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Purification and Partial Characterization of Intracellular Fructosyltransferase from a Novel Strain of *Aureobasidium pullulans*

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Abstract: The intracellular fructosyltransferase (FTase) of a novel strain of *Aureobasidium pullulans* (CFR 77) capable of producing 59% of fructooligosaccharides (FOS) within 9 h of reaction time was obtained by wet-milling, and then purified and characterized. The purified FTase revealed 2 bands of 147 and 170 KD; its activity was optimum at an approximate pH of 5.0 and temperature of 55 °C. The specific activity of the final purified material was 42, representing a purification factor of 79.44 and yield of 43%. The enzyme is very stable, retaining more than 80% of its original activity at the optimum reaction conditions after 12 h. Using the crude intracellular FTase, 59% of FOS was produced within 9 h of reaction time, which is a considerable reduction in the reaction time of 12-25 h that has been reported in the literature. The purified FTase yielded 59% of FOS within 3 h of reaction time.

Key Words: Fructosyltransferase, *Aureobasidium pullulans*, enzyme purification, fructooligosaccharides

Aureobasidium pullulans Suşundan Hücreiçi Fruktoziltransferaz Enziminin Saflaştırılması ve Kısmi Karekterizasyonu

Özet: Dokuz saatlik reaksiyon süresinde %59 fructosiltransferaz üretebilen yeni bir *Aureobasidium pullulans* (CFR77) suşunun hücre içi FOS'u wet-milling yöntemi ile elde edilmiş, saflaştırılmış ve karakterize edilmiştir. Saflaştırılan FTaz 147 ve 170 KD ağırlığında iki bant vermiştir, aktivitesi yaklaşık pH 5 ve 55 °C'de optimum olarak bulunmuştur. Sonuçta saflaştırılan materyalin özel aktivitesi 42'dir ve 79,44 saflaştırma faktörü, %43 kazanca sahiptir. Enzim stabildir ve optimum reaksiyon koşullarında 12 saatten sonra orijinal aktivitesinin %8'inden fazlasını geri kazanmaktadır. FOS'un %59'u 9 saatlik bir reaksiyon süresinde ham hücre içi FTaz kullanılarak üretilmiştir. Bu süre literatürde belirtilen 12-15 saatlik sürenin belirgin bir şekilde azalmasıdır. Saflaştırılan FTaz 3 saatlik reaksiyon süresinde %59 FOS kazanımını sağlamaktadır.

Anahtar Sözcükler: Fructosiltransferaz, *Aureobasidium pullulans*, enzim saflaştırma, fruktooligosakkaritler

Introduction

Fructosyltransferase (FTase) (E.C. 2.4.1.9) catalyzes the formation of fructooligosaccharides (FOS) from sucrose, which have broad applications in the food and pharmaceutical industries. Several microorganisms have been reported to possess FTase activity and produce FOS from sucrose (1). Amongst these is *Aureobasidium pullulans* CFR 77, which has been previously reported as a potential source of FTase (2). *Aureobasidium pullulans* has been a major source of FTase (EC 2.4.1.9) for the production of FOS from sucrose (3-7). Purification and characterization of FTase from various sources have been reported (8-12); however, this accumulated knowledge is confusing (12). The information differs from one source to another, from one microorganism to another, even from one strain to another, thereby making it imperative to purify the enzyme from each source. The present study deals with the purification and characterization of FTase obtained from *Aureobasidium pullulans* CFR 77. This strain showed very high enzyme productivity and FTase

activity, producing 59% of FOS within 9 h of reaction. The purification and partial characterization of the enzyme were undertaken as necessary steps towards understanding some of its properties.

Materials and Methods

Microorganism and culture conditions

The yeast-like fungus *Aureobasidium pullulans* CFR 77 was obtained from the Type culture collection of CFTRI, Mysore, India. It was maintained on slants of potato dextrose agar (Himedia Lab., India) at 4 °C. The inoculum was developed by transferring a loopful of mycelia from a 5-day-old slant of *A. pullulans* CFR 77 into the inoculum medium (1% sucrose, 0.2% yeast extract, pH 5.50). The flasks were incubated for 24 h at 30 ± 1 °C on a rotary shaker (Emenvee Rotary Shaker 48N3, Pune, India) at 250 rpm.

