



Enzymatic Synthesis of Sucrose-6-acetate by a Novel Immobilized Fructosyltransferase From *Aspergillus* sp. GX-0010

Qunliang Li ^{1*}, Xin Zhang ¹, Xiaobo Guo ¹, Pingjia Yao ² and Yuanan Wei ²

¹ School of Chemistry and Chemical Engineering, Guangxi University, Nanning, China

² The State Key Laboratory of Subtropical Bio-resource Conservation and Utilization, Guangxi University, Nanning, China

*Corresponding author: Qunliang Li, School of Chemistry and Chemical Engineering, Guangxi University, Nanning, China. Tel: +86-07713233718, Fax: +86-07713233718, E-mail: liqunliang231@163.com or liqunliang231@gxu.edu.cn

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Background: Sucralose is an ideal food sweetener and sucrose-6-acetate (s-6-a) is a key intermediate for synthesis of sucralose. Synthesis of s-6-a was studied by free fructosyltransferase (FTase) from *Aspergillus oryzae*. Because of the limitations of free enzyme in stability and reusability, a FTase obtained from the new isolated *Aspergillus* sp. GX-0010 was immobilized and investigated for the potential of s-6-a synthesis.

Objectives: The synthesis of s-6-a with sucrose and glucose-6-acetate (g-6-a) by immobilized fructosyltransferase (IFTase) from a novel *Aspergillus* sp. GX-0010 was studied, and its synthesis conditions were also optimized.

Materials and Methods: *Aspergillus* sp. GX-0010 was isolated. The effects of reaction time, ratio of g-6-a to sucrose, pH, substrate (sucrose and g-6-a) concentrations, IFTase concentration and temperature on the synthesis of s-6-a were investigated.

Results: IFTase was able to catalyze sucrose and g-6-a to synthesize the s-6-a. Thermal and pH stability of IFTase were promoted once compared to the FTase. The optimal condition for IFTase catalysis was obtained at 50 °C, 60 min reaction time, pH 6.5, 1:2 ratio of g-6-a to sucrose and 35.0 g.L⁻¹ concentration of enzyme. Under this optimal condition, a g-6-a conversion rate of 24.96% was reached.

Conclusions: This study showed IFTase has a great potential in the biosynthesis of s-6-a, a key intermediate of sucralose synthesis.

Keywords: *Aspergillus* sp. GX-0010, Immobilized fructosyltransferase, Sucralose, Sucrose-6-acetate

1. Background

Sucralose is an ideal food sweetener, which can be widely used to replace sucrose due to its pure sweetness and good heat stability. The preparation of sucrose-6-acetate (s-6-a) is a key step in synthesis of sucralose (1). Currently, the synthesis methods of s-6-a are mainly composed of chemical and enzymatic procedures. S-6-a can be produced with traditional chemical reaction of sucrose in pyridine with acetic anhydride at variable temperatures. This reaction also produces kinds of impurities, including monoacylates, some higher acylates, and toxic substances, which requires complicated separation techniques for the pure s-6-a (2). Enzymes, mainly fructosyltransferase (FTase) and lipase, have been used in sucralose synthesis. Cross-linked enzyme aggregates and immobilized

lipase have been used in the biosynthesis of s-6-a (3-4). Additionally, use of FTase in transferring a fructosyl residue on either sucrose or glucose-6-acetate (g-6-a) to produce s-6-a have been reported (5-8). The FTase that has been used to date is either under patent restrictions, e.g. *Bacillus megaterium* NCIB 11871 and *Aureobasidium pullulans*, or is limited for the reaction in time-cost (120 h) that makes the process non-economical (5, 9). The FTase from *Aspergillus oryzae* was recently purified and characterized, suggesting it having the potential to be a biocatalyst for the synthesis of s-6-a (10). In addition, a FTase was also reported for catalyzing sucrose and glucose-6-acetate to synthesize s-6-a (11), but its practical application is limited due to the poor stability and non-reusability.

