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Tagatose Production with pH Control in a Stirred Tank Reactor Containing Immobilized L-Arabinose Isomerase from *Thermotoga neapolitana*

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Abstract Chitopearl beads were used as immobilization supports for D-tagatose production from D-galactose by L-arabinose isomerase from *Thermotoga neapolitana* because chitopearl beads were more stable than alginate beads at temperatures above 60 °C. The pH and temperature for the maximum isomerization of galactose were 7.5 and 90 °C, respectively. In thermostability experiments, the half-lives of the immobilized enzyme at 70, 75, 80, 85, and 90 °C were 388, 106, 54, 36, and 22 h, respectively. The reaction temperature was determined to be 70 °C because the enzyme is highly stable up to 70 °C during the reaction. When the reaction time, galactose concentration, and temperature were increased, the pH of a mixture containing enzyme and galactose decreased by the Maillard reaction, resulting in decreased tagatose production. With pH control at 7.5, tagatose production (138 g/L) at 70 °C in a stirred tank reactor containing immobilized enzyme and 300 g/L galactose increased two times higher, comparing that without pH control.

Keywords L-Arabinose isomerase · Tagatose · Immobilization · *Thermotoga neapolitana* · Stirred tank reactor · pH control

Introduction

The sweetness of tagatose is 92% that of sucrose when compared in 10% solutions. Tagatose has a sucrose-like taste with no cooling effect or aftertaste, and it is similar to the polyols with its tooth-friendly property. However, tagatose has a less laxative effect unlike polyols [1]. Although the taste quality of tagatose is similar to sucrose, tagatose does not contribute to calorie production [2, 3]. As a result of these properties, tagatose is considered to be a potential reduced energy sweetener. It has also been shown to have numerous medical benefits such as the improvement of fetal development and the reduction in symptoms associated with type 2 diabetes, hyperglycemia, anemia, and hemophilia [4, 5].

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To produce tagatose effectively, a bioreactor containing immobilized enzyme or cell is used. The immobilized enzyme reactor can be used with the highly concentrated enzyme, whereas the immobilized cell reactor cannot be used at high cell concentrations because immobilized cells contain high levels of other cell materials, which have no tagatose-producing activity and cause steric hindrance [6]. Thus, the immobilized enzyme reactor has a higher tagatose titer, conversion yield, and volumetric productivity than the immobilized cell reactor does [6–9].

The pH of a mixture containing L-arabinose isomerase and galactose decreased with increasing reaction time, temperature, and galactose concentration, resulting in decreased tagatose production. Thus, to produce tagatose stably at high temperatures, the pH of the mixture should be controlled. In this study, by holding the pH steady in a stirred tank reactor, tagatose was produced using immobilized L-arabinose isomerase from *Thermotoga neapolitana* (TNAI).

Materials and Methods

Plasmid and Microorganisms

A recombinant *Escherichia coli* BL21(λDE3) harboring pET-22b(+)/L-arabinose isomerase (TNAI) gene from *T. neapolitana* was used for the source of the enzyme [10]. The cells were cultivated in a 7-L fermentor (Biotron, Bucheon, South Korea) with 5 L Luria–Bertani medium containing 50 μg/L ampicillin at 37 °C. When the absorbance of bacteria reached 0.8 at 600 nm, lactose was added with a final concentration of 20 mM, and TNAI was induced and expressed for 16 h at 16 °C. The harvested cells were disrupted by a Sonic Dismembrator (Fisher Scientific, USA).