Fermentation of *A. pullulans* CFR 77 and isolation of intracellular FTase

A 24-h-old inoculum (20% v/v) was transferred into 100 ml of fermentation medium consisting of the following (% w/v): sucrose: 20; yeast extract: 0.5; KH_2PO_4: 0.5; $NaNO_3$: 1.0; K_2HPO_4: 0.25; NaCl: 0.25; $MgSO_4.7H_2O$: 0.05; NH_4Cl: 0.5 (initial pH of 5.50). The culture was incubated for 24 h at 30 ± 1 °C on a rotary shaker at 250 rpm. At the end of incubation, the culture broth was centrifuged for 20 min at 6000 rpm and 4 °C using a Kubota 1920 refrigerated centrifuge (Kubota Corp., Tokyo, Japan) to obtain cells. The cells were resuspended in distilled water, to which 10% (w/v) glass beads (0.5-0.75 mm in diameter) were added. The cell-bead mixture was poured into the glass vessel of a Dynomill TYP KDL (Willy A. Bachofen, Basel, Switzerland), which operated for 15 min to break the cells. An Ultra Cryostat Remi RK 701 (Remi Instruments, India) was used to circulate cold water at 4 °C in the outer jacket of the Dynomill glass vessel to prevent the build-up of heat. The disrupted cell suspension was centrifuged for 20 min at 6000 rpm and 4 °C to obtain the cell-free lysate (crude FTase). The enzyme was stored at 4 °C until further use.

Enzyme assay

FTase activity of the enzyme preparations was assayed under pre-determined conditions by incubating 250 µl of enzyme with 750 µl of sucrose (60% w/v) in 0.1 M citrate buffer (pH 5.0) at 55 °C for 1 h in a shaking water bath (Haake SWB 20, Haake, Germany) at 100 rpm. At the end of incubation, the reaction was arrested by keeping the reaction mixture in boiling water for 15 min. The activity of the enzyme was determined based on the amount of glucose released using a glucose-oxidase kit (Span Diagnostics Ltd., Surat, India). FTase activity was defined as the amount of enzyme required to liberate 1 µmol of glucose under the specified conditions (3,13).

Production and analysis of FOS

Production of FOS was carried out using a reaction mixture, which consisted of 250 µl of enzyme and 750 µl of 60% (w/v) sucrose in citrate buffer (pH 5.0). Incubation took place at 55 °C for 18 h in a shaking water bath, with regular sampling at 3-h intervals. At the end of incubation, the reaction was arrested as before, and the products in the reaction mixture were analyzed by an HPLC LC-6A (Shimadzu, Japan) equipped with an RID 6A refractive index detector (Shimadzu, Japan), using an Aminopropyl column 250 × 4.6 mm SS Excil amino 5 µm (SGE, Australia). We injected 10 µl of 20-fold dilution of the reaction mixture into the HPLC using an injector syringe (Hamilton, Nevada, USA). The analysis was carried out at room temperature using acetonitrile/water (65:35) as the mobile phase at a flow rate of 1.0 ml/min. Data acquisition was performed on an AIMIL Chromatography Data Station (AIMIL, New Delhi, India) and processed on a computer using WINACDS software (AIMIL, New Delhi, India). The final yield of FOS was expressed as the percentage of conversion yield based on the initial concentration of sucrose. FOS standards, namely 1-kestose (GF_2), 1-nystose (GF_3), and 1-fructofuranosylnystose (GF_4), obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), were provided by Prof. Sosaku Ichikawa (Tsukuba University, Japan). Analytical grade glucose (HiMedia Lab., India) and sucrose (Ranbaxy, India) were also used for reference.

Purification of the enzyme

A summary of the steps involved in the purification of the enzyme is described below. Unless otherwise stated, all the steps were conducted at room temperature.

Ammonium sulfate precipitation

The intracellular enzyme was subjected to ammonium sulfate precipitation according to standard methods. The gradual fractionation of proteins was started from 0%-

30%, 30%-60%, and finally 60%-80%. At each stage, analytical grade ammonium sulfate (Ranbaxy, India) was slowly added to the crude enzyme with continuous stirring on a magnetic stirrer (Remi Instruments, India). An external jacket of cold water was used to maintain the temperature below 10 °C. At the end of each step of ammonium sulfate fractionation, the mixture was further stored at 4 °C for 2 h, after which it was centrifuged for 20 min at 12,000 rpm and 4 °C. The precipitate obtained from each fractionation was redissolved in a minimal amount of phosphate buffer (0.2 M, pH 7.0). Both precipitate and filtrate were assayed for FTase activity, as earlier described. Precipitates from 0%-30% and 30%-60% precipitation with high enzyme activity were pooled and dialyzed against 3 changes of phosphate buffer (20 mM, pH 7.0) to remove the ammonium salt. Dialysis was carried out using a cellulose dialysis membrane (Sigma, USA) 120 mm in diameter, with a cut off point of 12 KD.

