. Such mutants fail to grow on D-fructose, but did grow, albeit slowly, on mannose as the sole source of carbon, indicating that another route exists for the metabolism of mannose. Consistent with this observation is the finding that fructose

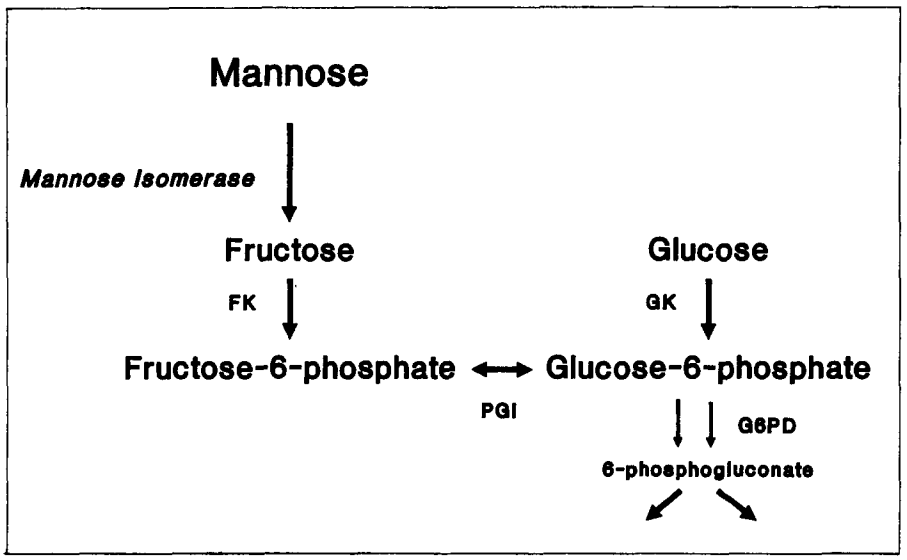

Fig. 1. Pathway for D-mannose utilization by *Pseudomonas cepacia*. Abbreviations: FK, fructokinase; GK, glucokinase; PGI, phosphoglucose isomerase; and G6PD, glucose-6-phosphate dehydrogenase.

is secreted at low levels by such mutants during growth on mannose and that the induction of alternate pathways indicates an intracellular production of D-glucose and/or glucose-6-phosphate. Although such mutants are capable of producing elevated levels of mannose isomerase, for the purposes of this study we used the wild-type strain 249 as the source of the enzyme. This is owing to the relatively slow growth of the mutant strains on mannose (115–175 min generation time vs 85 min for the wild type) and the possible problem of revertants. Fermentations (1.5 L) using *P. cepacia* 249 were generally complete in less than 24 h.

Initial preparation of mannose isomerase for any use required disruption of the cells and removal of cell debris by centrifugation. Crude extracts (10 mL) from 1.5 L fermentation contained approximately 4000 U of activity/mL. For initial studies of the soluble and immobilized enzyme, the mannose isomerase was partially purified through precipitation of nucleic acids by streptomycin sulfate, ammonium sulfate precipitations, and chromatography using DEAE Sephadex column chromatography (Fig. 2). The resultant enzyme preparation (208 U/mL), obtained by combining the peak fractions from the DEAE Sephadex chromatography, was used for the determination of pH optimum (Fig. 3) and K_m of the enzyme for D-mannose (Fig. 4), as well as for the initial immobilization studies.

Immobilization of the semipurified enzyme preparation proved quite efficient with >90% of the enzyme activity immobilized and a coupling efficiency of about 100%. Following the immobilization of the enzyme onto the support, the solution was removed and the immobilized enzyme washed with 0.1*M* Tris buffer, pH 6.6. No mannose isomerase activity