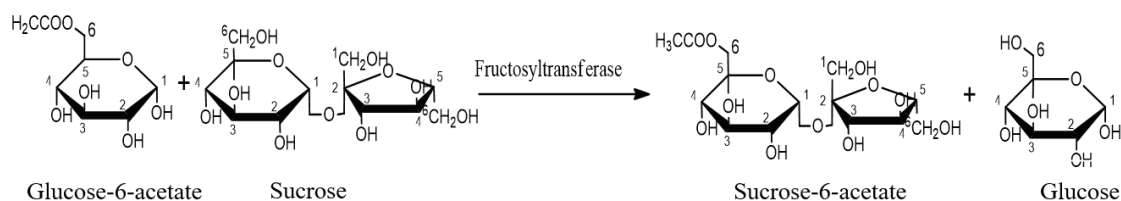


Figure 1. The synthesis of s-6-a catalyzed by FTase

2. Objectives

Aspergillus sp. GX-0010 was obtained in our laboratory. In preliminary experiments, FTase from *Aspergillus* sp. GX-0010 was able to catalyze sucrose and g-6-a to synthesize s-6-a, a key intermediate for producing sucralose, as shown in **Figure 1**. Considering the advantages of immobilized enzymes in enzyme activity, storage stability, and efficient separation over the free enzymes (12-13), an immobilized fructosyltransferase (IFTase) from *Aspergillus* sp. GX-0010 was used as a catalyst to synthesize s-6-a and the synthesis conditions were also optimized.

3. Materials and Methods

3.1. Reagents

The standard samples of fructose and sucrose were from Sigma Co. Ltd. S-6-a was purchased from Toronto Research Chemicals Inc., Canada. The g-6-a was prepared in our laboratory.

3.2. Microbial Strain and Cultivation

Aspergillus sp. GX-0010 obtained by mutation in our laboratory was inoculated into 250 mL culture medium containing sterile sucrose 24.0 g.L⁻¹, yeast extract 5.0 g.L⁻¹, and K₂SO₄ 1.0 g.L⁻¹ (pH 6.5) in a 500 mL flask and incubated at 28 °C for 32 h with shaking speed of 150 rpm.

3.3. Preparation of Immobilized Enzyme with Chitosan Gel in Glutaraldehyde

The mycelium was collected and washed by phosphate buffer (pH 6.5), then disrupted by bead mill. The cell debris was removed by centrifugation at 4 °C, 8944 ×g for 60 min. Crude enzyme obtained from supernatant was primarily purified by Ultrafilter at 4 °C. The crude FTase was immobilized according to (14). Briefly, 1.0 g chitosan was dissolved in 2% (m/v) acetic acid for 12 h, then filtered with filter paper. The filtrate was diluted by 4 times in volume, and neutralized with 2.0 mol.L⁻¹ NaOH. Chitosan-gel was precipitated via vigorous agitation and washed to neutrality with distilled water, then dehydrated via suction filtration. Chitosan-gel was mixed with crude FTase, and the mixture was added to a flask containing 20 mL glutaraldehyde solution (0.2 % w/v) and shaken

at 40 rpm for 8 h at 25 °C. After cross-linking reaction, the prepared of IFTase was washed with distilled water to remove excess glutaraldehyde, then stored in a 0.1 mol.L⁻¹ sodium acetate buffer (pH 5.5) at 4 °C.

3.4. Catalytic Conditions of IFTase

Catalytic conditions including the molar ratio of g-6-a to sucrose, substrate concentrations, reaction time, pH, and temperature were optimized. All reaction volume was in 50 mL phosphate buffer. To investigate the reusability potential, IFTase was separated from the products by filtration and washed two times with 0.1 mol.L⁻¹ sodium acetate buffer (pH 5.5) and dried under vacuum.

3.5. Definition of the Activity Unit of the Immobilized Enzyme

One unit (1 U) of the immobilized enzyme activity was defined as the amount of enzyme that required for catalyzing 1 μmol g-6-a per minute. The activity of IFTase was the unit of μmol of s-6-a/min via per IFTase (g), abbreviated as U.g⁻¹.