Ion-exchange chromatography

Diethylaminoethyl (DEAE) cellulose (Sisco Research Laboratories, India) was used in this study. The resin was packed into a column (18 × 3 cm) after activation and then equilibrated with phosphate buffer. The dialyzed sample (4.0 ml) was loaded onto the column and eluted with phosphate buffer (20 mM, pH 7.0). Adsorbed proteins were eluted with a linear gradient of 0.1-0.5 M NaCl, at a flow rate of 10 ml/h using a peristaltic pump (LKB-Pump P-1, Pharmacia, Sweden). Fractions were collected on a fraction collector (LKB FRAC-100, Pharmacia, Sweden). The fractions were assayed for FTase activity. The protein content was measured at the OD of 280 nm. The active fractions were pooled and lyophilized using a Heto Dry Winner DW 3 (Heto-Halten, Denmark). The lyophilized protein was resuspended in a minimal amount of phosphate buffer (20 mM, pH 7.0) and stored at 4 °C until further use.

Gel filtration chromatography

The lyophilized sample was dissolved in 1.0 ml of phosphate buffer (pH 7.0) and then loaded on a Sephadex G200 column (95.0 × 1.75 cm) previously equilibrated with 20 mM phosphate buffer (pH 7.0). Proteins were eluted with the same buffer at the flow rate of 10 ml/h and fractions were collected as earlier described. The FTase activity and protein content of each fraction were determined. The active fractions were pooled, lyophilized, and stored at 4 °C until further use.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (14) in a tris-glycine buffer system; 10% acrylamide separating gel was used and the molecular weight markers applied were from the Sigma molecular weight calibration kit (Sigma Aldrich, USA). Electrophoretic analysis was carried out using a Genei electrophoresis kit (Genei Pvt Ltd., Bangalore, India). A silver staining technique (15) was used to stain the proteins in the gel.

Determination of molecular weight

The mixture of standard reference molecular weight markers (Sigma Aldrich, USA) was applied on SDS-PAGE. The molecular markers were carbonic anhydrase (29,000), ovalbumin (egg albumin) (45,000), bovine albumin (66,000), phosphorylase b (97,400), and alcohol dehydrogenase (150,000). The mobility of each protein band was determined as the ratio of distance moved by each protein band to the distance moved by the tracking dye. The logarithm of the molecular weight was plotted against the mobility to obtain the regression equation. The molecular weight of the purified protein was then obtained by interpolation.

Characterization of the enzyme

The purified FTase was characterized to determine the effect of pH and temperature on its activity. Similarly, the pH and temperature stability of the FTase were determined.

Effect of pH on FTase activity and stability

Citrate buffer was used to prepare substrates with pH values of 4.0, 5.0, and 6.0, whereas substrates of pH 7.0 and 8.0, and 9.0 and 10.0 were prepared using sodium phosphate and bicarbonate buffers, respectively. The FTase activity of the enzyme was determined at the various pH values, while the stability of the enzyme at optimum pH was determined by incubating with the substrate for 12 h. The residual activity of the enzyme was determined as earlier described.

Effect of Temperature on FTase activity and stability

The FTase activity of the enzymes was determined at the optimum pH (pH 5.0) at temperatures of 30, 40, 50, 55, 60, and 70 °C. The stability of the enzyme at the optimum temperature was determined by incubation with

the substrate for 12 h. The residual FTase activity was determined as earlier described.

Results and Discussion

Preliminary studies showed that *A. pullulans* CFR 77 was capable of producing FTase when grown on sucrose-based medium for 24 h. The result of ion-exchange chromatography of the crude enzyme on DEAE cellulose is presented in Figure 1. At the end of this step, most of the contaminating proteins were removed. Subsequently, chromatography on Sephadex G200 (Figure 2) was carried out. The results of the purification procedure are presented in Table 1.