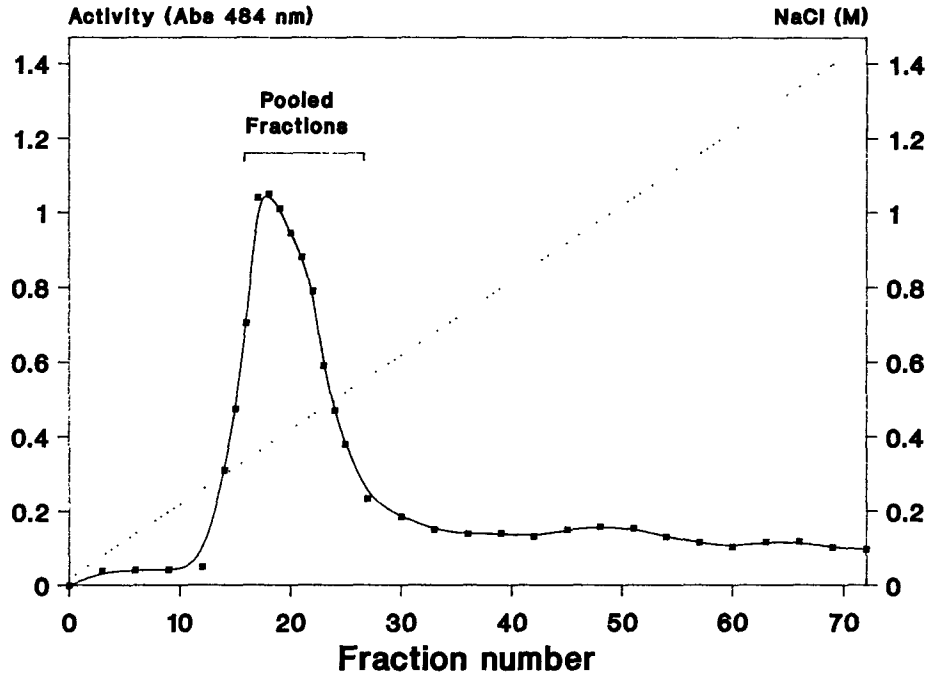

Fig. 2. Purification of mannose isomerase by DEAE-Sephadex column chromatography. The enzyme was eluted using a gradient of NaCl (■). Fractions (2 mL) showing significant activity (14–25) were pooled and used in later experiments.

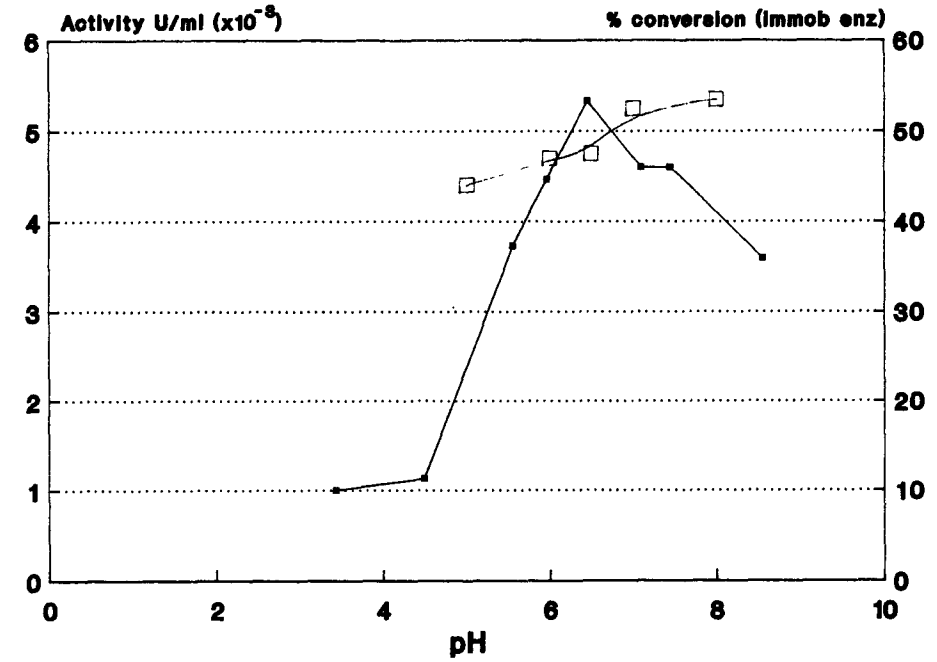

Fig. 3. Effect of pH on the activity of the immobilized and soluble mannose isomerase: soluble enzyme (■—■); immobilized enzyme (□—□). Immobilized enzyme activity is expressed as percent conversion of fructose to mannose under standard conditions after 120 min.