3.6. Analyzing Methods

The catalyzing reaction of IFTase for sucrose was measured by HPLC (Waters 600 HPLC pump, Waters 717 plus Autosampler, Alltech 2000 ELSD Detector). The analysis column was Hypersil NH₂ (4.6 × 300 mm). Mobile phase was the mixture of acetonitrile: water (75:25). Flow rate was 1.0 mL.min⁻¹ in total of 10 μL. Column temperature was set to 30 °C and run time for the elution was 15 min.

The contents of different components were determined by the normalization method according to the HPLC peak. The theoretical and measured contents of s-6-a were detected according to the contents of g-6-a. The molar conversion rate of g-6-a was calculated based on the following formula (11).

$$\begin{aligned}
 g-6-a \text{ molar conversion rate} &= \frac{n(s-6-a)_{\text{measure values}}}{n(s-6-a)_{\text{theoretical values}}} \\
 &= \frac{w(g-6-a)/M(g-6-a)}{w(g-6-a)/M(g-6-a) + w(s-6-a)/M(s-6-a)} \times 100
 \end{aligned}$$

In formula, n : number of moles, w : mass number, M : molar mass.

3.7. Statistical Analysis

Data, except for HPLC, was the mean value of three replicates. The coefficient of variation was controlled until it did not exceed 5%. The graphs were drawn via Origin 8.0.

4. Results

4.1. The Identification of Standard Samples and Catalyzing Products

The retention time of the standard samples of g-6-a, sucrose and s-6-a was detected by HPLC (Fig. 2A). Linear equation in terms of the correlation between concentration of standard s-6-a and its peak area was conducted. The peak areas of serial dilutions of the standard s-6-a (1.0, 2.0, 3.0, 4.0 and 5.0 mg·mL⁻¹) were 238836, 593824, 867463, 1199921 and 1527864 mV·S respectively determined by HPLC. The linear equation was $y = 318415x - 69664$, ($R^2 = 0.9988$).

After enzyme inactivated in boiling water for 10 min, cooled, centrifuged and diluted, the reaction samples were analyzed by HPLC in the same chromatographic conditions as standard samples did. The g-6-a, s-6-a and sucrose were identified in the catalyzing solution by FTase based on the retention time in Figure 2B in which the g-6-a was the remnant reaction substrate and the sucrose derived from the culture medium and the reaction substrate. The peak at retention time of 6.087 min indicated that s-6-a was successfully synthesized and the yield of s-6-a can be calculated according to its peak area.

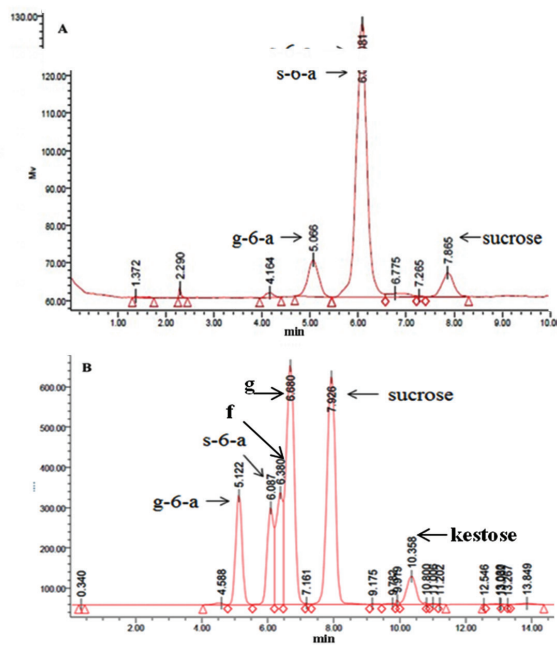


Figure 2. The HPLC spectrum of standard samples (A) and catalyzing products (B). g-6-a: glucose-6-acetate, s-6-a: sucrose-6-acetate, g: glucose, f: fructose.

