Microbial Fructosyltransferase: Production by Submerged Fermentation and Evaluation of pH and Temperature Effects on Transfructosylation and Hydrolytic Enzymatic Activities

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Abstract**—***Fructosyltransferases (FTase, E.C. 2.4.1.9) are enzymes that catalyze transfructosylation reactions obtaining, as final product, fructose oligomers. In terms of industrial production, the use of microbial enzymes is interesting, especially those produced by Aspergillus sp. The aim of the work was to study the production of FTase by submerged fermentation and evaluation of pH and temperature effects on fructosyltransferase activity. The enzyme was produced by Aspergillus oryzae IPT 301 in a 10-liter bioreactor with growth medium containing sucrose as main carbon source. The assay was performed at 800 rpm, 30 °C, 0.75 vvm and pH 4.5. Transfructosylation (At) and hydrolytic (Ah) activities were determined in the temperature range from 35 to 65 °C and pH range from 3.5 to 6.0. It was observed that mycelial is increases with temperature, holding the maximum value at 50 – 65 ºC, while the optimum pH value were 5.0. The optimum temperature for extracellular At ranged from 55 to 65 °C and the optimum pH ranged from 5.0 to 6.0. Furthermore, the optimum temperature for mycelial Ah was 65 °C and optimum pH 4.5 - 5.0. For extracellular Ah, the optimum temperature and optimum pH were 55 to 65 °C and 3.5 to 6.0, respectively.*

Keywords**—*****Aspergillus oryzae, Bioreactor, Fructooligosaccharides, Fructosyltransferase.***

I. INTRODUCTION

Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety [1,2]. They are mainly composed of 1-kestose (GF2), nystose (GF3), and 1-β-fructofuranosyl nystose (GF4), in which fructosyl units (F) are bound at the β (2→1) position of the sucrose molecule (GF) [2,3,4,5]. FOS with low polymeric degree has better therapeutic properties than those with a high polymeric degree [6]. These fructose oligomers are classified as prebiotics and have numerous beneficial properties for human health [1,2,7]. They are non-cariogenic sweeteners of low caloric value, safe for diabetes, as they are not hydrolyzed by the gastro-intestinal enzymes, selectively promoting the growth of Bifidobacterium in the colon [8,9,10] thus helping to eliminate microorganisms which are pathogenic to human health and preventing colonic carcinogenesis [11,12], and increase the adsorption of calcium and magnesium [13]. In addition, FOS favor the reduction of plasma levels of cholesterol, triglycerides and phospholipids [14,15,16]. They are about 0.4 and 0.6 times as sweet as sucrose and have been widely utilized in food and pharmaceutical industries as a functional sweetener [2,6,17].

Although FOS can be produced by the action of enzymes present in some plants, the commercially available FOS are produced mainly by enzymatic synthesis from sucrose by microbial enzymes called β-fructofuranosidases (FFases, E.C.3.2.1.26) and fructosyltransferases (FTases, E.C.2.4.1.9) [18,5] which have been found in several fungal strains, such as *Penicillium* sp. [19,20,21,22], *Aureobasidium* sp. [23,24,25], *Fusarium* sp. [26,27,28,29] and mainly *Aspergillus sp*. [3,6,9,17,18,30,31,32,33,34,35,36]. FOS-producing enzymes from microorganisms are excreted either outside the cell as extracellular enzymes or retained within the cell as intracellular enzymes [9,35,37]. FFase enzymes catalyse hydrolytic and transfructosylating reactions, but their ability for transfer is only with high sucrose amounts. On the other hand, FTases possess transfructosylating activity, acting on the β(2→1) link of sucrose and transfer a fructose molecule to an acceptor such as another sucrose molecule, leading to the generation of FOS with a different chain length and release of glucose in the reaction as a by-product [22,36,38]. FTases shows a little affinity towards water as an acceptor, which means that the hydrolases activity of enzyme is relatively low [38].