Preparation of Enzyme

The cells debris was removed by centrifugation at 15,000×*g* for 20 min, and the supernatant obtained was added with 5 mM $MnCl_2$ and 1 mM $CoCl_2$ and then were heated at 80 °C for 20 min. The suspension was centrifuged at 15,000×*g* for 20 min to remove denatured *E. coli* protein. The enzyme solution was concentrated using an ultrafiltration membrane (10,000 MWCO, Vivascience, Germany). The resulting solution was considered as partially purified enzyme and used for free and immobilized enzymes. The resulting solution was applied onto Hi-Trap Q HP column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl buffer (pH 7.0). The column was washed with 50 mL of the same buffer containing 0.2 M NaCl and eluted with the Tris–HCl (pH 7.0) buffer containing 0.5 M NaCl, and the fractions containing the enzyme were collected. The collected enzyme solution was dialyzed and then applied to Resource Q column (Amersham Biosciences) equilibrated with 50 mM Tris–HCl buffer (pH 7.0). The enzyme was eluted with the same method of the Hi-trap Q HP column. The collected enzyme solution was analyzed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis as a single band and used as a purified enzyme. The purified enzyme was concentrated by Amicon Ultra-15 (Millipore, Billerica, MA). One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol tagatose per min at 70 °C and pH 7.5.

pH Decrease During Galactose Isomerization

The partially purified enzyme of 2 g/L was mixed with galactose (300, 400, 500 g/L) and adjusted to pH 7.0. The mixtures were incubated for 60 h at several temperatures of 70, 75, and 80 °C. A sample was withdrawn at time intervals, and the pH of the mixture was measured.

Protein extracts of cultivated *E. coli* BL21 (λDE3) harboring pET-22b(+) without the L-arabinose isomerase gene were prepared using the same method used for L-arabinose isomerase. The *E. coli* protein, partial purified enzyme, and purified enzyme with the same concentration of 2 g/L were mixed with 300 g/L galactose, and the mixture was incubated at 70 °C for 60 h.

Immobilization Methods

TNAI (25 mg) was absorbed on 1 g (wet weight) of chitopearl BCW 2510 beads (Fuji Spinning, Tokyo, Japan), and the mixture was stirred at 4 °C for 3 h. The beads were then collected by filtration and washed three times with 50 mM potassium phosphate buffer (pH 7.5) [11].

The enzyme was mixed in sodium alginate with its final concentration of 2.0% (w/v; Yakuri Pure Chemicals, Kyoto, Japan), and the mixture was dropped into an excess of 0.2 M $CaCl_2$ using a syringe pump (74900, Cole-Parmer, Vernon Hills, IL) [7]. An initial mass of 16 U enzyme was used with 2 mL of chitopearl or alginate beads. The reactions were performed with 5 g/L galactose and 8 U/mL immobilized enzyme at 60, 65, 70, and 75 °C until tagatose production reached a plateau. To compare between chitopearl and alginate beads as immobilization supports, tagatose production using chitopearl beads was performed in 50 mM potassium phosphate buffer (pH 7.5) for 40 h, and that using alginate beads was performed 50 mM piperazine-*N*,*N*′-*bis*(2-ethanesulfonic acid) buffer (pH 7.5) for 40 h at 70 and 75 °C and for 75 h at 60 and 65 °C.

Effects of pH and Temperature on Tagatose Production

Unless otherwise stated, the reactions were performed in 50 mM potassium phosphate buffer (pH 7.5) containing 10 g/L galactose and 0.8 U/mL immobilized TNAI on chitopearl beads at 70 °C for 20 min. To determine the conditions necessary for maximum enzymatic activity, the temperature was varied from 50 to 95 °C and the pH from 4.0 to 9.0. The buffer systems used were 50 mM citric acid (pH 4.0–6.0), 50 mM potassium phosphate (pH 6.0–8.0), and 50 mM Tris–HCl (pH 8.0–9.0).

Effects of Thermal Stability on Tagatose Production

To test the thermostability of the enzyme, the temperature was raised from 70 to 90 °C for varying periods of time. A sample was withdrawn at time interval and was assayed. The experimental data for enzyme inactivation were fitted to a first-order curve, and the half-lives ($t_{1/2}$) of the immobilized enzymes on chitopearl beads were calculated using SigmaPlot 9.0 software (Systat Software, San Jose, CA).