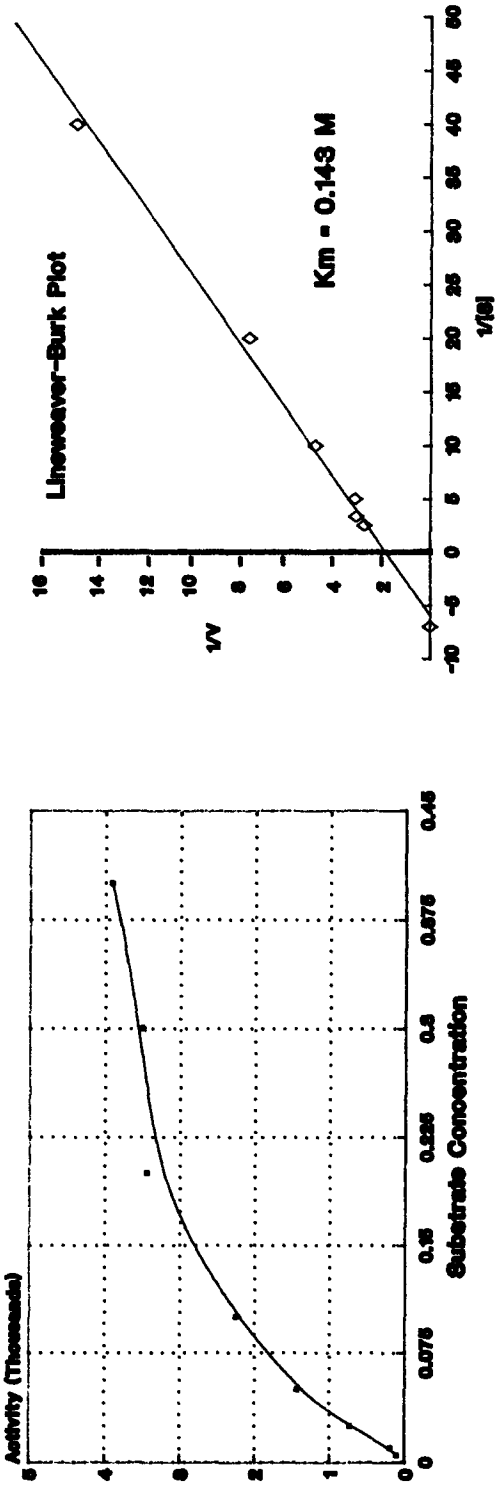

Fig. 4. Determination of the K_m of mannose isomerase for mannose using the soluble enzyme.