Even though other manuscripts have presented data on FOS and FTases production, there is a scarcity of works on the production of these components by submerged fermentation in a pilot reactor, as well as regarding the screening of optimal pH and temperature for extracellular and mycelial FTase activities produced by *Aspergillus oryzae*. Thus, the aim of the

present paper was the production of fructosyltransferase by *Aspergillus oryzae* IPT 301 performed by submerged fermentation using batch reactor with growth medium containing sucrose as main carbon source. The paper also reports the influence of pH and temperature on mycelial and extracellular activities of the enzyme by these fungi. The microorganism was used at first for amylase [39] and pectinase [40] production. After these initial applications, the fungus was tested for fructosyltransferase production at the Institute for Technology Research of São Paulo (IPT/SP, Brazil), obtaining positive results.

II. MATERIAL AND METHODS

2.1 Microorganism and Cultivation Conditions

The fungal strain *Aspergillus oryzae* IPT 301 from the Institute for Technology Researchof São Paulo (IPT/SP - Brazil) culture collection was used for fructosyltransferase production. This fungal strain was used since it has previously shown the best results of enzyme production [9]. For inoculum preparation, the fungal strain was grown on malt extract agar plates for 7 days at 30 ^{0}C; the conidia were suspended in saline solution (NaCl 0.95%, w/v and Tween-80 0.1 %, v/v) and diluted to obtain a spore suspension containing around 108 spores.mL^{-1}. Experiments were performed in a bioreactor containing 10 L of culture medium, with the following composition (g.L^{-1}): sucrose 320.500; yeast extract 2.107; urea 7.130; $MgSO_4.7H_2O$ 0.500; K_2HPO_4 5.000; $MnCl_2.4H_2O$ 0.030; $FeSO_4.7H_2O$ 0.010. 5 mL of polypropylene glycol were used as antifoam. The bioreactor was inoculated with 100 mL of spore suspension and fermentation was performed at 30 ^{0}C and 800 rpm for 63 h. Culture medium pH was adjusted to 4.5 before sterilization and maintained during fermentation using $H_2SO_4$2 mol.L^{-1}and NaOH 4 mol.L^{-1}. The aeration system was 0.75 vvm (volume of air per volume of medium per minute) with air flow rate of 6.0 L.min^{-1}. Samples for analysis were collected and filtered using filter paper (Whatman No 1). In the filtered broth, the residual sugar (sucrose, glucose, fructose and fructooligosaccharides), pH and extracellular enzyme activity were measured. Cell mass concentration and mycelial enzymatic activity were determined using the biomass produced. Fermentation experiments were performed in duplicate.

2.2 Cell mass Concentration

Cell mass concentration was determined by dry cell weight per volume (g.L^{-1}). The cell mass obtained by vacuum filtration of the fermentation broth was washed with distilled water and dried at 105 ºC for 4 h.

2.3 Enzymatic activity

The extracellular and mycelial enzymatic activities were determined as follows: 0.1 mL of suitably diluted supernatant or 0.05 g of cells was mixed with 3.7 mL of 636 g.L^{-1} sucrose and 1.2 mL of 0.2 mol. L^{-1} tris-acetate buffer pH 5.0. The enzymatic reaction was conducted in a rotary shaker at 55 ^{0}C and 190 rpm for 60 min and stopped by heating the mixture in boiling water for 10 min [6,9,41]. The units of fructosyltransferase (At) and hydrolytic (Ah) activities were defined as the amount of enzyme that produces a 1 µmol of FOS or releases a 1 µmol of fructose, respectively, per minute under the chosen experimental conditions.

2.4 Sugars and FOS concentration

Sugar and FOS concentration in the samples were analyzed using high performance liquid chromatography (HPLC) with a Rezex RCM-Monosaccharides Ca^{2+} (8%) (300 x; 7.8 mm, Phenomenex®) Ion-Exclusion column, and a system composed of a 510 pump, a differential refraction index detector and a data processor with register, all from Waters®, USA. The samples were eluted with Milli-Q water at the flow rate 0.6 mL.min^{-1}, maintaining column temperature at 85 ^{0}C.