Bioreactor Operation

The effect of galactose concentration on tagatose production was investigated in a 1-L bioreactor (Biotron) containing 300 mL of 100, 200, 300, 400, and 500 g/L galactose. The reactions were performed at 70 °C with 4.0 U/mL enzyme until tagatose production reached a plateau. The reaction times for free and immobilized TNAI on chitopearl beads without and those with pH control were 12, 24, 24, and 96 h, respectively.

Tagatose production was performed in the bioreactor with 50 mM potassium phosphate buffer (pH 7.5) containing 4.0 U/mL immobilized enzyme on chitopearl beads and 300 g/L galactose. The reaction temperature, pH, and agitation speed were maintained at 70 °C, 7.5, and 70 rpm, respectively.

Analytical Methods

All of the beads were round and homogeneously distributed. The protein contents were determined by the Bradford method using bovine serum albumin as a standard. The concentrations of tagatose and galactose were determined by high-performance liquid chromatography (SCL-10A, Shimadzu, Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu) using a Shodex Asahipak (NH2P-50 4E, Showa Denko, Tokyo, Japan) column, which was eluted with 70% (v/v) acetonitrile at a flow rate of 0.8 mL/min at 30 °C.

Results and Discussion

Tagatose Production by Immobilized TNAI on Alginate and Chitopearl Beads

Alginate beads have been used to immobilize enzymes and cells for tagatose production [6–9], and enzymes immobilized on chitopearl beads have been used for the production of rare sugars such as D-arabinose, D-allose, L-gulose, and L-talose [12–14] and the production of oligosaccharides [15, 16]. The optimum temperature and heat stability of enzyme were increased by the immobilization on chitopearl beads [17]. An inulin fructotransferase from *Arthrobacter pascens* immobilized on chitopearl beads was stable up to 75 °C [18].

To compare between alginate as references and chitopearl beads as immobilization supports, two beads were used for the immobilization of TNAI as a hyperthermophilic L-arabinose isomerase at temperatures ranging from 60 to 75 °C (Fig. 1). The conversion of galactose into tagatose using the alginate beads was almost the same as that using the chitopearl beads at 60 °C. When the temperature was raised to 75 °C, however, tagatose production increased on the chitopearl beads but decreased on the alginate beads. Thus, chitopearl beads were selected as the immobilization support for further study.

In general, immobilized cells in alginate beads were incubated at temperatures of cell growth as 30 or 37 °C [19–21], and immobilized enzymes in the beads were reacted below 60 °C [22–24]. Above 60 °C, immobilized enzymes exhibit decreased stability compared to free enzymes because of their higher activation energies [25–27], suggesting that alginate beads are not suitable for enzyme immobilization at temperatures exceeding 60 °C.

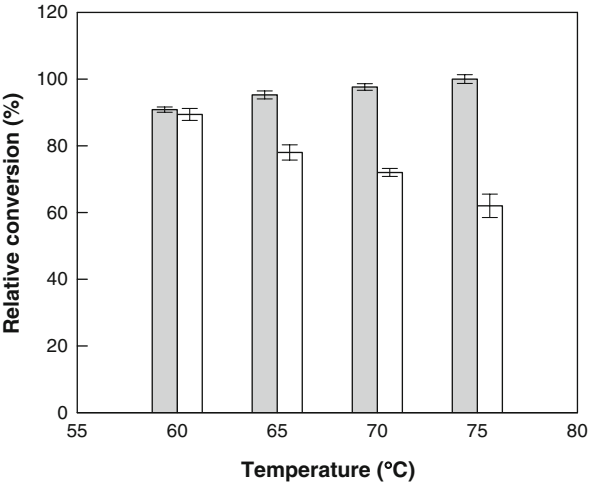

Fig. 1 Tagatose production by immobilized enzymes on chitopearl (*shaded bars*) and alginate (*unshaded bars*) beads. The relative activity of 100% produced by immobilized enzyme on chitopearl was 2.8 g/L of tagatose production after 40 h from 5 g/L galactose. Data represent the means of three separate experiments

Effects of pH and Temperature on Tagatose Production

The galactose isomerization activity of immobilized TNAI on chitopearl beads was examined at temperatures ranging from 50 to 95 °C (Fig. 2). Maximum activity was observed between 90 and 95 °C; the activity for free TNAI was at its maximum at 95 °C [10].