The elution profile of Sephadex G200 gel filtration revealed a broad peak. The specific activity of the final purified material was 42 U/mg protein, representing a purification factor of 79.44 and yield of 43%. After analytical polyacrylamide gel electrophoresis, there still appeared to be the presence of a contaminating hydrolytic enzyme in the purified material (Figure 3). A similar observation was reported by L'Hocine et al. (12) on the FTase of *Aspergillus niger* AS0023, in which FTase and invertase (hydrolytic enzyme) were eluted as a broad band peak in the chromatographic steps. Electrophoresis on PAGE indicated that the 2 bands of proteins had different mobilities; the first one was equivalent to a molecular mass of 147 kD and the second was 170 kD,

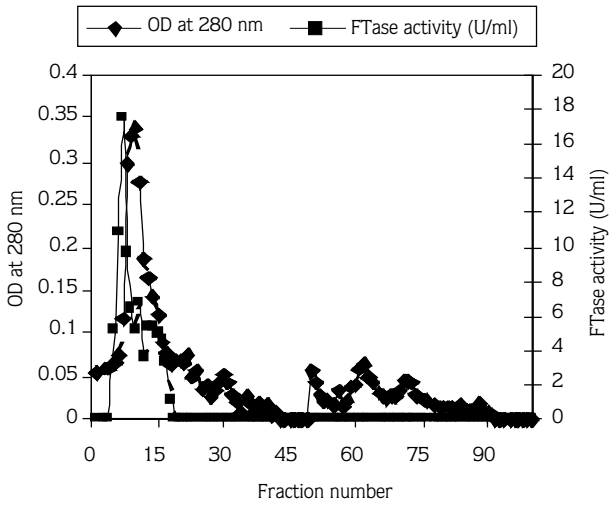

Figure 1. DEAE cellulose column chromatogram of the ammonium sulfate fraction.

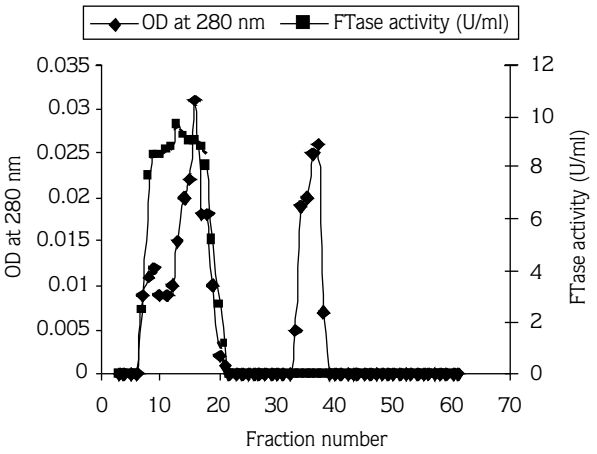

Figure 2. Sephadex G200 column chromatogram of the DEAE cellulose fraction.

Table 1. Purification steps of FTase from *A. pullulans* CFR 77.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	% Yield
Crude	100	616.8	11667	0.5287	-	100
$(NH4)_2SO_4$ fractionation	3	521.49	98.07	5.32	10.06	84.55
IEC*	38	380	10.846	35.04	66.27	61.61
GFC**	25	266.5	6.3452	42	79.44	43.21

* ion-exchange chromatography; ** gel-filtration chromatography.

as obtained from the regression equation (Figure 4). These molecular masses were in the range of those reported for the FTase of *Aspergillus niger* AS0023 (12), but considerably lower than 318 kD and 346 kD for *Aureobasidium* sp. ATCC 20524 (8), and 340 kD for the ß-fructofuranosidase from *Aspergillus niger* (16). Previous studies on FOS-producing enzymes have reported that it was a ß-fructofuranosidase (invertase) with high FTase activity at high sucrose concentration (17-19). FTase without hydrolytic activity has been isolated mainly from higher plants (20,21). The only report on such an FTase from a microbial source was that by L'Hocine et al. (12), in which a series of chromatographic procedures was used to separate the 2 enzymes. In the present study, it was impossible to separate the 2 protein bands through the chromatographic methods herein reported.

The effects of pH and temperature on the activity of the purified FTase are as shown in Figures 5 and 6. The

Figure 3. SDS-PAGE of purified FTase detected by silver staining (P: purified FTase; M: molecular markers; C: crude FTase).