2.5 Temperature and pH effects on enzymatic activity

After 63 h of fermentation, samples of the culture medium were collected from the bioreactor and vacuum-filtered. Biomass (mycelium) containing mycelial FTase and permeate (filtered broth) containing extracellular FTase were used for enzymatic characterization assays at different pH values (3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) at 55 °C and distinct temperatures (35, 40, 45, 50, 55, 60 and 65 °C) at pH 5.0. All experiments were performed using a reaction mixture composed of 3.7 mL of 636 g.L^{-1} sucrose, 1.2 mL of 0.2 mol.L^{-1} tris-acetate buffer and 0.1 mL of appropriately diluted enzyme solution (extracellular activity) or 0.05 g of cells (mycelial activity). Enzymatic reaction was performed for 60 min at pH and temperatures tested in a rotary shaker at 190 rpm and stopped by heating the mixture in boiling water for 10 min. All experiments were performed in triplicate.

III. RESULTS AND DISCUSSION

3.1 Production of FTase by submerged fermentation

Fig. 1 shows a decrease of approximately 50% in sucrose concentration and 112 $g.L^{-1}$ FOS production in 17 h of process. In this period, glucose and fructose were produced in small amounts. Although an initial hydrolytic activity is observed, resulting in glucose and fructose production, the high content of FOS indicates favorable pH, temperature and agitation for the production of this sugar by submerged fermentation.

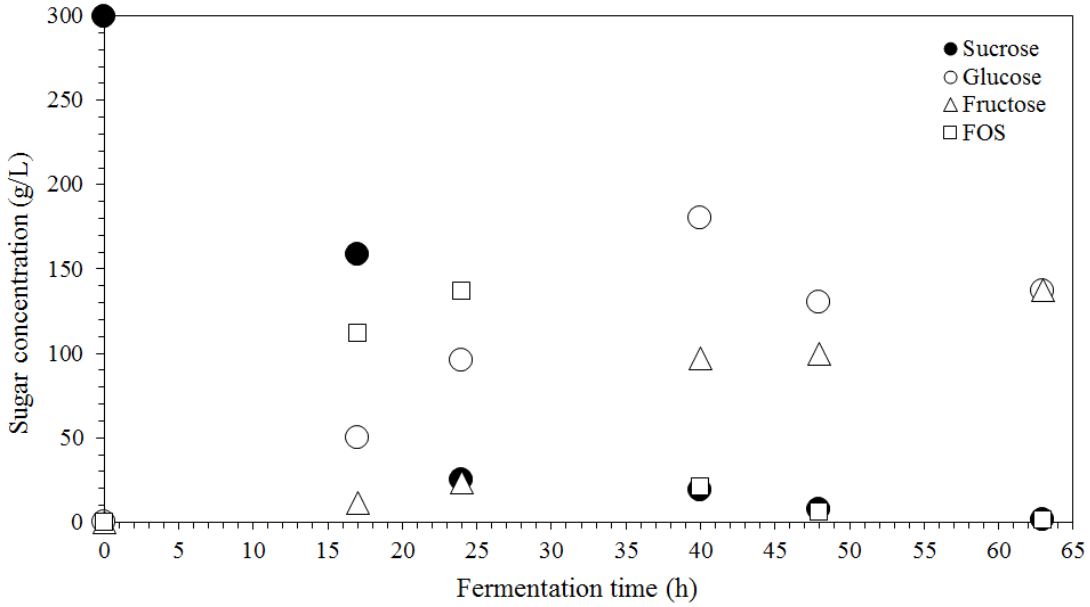

FIGURE 1: Sugar concentration (sucrose, glucose, fructose and FOS) quantified during fermentation time in 10-liter batch reactor using Aspergillus oryzae IPT-301. Fermentation was performed at 30 ^{0}C and 800 rpm agitation for 63 h, culture medium pH 4.5 and 0.75 vvm aeration with air flow rate of 6.0 $L.min^{-1}$.