The pH for maximum activity was estimated to be in the range of 4.0 to 9.0 at 70 °C (Fig. 3); peak activity was obtained at pH 7.5 for the immobilized TNAI. As the pH was lowered below 7.0, the activity of the enzyme significantly decreased, suggesting that the pH should be maintained above 7.0 for effective tagatose production. In comparison, free TNAI exhibits maximum activity at pH 7.0 [10].

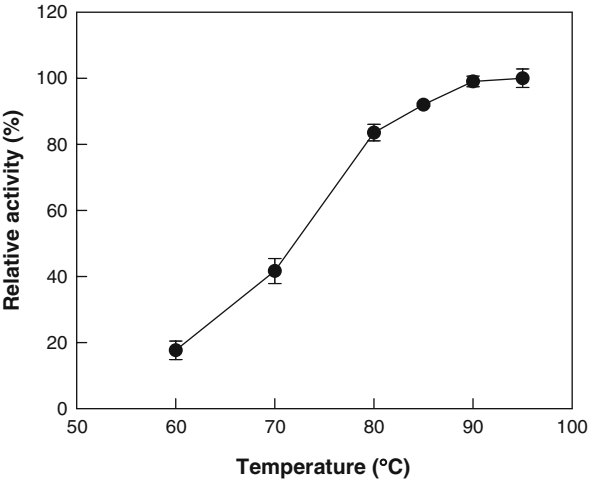

Fig. 2 Effect of temperature on tagatose production by immobilized enzyme on chitopearl beads. The reactions were performed in a 50-mM potassium buffer (pH 7.5) containing 10 g/L galactose and 0.8 U/mL immobilized enzyme for 20 min. Data represent the means of three separate experiments

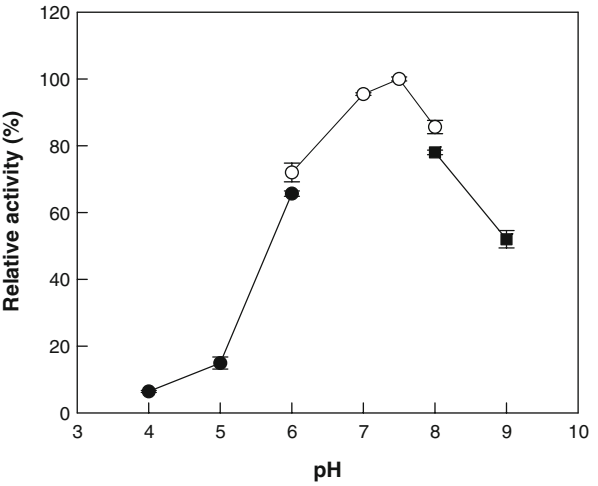

Fig. 3 Effect of pH on tagatose production by immobilized enzyme on chitopearl beads. The reactions were performed in a buffer system containing 10 g/L galactose and 0.8 U/mL immobilized enzyme at 70°C for 20 min. The buffer systems were 50 mM citric acid (pH 4–6; *filled circles*), 50 mM potassium phosphate (pH 6–8; *empty circles*), and 50 mM Tris–HCl (pH 8–9; *filled squares*). Data represent the means of three separate experiments

Thermal Stability of Immobilized TNAI

Enzyme stability is an important factor in the commercial application of enzymatic bioconversion. To assess its commercial potential, the temperature stability of immobilized TNAI was investigated.

Thermal inactivation of the enzyme was examined by measuring enzymatic activity over time at pH 7.5 from 70 to 90 °C (Fig. 4). The half-lives of the enzyme at 70, 75, 80, 85, and 90 °C were 388, 106, 54, 36, and 22 h, respectively. The half-life of free TNAI is 2 h at 90 °C [10]. Thus, the half-life of the immobilized TNAI at 90 °C was 11 times higher than that of the free TNAI. The reaction temperature was determined to be 70 °C because the enzyme is highly stable up to 70 °C during the reaction.