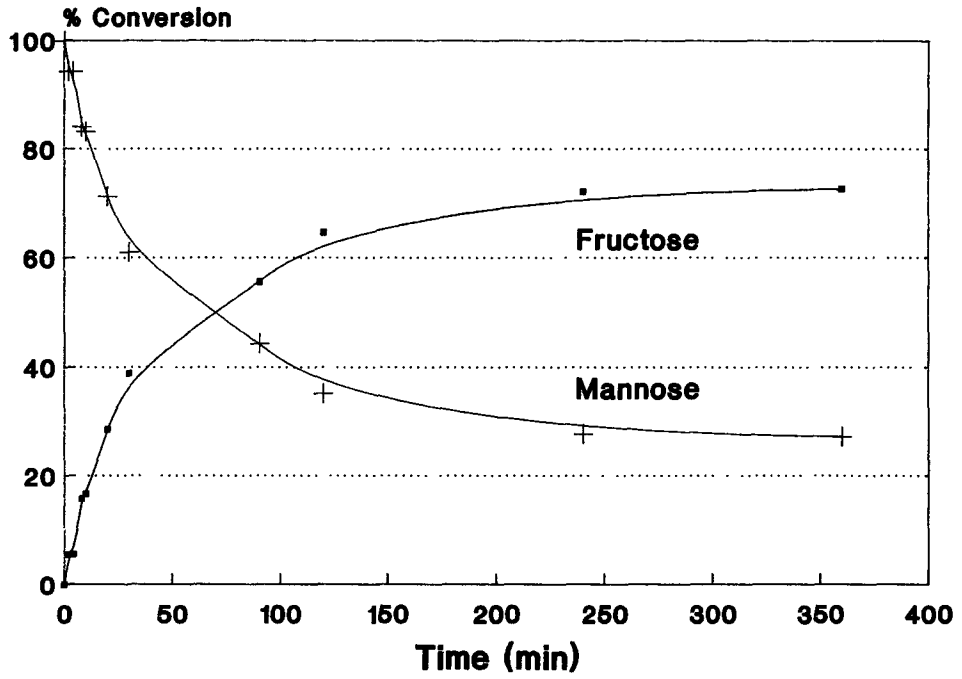

Fig. 5. Conversion of D-mannose to D-fructose by immobilized mannose isomerase. The conversion was measured using 0.1*M* mannose as substrate with the immobilized enzyme in a batch recycle mode.

could be detected in the wash solutions, indicating that the enzyme was stably immobilized. With the bioreactor at 40°C, the conversion of mannose to fructose was determined as a function of time, as shown in Fig. 5. The equilibrium, typically at about 65% fructose and 35% mannose, was identical to that obtained with the soluble enzyme under the same conditions (data not shown).

The level of activity of the immobilized enzyme used for these experiments was quite low owing to the amount of enzyme available for immobilization after purification. To develop a reasonable process for the conversion of mannose to fructose, it was necessary to have a simpler, more efficient method for recovery of enzyme and immobilization. To accomplish this, enzyme was offered for immobilization at various stages of purification. It was found that only the initial removal of cell debris after cell disruption was necessary. As shown in Table 2, high levels of immobilization were obtained when the enzyme was offered for immobilization as a crude extract, an ammonium sulfate concentrate, or semipurified through DEAE Sephadex. In each case, the mannose isomerase was immobilized preferentially, i.e., the ratio of the immobilized mannose isomerase to total protein immobilized was always greater than one and greater than two when the enzyme was offered in great excess. The most efficient method for preparation and immobilization of the enzyme was using streptomycin sulfate and ammonium sulfate precipitation to achieve

Table 2
Immobilization of Mannose Isomerase[a]

	Mannose Isomerase, U[b]			Protein, mg		
	Offered	Immobilized	%	Offered	Immobilized	%
Crude Extract	6426	5656	88	90	34	37
Crude Extract	28555	13733	48	400	77	19
Ammonium Sulfate	17104	15133	89	52	33	64
DEAE Sephadex	770	739	96		nd	

[a] Activity and total protein immobilized per gram of support.
[b] One unit of activity is the amount of enzyme required for production of 1 μmol of mannose/min at 40°C.

a partial purification. It was interesting that the best recovery of enzyme occurred when the streptomycin sulfate was added prior to removal of cell debris. Thus, only one centrifugation step was necessary to recover the extract prior to ammonium sulfate precipitation. This became the standard method for preparation of the enzyme for immobilization.

Since such immobilized enzyme preparations obviously contain a variety of enzymes immobilized onto the support, it was necessary to demonstrate that these other enzyme activities do not interfere with the conversion of mannose to fructose. This does not appear to be a problem, as judged by the rate and extent of conversion of mannose to fructose (65% fructose). After a prolonged period of time with the immobilized enzyme in a batch recycle mode, the levels of mannose and fructose did not change, showing that other enzymes that may catalyze the breakdown of these products are not a problem.