4.2. Effect of Temperature and pH on FTase and IFTase

The effect of temperature on the FTase and IFTase activities was investigated by incubating enzymes at various temperatures (30-60 °C) in buffer solution (pH 5.5). The appropriate temperature for IFTase was between

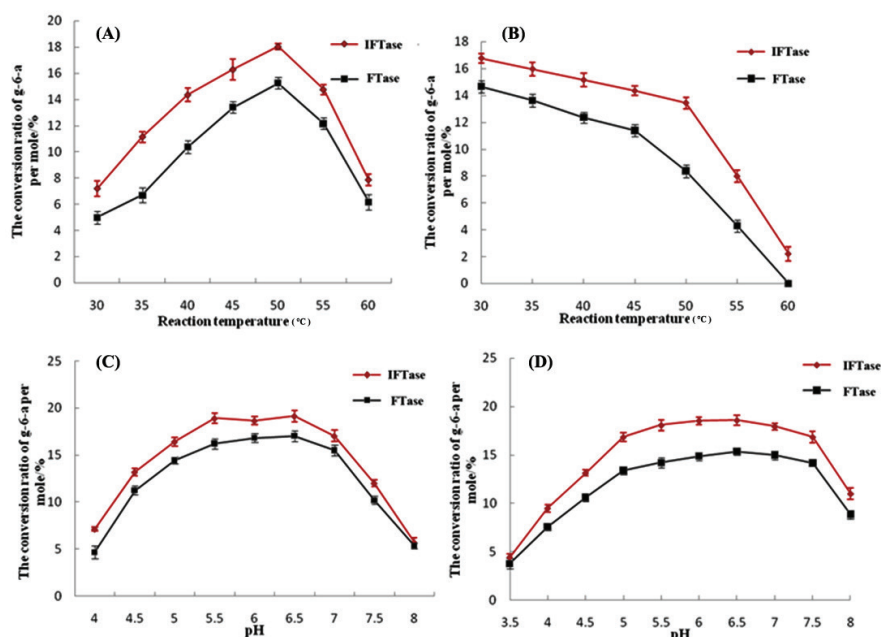


Figure 3. Effect of temperature and pH on the enzymes' activities and stabilities. A, the effect of temperature on enzymes' activities; B, the effect of temperature on enzymes' stabilities; C, the effect of pH on enzymes' activities; D, the effect of pH on enzymes' stabilities.