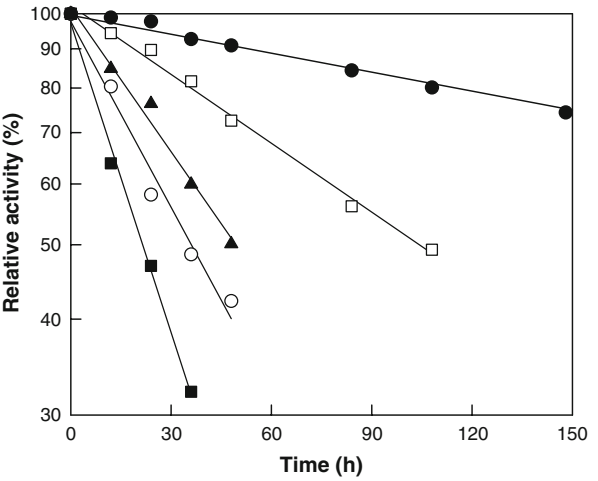

Fig. 4 Thermal inactivation of immobilized enzyme on chitopearl beads for tagatose production at each temperature. The reactions were performed in a 50 mM potassium buffer (pH 7.5) containing 10 g/L galactose and 0.8 U/mL immobilized enzyme at 70 °C for 20 min. Temperature was 70 (*filled circles*), 75(*empty squares*), 80 (*filled triangles*), 85 (*empty circles*), and 90 °C (*filled squares*)

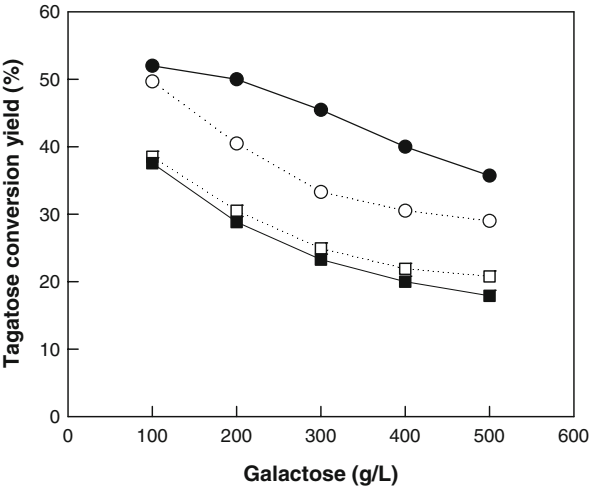

Fig. 5 Effect of substrate concentration on tagatose production in a stirred bioreactor reactor. The reactions were performed in 50 mM potassium phosphate (pH 7.5) containing galactose and 4.0 U/mL enzyme at 70 °C until tagatose production reached a plateau. Tagatose conversion yields produced from different substrate concentrations in the bioreactor contained free (*empty squares*) and immobilized enzymes (*filled squares*) without pH control and free (*empty circles*) and immobilized enzymes (*filled circles*) with pH control at 7.5

pH Decrease During Galactose Isomerization

The pH of the partially purified enzyme mixed with galactose decreased as the reaction time, galactose concentration, and temperature increased. When a mixture containing 2 g/L partially purified enzyme and 300 g/L galactose was incubated at 75 °C, the initial pH of 7.0 decreased to 6.6 after 10 h, 5.5 after 35 h, and 4.5 after 60 h. The galactose concentration in the mixture containing 2 g/L partially purified enzyme was then varied; after 12 h, the initial pH of 7.0 decreased to 6.5 at 100 g/L galactose, 6.1 at 300 g/L galactose, and 5.5 at 500 g/L galactose. The incubation temperature of the mixture was also varied; after 12 h, the initial pH of 7.0 decreased to 6.5 at 70 °C, 6.1 at 75 °C, and 5.6 at 80 °C. The *E. coli* protein, partial purified enzyme, and purified enzyme with the same concentration mixed with galactose were incubated at 70 °C for 60 h. The pH values in all cases exhibited the same pattern in pH decreases regardless of protein kinds.