The maximum FOS concentration was obtained at about 24 h of fermentation, when its content was 137 $g.L^{-1}$ and the yield was 45.8%. For the same period, the content of sucrose decreased from 300 $g.L^{-1}$ to 25 $g.L^{-1}$, and with the content of fructose reaching 25 $g.L^{-1}$. Glucose concentration also doubled, rising from 50 $g.L^{-1}$(17 h of fermentation) to 100 $g.L^{-1}$(24 h). Elapsed 40 h of process and comparing with the results after 24 h, glucose and fructose contents increased, respectively, about 87.5% and 415%. Simultaneously, FOS content decreased to 21 $g.L^{-1}$and sucrose concentration decreased about 75%. This fermentative behavior indicates FOS consumption by the fungal metabolism, once sucrose content is considerably low, and a concomitant increase in hydrolytic activity. The fermentation was conducted in an acid culture medium maintained at pH 4.5 throughout the enzyme production process.

For 48 h and 63 h of process, it was observed that the amounts of sucrose and FOS were almost depleted from the culture medium. It is believed that the molecules of these oligomers were hydrolyzed by the action of hydrolytic enzymes produced during fermentation. It is presumed that the high hydrolytic activity of the medium favored a considerable rise in the concentration of glucose and fructose monomers in the final fermentation steps.

[2] and [9] reported a partial hydrolysis and consumption of the initial sucrose content in the culture medium for growth and maintenance of the cellular metabolism, whereas the remaining sucrose is subjected to the action of the enzymeβ-fructofuranosidase produced by the growing microorganisms, generating FOS, glucose and fructose. Considering only the extracellular enzymes, FTase activity increased as a function of fermentation time, from 6 $U.mL^{-1}$in 17 h to a maximum of 36 $U.mL^{-1}$in 40 h, as showed in Fig. 2. After this period, the activity of this enzyme was almost constant, indicating a slight trend for decrease to 29 $U.mL^{-1}$, keeping approximately this same value until 63 h of fermentation. These results indicate an increase in FTase activity, concomitant to a decrease in sucrose content until 24 h, and a decrease in FOS content as a function of the higher hydrolytic activity after this time. This behavior is probably related to a predominant transfructosylation activity in the first stages of the process, and a sequential hydrolytic activity, resulting in the breakdown of FOS molecules.

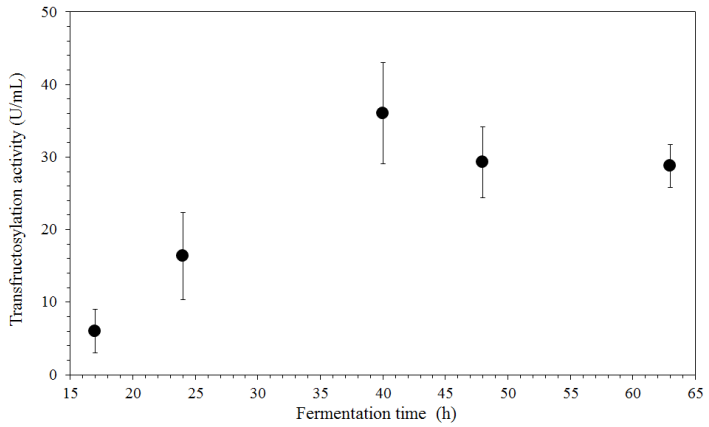

FIGURE 2: Extracellular transfructosylation activity obtained during the fermentation time in 10-liter batch reactor using *Aspergillus oryzae* IPT-301. Fermentation was performed at 30 ^{0}C and 800 rpm agitation for 63 h, culture medium pH 4.5 and 0.75 vvm aeration with air flow rate of 6.0 L.min^{-1}.