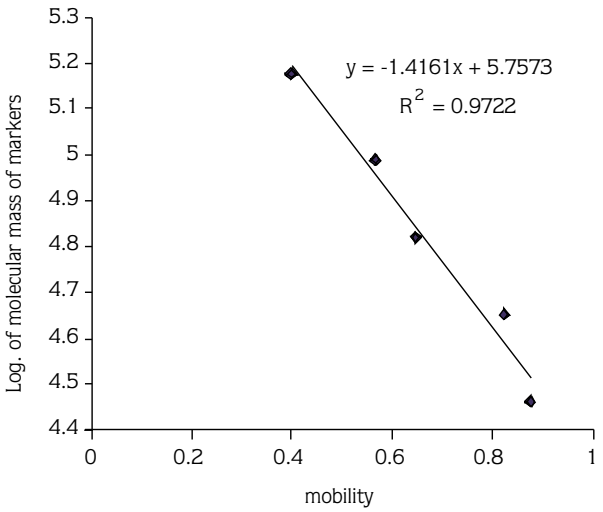

Figure 4. Plot of the logarithm of the molecular mass of markers against their mobility.

enzyme was most active at the approximate pH of 5.0. It had broad activity of at least 80% within the pH range of 4.0-7.0. The activity decreased drastically at pH > 7.0, showing almost no activity. The optimum temperature for the activity of the enzyme was found to be 55 °C. Activity of ≥ 80% was obtained at 60 °C, after which the enzyme lost its activity at 70 °C. At the optimum pH of 5.0 and optimum temperature of 55 °C it was discovered that the enzyme retained over 80% of its original activity after 12 h of reaction (data not shown).

The crude intracellular FTase of this novel strain of *A. pullulans* grown for 24 h in the fermentation medium yielded 56% of FOS within 18 h of reaction. The maximum amount of 59% of FOS was produced within 9 h of reaction (Figure 7), indicating a considerable reduction in the reaction time of 12-25 h reported in the literature (Table 2). The typical HPLC profile of the reaction mixture is as shown in Figure 8. The amount of FOS produced is consistent with the work by Yun and Song (22) and Sangeetha et al. (2); however, it is greater than that reported by Shin et al. (6). The purification of the intracellular FTase of this organism was undertaken in view of its novelty, producing FTase in the shortest fermentation time to be reported for any strain of *A. pullulans* and giving a yield of 59% of FOS within 9 h of reaction, also being the shortest reaction time to be reported regarding the enzymatic production of FOS (Table 2). The purified enzyme produced 59% FOS within 3 h of reaction.

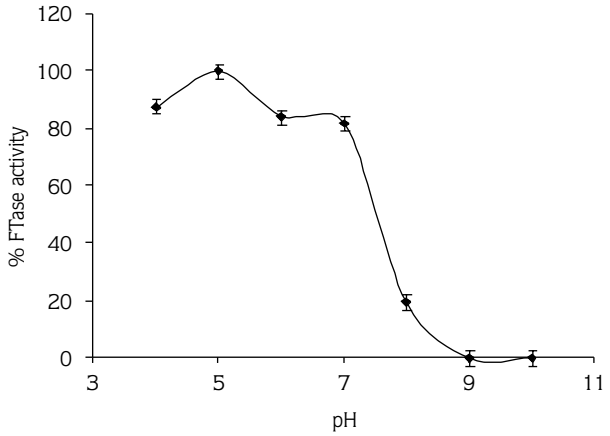

Figure 5. The effect of pH on the FTase activity of the purified enzyme.

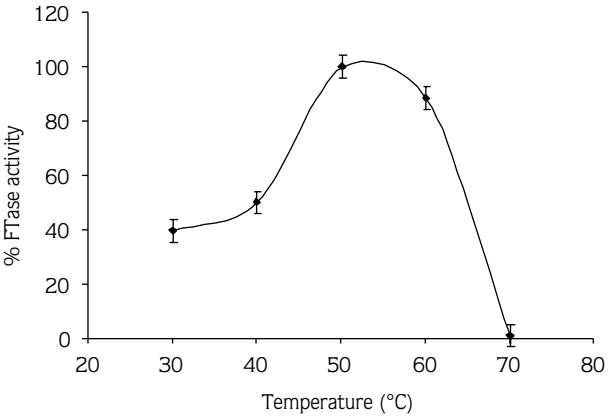

Figure 6. The effect of temperature on the FTase activity of the purified enzyme.

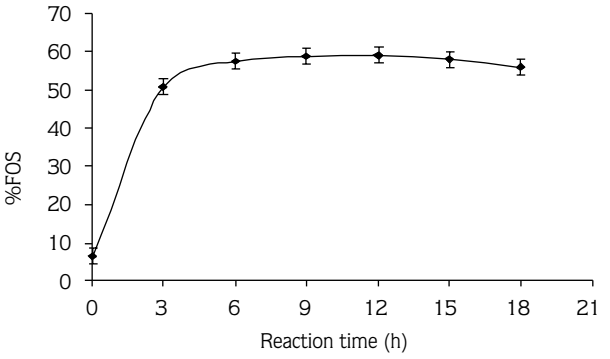

Figure 7. The time course of FOS produced by intracellular FTase of *A. pullulans* grown for 24 h.