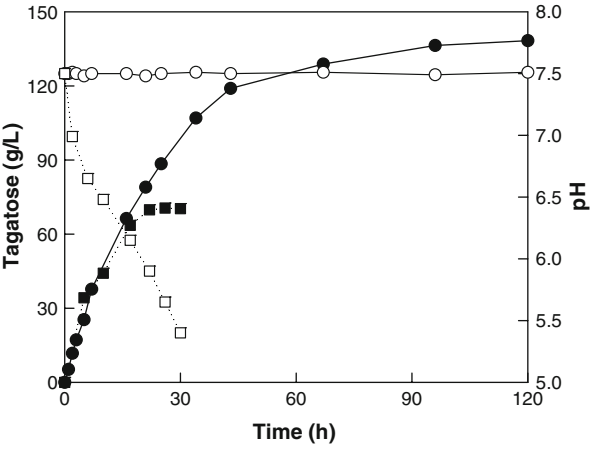

Fig. 6 Tagatose production from 300 g/L galactose in the stirred bioreactor contained immobilized enzymes without and with controlling pH. Tagatose production without (*filled squares*) and with pH control (*filled circles*) and pH profiles without (*empty squares*) and with pH control (*empty circles*)

The Maillard reaction involved in the condensation was between a carbonyl group of sugar and an amino group of protein [28]. The decrease in pH occurring in the Maillard reaction has been reported [29, 30]. When the reaction time, galactose concentration, and temperature increased, the pH in the mixed solution containing protein (or amino acid) and galactose decreased. Thus, the decrease of pH at high temperature and high galactose concentration during the galactose isomerization was caused by the Maillard reaction. The decrease of pH in the earlier stage may be due to the loss of the basic amino groups [31]. However, in the later stage, after Amadori rearrangement, products with various acidities such as glyoxal, pyruvaldehyde, and furfural were accumulated, which resulted in the decrease of pH [29].

Tagatose Production in a Bioreactor Containing Immobilized Enzyme with pH Control

A packed-bed bioreactor could not be applied to tagatose production because continuous feeding of galactose solution at pH 7.5 did not overcome the decrease in pH at high temperature and high galactose concentration caused by the Maillard reaction. In a stirred tank reactor, tagatose production using the free or immobilized enzyme was performed by varying substrate concentration with and without pH control to investigate the effect of pH on tagatose production (Fig. 5). When the pH was maintained at 7.5, the tagatose conversion yield of the free and immobilized enzymes was approximately 50 and 52% at 100 g/L galactose and 29 and 36% at 500 g/L galactose, respectively. Without pH control, the amount of tagatose produced by the free and immobilized enzymes was quite similar (within 3%) as a lower yield than that with pH control. The lower amount of tagatose produced without pH control was due to the decreased yield resulted from a significant decrease in enzymatic activity below pH 7.0 (Fig. 3). The immobilized enzyme compared to the free enzyme produced more product when the pH was held constant because of enhanced stability of the immobilized TNAI. As shown in Fig. 6, the free and immobilized enzymes produced the same amount of product up to 20 h. However, after 20 h, the free enzyme leveled off, whereas the immobilized enzyme continued to produce tagatose for up to 96 h.

Conclusions

Chitopearl beads were selected as immobilized supports for tagatose production because the alginate beads were unstable above 60 °C. At high temperatures, using immobilized L-arabinose isomerase on chitopearl beads, the pH of a mixture containing enzyme and galactose decreased because of the Maillard reaction, resulting in decreased tagatose production. This problem was overcome by using a stirred tank reactor with pH control. At a constant pH, the immobilized enzyme produced approximately two times tagatose from 300 g/L galactose at 70 °C compared to that without pH control. When the pH was controlled, immobilized L-arabinose isomerase compared to free L-arabinose isomerase produced more tagatose because of the enhanced stability of the immobilized enzyme.

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