The immobilized enzyme preparation was also tested in the presence of other sugar substrates. For such experiments, a mixed sugar feed was prepared approximating the ratio of the hemicellulose-derived sugars identified in sulfite waste liquor streams (*6*) containing (in grams per liter of 0.2*M* Tris buffer, pH 7) galactose (15.9), mannose (40.3), glucose (15.9), arabinose (8.0), and xylose (15.2). Under these conditions, the immobilized enzyme catalyzed the conversion at an average rate of 55% of the rate of conversion under optimal conditions with mannose as the sole substrate (Fig. 6). No new products other than fructose (derived from mannose) were identified by HPLC. The specificity of the immobilized enzyme was confirmed by removing small aliquots of the immobilized enzyme (0.4 g) and incubating them at 40°C in 5 mL of 0.1*M* solutions of the following sugars: D-galactose, L-arabinose, L-rhamnose, D-xylose, D-lyxose, D-xylu-

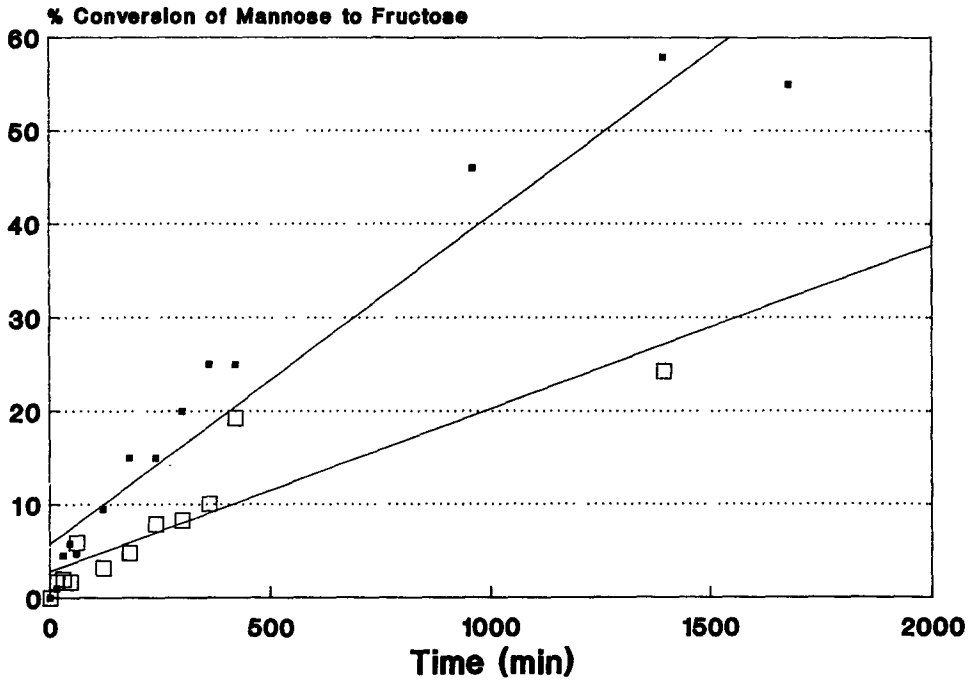

Fig. 6. Effect of mixed substrate in the conversion of mannose to fructose. Production of fructose by the immobilized mannose isomerase operating in a batch recycle mode was determined with 4.03% (w/v) D-mannose as substrate (■—■) or a mixed substrate consisting of 4.03% D-mannose, 1.59% D-galactose, 1.59% D-glucose, 0.8% L-arabinose, and 1.52% D-xylose (□—□).

lose, L-mannose, and D-mannose (control). After 18 h of incubation, there was a 68% loss of mannose (owing to fructose conversion) in the control, but no detectable loss of other sugars in the test samples.

The pH of the feed had little effect on the activity of the immobilized enzyme over the range of pH 5–8. This was a broader range of maximum activity than found for the soluble enzyme, as shown in Fig. 3. The optimum temperature for the enzyme was also examined, as shown in Fig. 7. Thus, it appeared that the conditions chosen for standard operation of the immobilized enzyme reactor (40°C, pH 7) afforded high levels of activity. To determine the stability of the enzyme under these conditions, the immoblized enzyme was run in a batch recycle configuration and, at various time intervals, modified for single-pass operation. One hundred bed volumes of feed were pumped through the reactor before sampling for product measurement. Under these conditions, the enzyme was found to retain greater than 54% of its initial activity over a period of 4 d. The soluble enzyme (as a preparation identical to that used for the immobilization) retained about 55% of its activity over the same time period, indicating that immobilization of the enzyme did not significantly affect its stability at these temperatures.