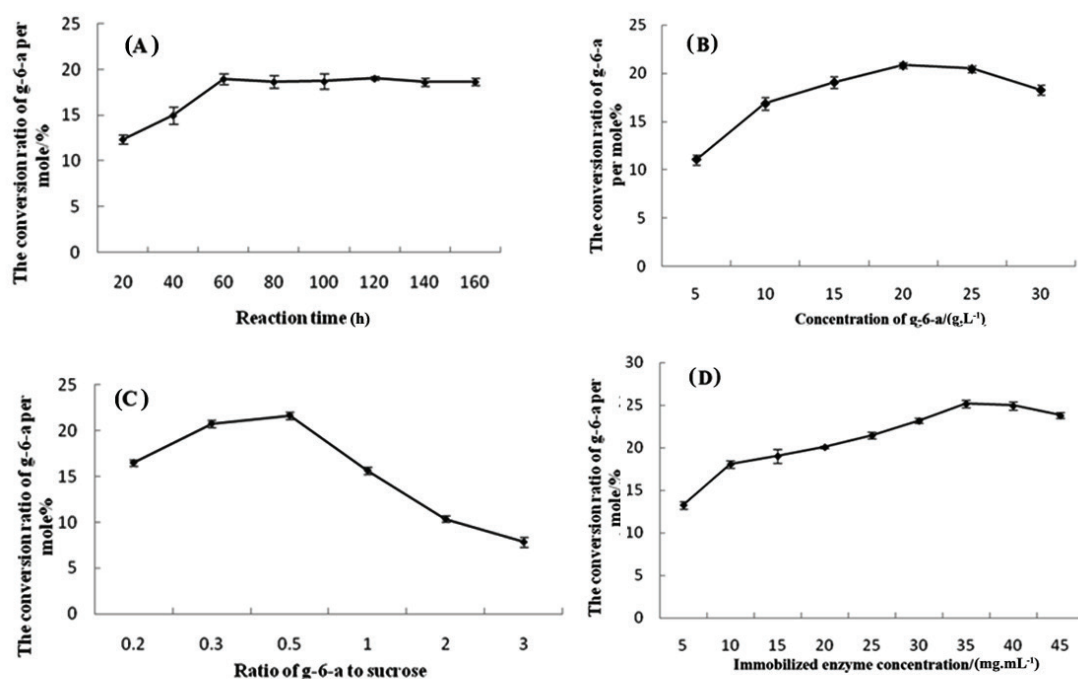


Figure 4. The optimization for IFTase catalyzing reaction. A, optimization of reaction time; B, optimization of g-6-a concentration; C, optimization of reactants ratio; D, optimization of IFTase concentration.

40-50 °C and the optimum temperature was at 50 °C (Fig. 3A). FTase had the higher activity between 45-50 °C. An extended appropriate temperature range of IFTase was observed compared to FTase. The thermal stability of FTase and IFTase was assessed by incubating the enzymes at various temperatures (30-60 °C) in buffer solution at pH 5.5 for 60 min (Fig. 3B). The conversion of g-6-a in IFTase was higher than FTase during the different temperature range, indicating that thermal stability of IFTase was promoted relative to FTase.

The effect of pH on the FTase and IFTase activity was studied by incubating the enzymes in pH range of 4-8 at 50 °C (Fig. 3C). The appropriate pH range for both FTase and IFTase was 5.0-7.0 and the optimal pH was 6.5. The enzymes were incubated at 50 °C in buffer solution at pH 3.5-8.0 for 60 min to assess the pH stability of FTase and IFTase activity which were stable at pH 5.0-7.5, then declined when pH was above 7.5 (Fig. 3D). The pH stability of IFTase was enhanced after immobilization.

4.3. Effects of Catalytic Conditions on g-6-a Conversion Rate Via IFTase

The conversion rate of g-6-a to s-6-a was used as an index to optimize catalytic time, concentration of g-6-a, ratio of g-6-a to sucrose and concentration of IFTase (Fig. 4).

The effect of catalytic time on the conversion rate of g-6-a was investigated in the range from 20 to 160 min at 50 °C, pH 6.5, 15 g.L⁻¹ of g-6-a, 1:3 of g-6-a to sucrose (m/m) and 20 g.L⁻¹ of IFTase concentration. Result revealed that conversion rate of g-6-a was enhanced gradually with increasing time from 20 to 60 min, and reached the peak at 60 min (Fig. 4A). The effect of g-6-a concentration on the conversion rate of g-6-a was investigated in the range from 5 to 30 g.L⁻¹ at 50 °C, pH 6.5, 60 min, 1:3 of g-6-a to sucrose (m/m) and 20 g.L⁻¹ of IFTase concentration. When the g-6-a concentration increasing from 5 to 20 g.L⁻¹, the conversion rate of g-6-a was promoted gradually, then reached the maximum of 20.8% (Fig. 4B). The ratio of g-6-a to sucrose affected the conversion of g-6-a significantly certificated by the catalytic reaction at 50 °C, pH 6.5, 60 min, 20 g.L⁻¹ of g-6-a and 20 g.L⁻¹ of enzyme concentration. At the beginning, the conversion rate of g-6-a increased and then decreased with inclining of the ratio of g-6-a to sucrose. The maximum conversion rate was observed at the ratio of 1:2 (Fig. 4C). The result in Figure 4D revealed that the conversion rate of g-6-a increased gradually, when IFTase concentration was between 5.0 to 45.0 g.L⁻¹ with the catalytic reaction at 50 °C, pH 6.5, 60 min, 20.0 g.L⁻¹ of g-6-a and 1:2 of g-6-a to sucrose. The conversion rate of g-6-a decreased gradually after it above 35.0 g.L⁻¹.