Only a few reports exist regarding the production of FTase and FOS in bioreactors. [42] reported the maximum production of FTase, 11 U.mL^{-1}, for 12 h of fermentation at 37 °C using *Bacillus macerans* as a production source of the enzyme. Sucrose (15 g.L^{-1}) was used as carbon source. Additionally, aeration (1.0 vvm), agitation (400 rpm) and pH 5.5 of the culture medium were controlled in the bioreactor. [41] reported on FTases produced with low transfructosylation activity (0.053 U.mL^{-1}) from *Penicillium citrinum* using 200 g.L^{-1} of sucrose in the culture medium, with pH 6.0. Fermentation was performed for 36 h at 28 °C and the bioreactor was maintained at 1.5 vvm aeration and agitation at 500 rpm. [43] reported a maximum FOS yield of 62.8% in a continuous process, with the biomass harvested after 48 h of *Aspergillus sp*. N74 growth using a mechanically-agitated airlift reactor. In this fermentation, an initial sucrose concentration of 70% (w/v) was employed, twice that used in the present work, at 60 °C. In spite of the higher FOS yield obtained by these authors, the maximum transfructosylation activity obtained was about 18.5 U.mL^{-1} lower than that obtained in the present work. Therefore, the extracellular FTases reported by the authors were observed to show a lower transfructosylation activity when compared to the present study. The results suggest that *Aspergillus oryzae* IPT-301 can be viewed as a potential microorganism for the production of FTases using bioreactors, according to the experimental conditions presented in this work.

Fig. 3 presents a maximum mycelial FTase activity of 2900 U.g^{-1}dry cell in the initial stages of the process for 17 h of fermentation. From this time, mycelial enzyme activity ranged between 2000 U.g^{-1}and 2600 U.g^{-1}dry cell, until 63 h of fermentation. The highest mycelial FTase activity at the beginning of the fermentation is coincident with the minimum extracellular activity time, possibly indicating a requirement of this type of enzyme, adhered to the mycelium, for the initial biomass development. Presumably, elapsed 24 h of fermentation, after the initial biomass development, the fungus starts to produce concomitantly extracellular FTase in large scale. From this fermentation period, mycelial and extracellular enzymes show relatively high activities, aiming to support the cell metabolism when sucrose, the primary carbon source, content is low.

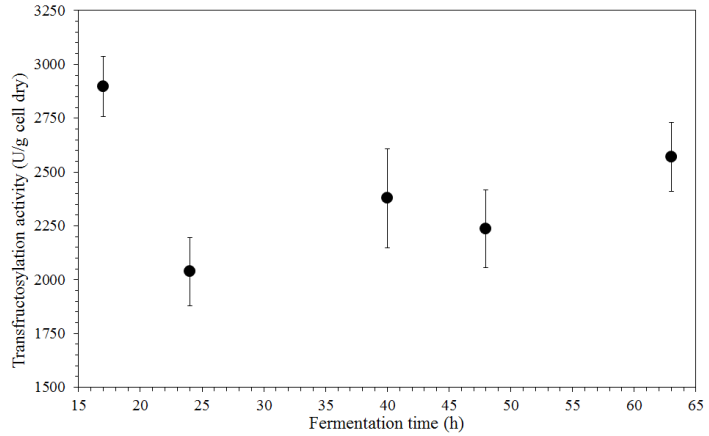

FIGURE 3: Mycelium transfructosylation activity obtained during the fermentation time in 10-liter batch reactor using *Aspergillus oryzae* IPT-301. Fermentation was performed at 30 ^{0}C and 800 rpm agitation for 63 h, culture medium pH 4.5 and 0.75 vvm aeration with air flow rate of 6.0 L min^{-1}.

3.2 Evaluation of pH and temperature effects on FTase activities

Transfructosylation and hydrolytic activities were higher, and constant, at temperatures from 55 °C to 65 °C, as was observed from screening of the extracellular FTase activity as a function of temperature, presented in Fig. 4. In this temperature range, transfructosylation activity was about 155 U.mL^{-1} and at 55 °C the maximum (At/Ah) ratio was observed, about 21. This temperature range is different from that used for microbial cultivation, indicating the advantages of using the isolated FTase, and not the fungus, for FOS production. Some studies, such as [44], stated that the optimum temperature for FTase transfructosylation activity was obtained between 50 and 60 °C.