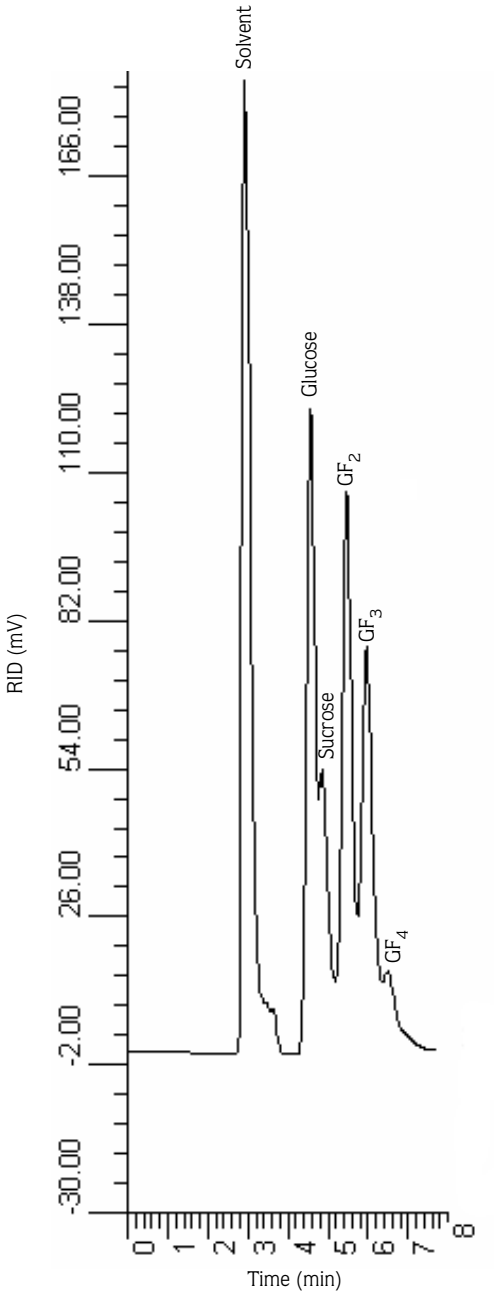

Figure 8. A typical HPLC chromatogram of the reaction mixture at the end of 18 h of incubation, indicating monosaccharide, disaccharide, and oligomers.

Although the enzyme was not purified to homogeneity, this study demonstrated some of the attributes of the enzyme, which could be improved upon in subsequent studies. Some classical attributes of the enzyme, which has made it first amongst equals in the class of FTases, are worth investigating further.

Table 2. Comparison of some parameters obtained for different strains of *Aureobasidium pullulans* in the present study and the literature.

Strain; fermentation time; growth temperature; inoculum size	Source of enzyme	Enzyme-substrate ratio; sucrose concentration	Reaction time; Temperature; pH	% FOS produced	References
Aureobasidium sp ATCC 20524; 48 h; 30 °C; NS	Intracellular	1:4; 75%	NR	NR	Hayashi et al. (8)
A. pullulans KFCC 10524; 96 h; 28 °C; 0.5%	Intracellular	10:1; 40%	25 h; 55 °C; 5.5	58%	Yun and Song (22)
A. pullulans ATCC 9348; 72 h; 28 °C; NS	Immobilized cell	3:1; 60%	24 h; 55 °C; 6.2	44%	Shin et al. (6)
A. pullulans KCCM 12017; 72 h; 28 °C; 10%	Intracellular (cells)	10:1; 41%	24 h; 55 °C; 5.5	53%	Shin et al. (23)
A. pullulans CCY 27-1-94; 40-60 h; 28 °C; 1%	Extra &Intracellular	1:19; 70%	NR; 55 °C; 5.5	NR	Vándákova et al. (7)
A. pullulans CFR 77; 48h; 30 ± 1°C, 10%	Extracellular and culture broth homogenate	1:1.5; 55% and 80%	12-24 h; 55 °C; 5.5	54%-57%	Sangeetha et al. (24)
A. pullulans CFR 77; 24h; 30 ± 1°C; 20%	Intra & extracellular	1:9; 60%	9 h; 55 °C; 5.15	56%-59%	Present study

Different media compositions were used by the investigators; NR: not reported; NS: not stated.

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