4.4. Storage Stability and Reusability of IFTase

The IFTase was kept at 4 °C and maintained 90.1% of its initial activity after 6 months storage. In the first 30 days, the activity of IFTase decreased slightly suggesting IFTase had a good storage stability (data was not shown). The reusability of IFTase in synthesizing s-6-a was profiled (Fig. 5). A higher of g-6-a conversion ratio at 92.3% compared to the initial conversion ability was maintained after 4 cycles of IFTase reusing. The conversion of g-6-a decreased by 50.5% after 15 cycles of IFTase reusing, suggesting the reutilized cycle of IFTase was 15. The result indicated that IFTase had a good storage stability and reusability, which was a foundation for its large-scale industrial application.

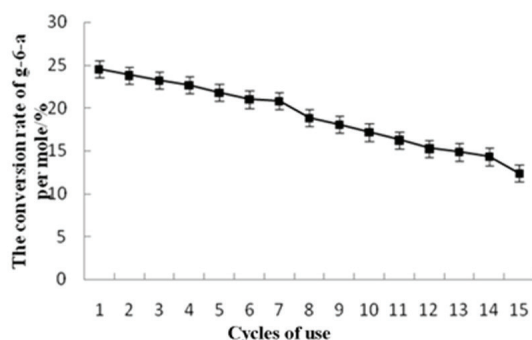


Figure 5. The effects of recyclable use on IFTase activity. All the reaction conditions of the enzyme recyclable use are described in section 3.4 in Materials and Methods. The results showed that the conversion of g-6-a decreased only by 50.5% after 15 recyclable times of IFTase.

5. Discussion

In this study, the activity and optimal conditions of IFTase from *Aspergillus* sp. GX-0010 were investigated. The results indicated that IFTase could catalyze the sucrose and g-6-a to synthesize the s-6-a. The thermal and pH stability of IFTase were improved compared to the FTase. The optimal ratio of g-6-a to sucrose of 1:2 was in accordance to the result (1). A higher ratio of g-6-a to sucrose restrained the production of fructose, resulting from the inhibition of substrate to IFTase (Fig. 4C). A steric hindrance from IFTase might have existed which led to a decrease in the g-6-a conversion rate, when the enzyme concentration above 35.0 g.L⁻¹ (Fig. 4D). The optimal condition was obtained as follows: 50 °C, 1 h reaction, pH 6.5, ratio of g-6-a to sucrose of 1:2, and enzymatic concentration of 35.0 g.L⁻¹. Under these optimal conditions, the g-6-a conversion rate reached 24.96%. This yield is comparable to the two related reports of s-6-a synthesis using FTase from *Aspergillus* (10-11). In one report, a novel FTase from *Aspergillus oryzae* ZZ-

01 was purified and characterized. The purified enzyme led to an overall conversion of 32.0% rate using g-6-a and sucrose as substrates under the optimal conditions (10). In the other report, Han *et al.* illustrated the crude FTase from *Aspergillus oryzae* catalyzed s-6-a synthesis with a g-6-a molar conversion rate of 26.9% (11). However, bad stability and no-reusability limited the application of free enzyme. The stability and reusability of IFTase in this work showed that the immobilized enzyme overcame the disadvantages of free enzymes.

Notably, IFTase displayed both conversion and hydrolysis activity in a sucrose reaction system, leading to fructose and glucose formation. The produced fructose can be transferred to sucrose form fructooligosaccharides as well (14-16). Therefore, the sucrose can be a competitive inhibitor of g-6-a in the reaction for synthesizing s-6-a. How to inhibit the kestose production for increasing s-6-a yield will be concerned in the future study.

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Authors' Contribution

Qunliang Li, Xin Zhang, Pingjia Yao and Yuanan Wei designed the experiments. Xin Zhang performed the experiments. Qunliang Li, Xin Zhang, and Xiaobo Guo analyzed the data and wrote the manuscript.

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