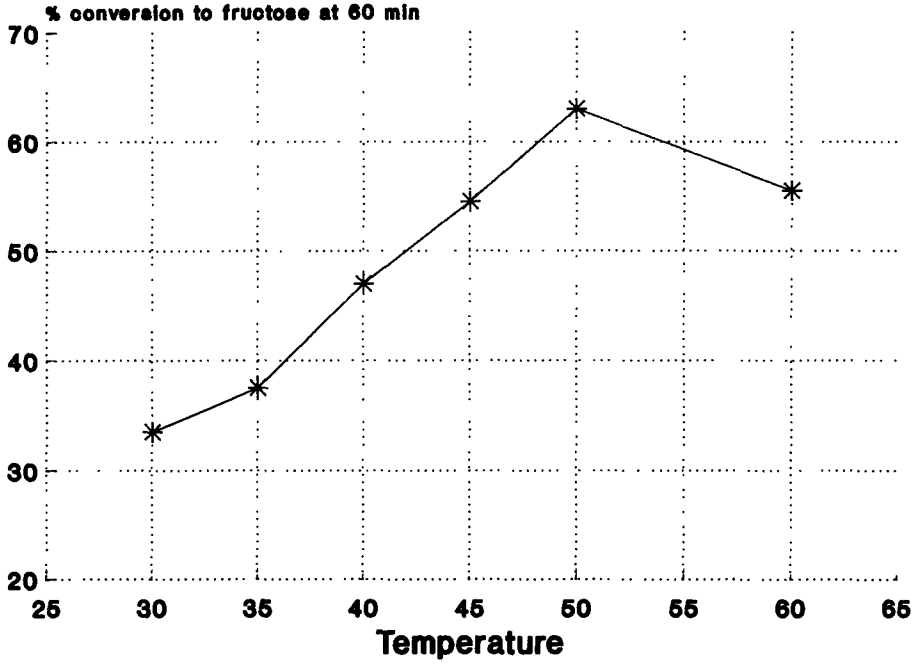

Fig. 7. Temperature optimum of the immobilized mannose isomerase.

DISCUSSION

The results summarized in Table 3 demonstrate that the mannose isomerase from *P. cepacia* can be efficiently immobilized and that the immobilized enzyme can be used to directly produce a product containing a high concentration of fructose, significantly higher than possible with conventional enzyme technology. The fact that mannose isomerase catalyzes a reaction with this favorable equilibrium has been described previously; however, here it is shown that the mannose isomerase from *P. cepacia* can be efficiently immobilized as a relatively crude enzyme preparation, thereby greatly lowering the potential cost of such a catalyst. Despite the coimmobilization of a variety of enzymes from this organism, the immobilized catalyst retains its specificity for the isomerization of mannose to fructose without detrimental side reactions. The stability of the immobilized enzyme at the chosen pH and temperature is quite good; however, for the isomerization of mannose to fructose to be an economical process, such items are critical. So far, no further attempts have been made to examine ways to further stabilize the enzyme.

Several earlier observations have suggested that significant increases in the yield of mannose isomerase could be achieved using various mutant strains of the bacteria, specifically mutants deficient in fructokinase (*11*). Interestingly, the first report of mannose isomerase produced by *P. sac-*

Table 3
Performance of the Immobilized Mannose Isomerase

Enzyme yield (U/mL of fermentation volume)	>25 U
Recovery of enzyme (from crude extract to immobilization)	>80%
Immobilization efficiency	90%
pH Optimum	5–8
Product yield (% fructose from mannose)	65%
Immobilized enzyme stability (% activity at 40°C after 4 d)	54%
Other products from mannose	none detected

charophila (*1*) identified mannose isomerase as only being produced by mutant strains that were capable of utilizing fructose as the sole source of carbon. In the course of this study, we were able to increase fermentation yields significantly by elevating the concentration of mannose in the medium. It is likely that other relatively minor modifications will yield increases in enzyme production. Thus, the greatest limitation in a process for the conversion of mannose to fructose will likely be the establishment of efficient processes for the recovery of mannose from the pulping waste liquors. Without the development of this large potential feedstock, mannose isomerase might be more likely to find use as a method for the conversion of fructose to mannose for use as a specialty chemical.

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