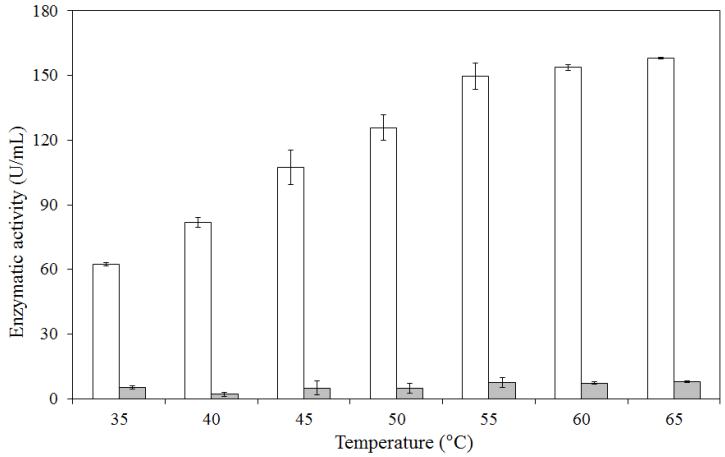

FIGURE 4: Effects of temperature on extracellular transfructosylation activity (unfilled bars) and extracellular hydrolytic activity (gray bars). Experimental conditions: activities were performed in rotary shaker at 190 rpm agitation for 60 min using 0.2 mol.L^{-1}tris-acetate buffer (pH 5.0) and 636 g.L^{-1} sucrose as substrate.

The ratio between transfructosylation and hydrolytic activities (At/Ah) can be considered as the most important criteria when evaluating FOS production from different microbial enzyme [9,45]. When high ratio values (At/Ah) are obtained, FTase exhibits greater transfructosylation activity in the reaction medium. Therefore, high transfructosylation activity allows a high conversion of sucrose to FOS while a high (At/Ah) is required to avoid FOS molecule hydrolysis [9,45,46]. In addition, for the industrial FTase application, high ratio (At/Ah) values are preferred associated with the potential production of the enzyme to achieve efficient FOS synthesis [9,47,48,49].

As shown in Fig. 5, FTase transfructosylation activity for mycelial enzymes was higher, and very similar at the temperature range from 50 °C to 65 °C, presenting values of about 2700 U.g^{-1} dry cell. The hydrolytic activity increased as a function of temperature from 70 U.g^{-1} dry cell, at 35 °C to 350 U.g-^{1} dry cell at 65 °C and the maximum (At/Ah) was about 17 at 55 °C.

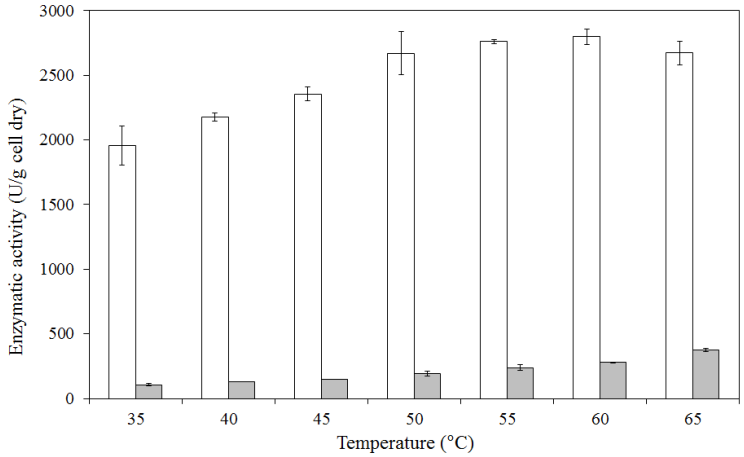

FIGURE 5: Effects of temperature on mycelium transfructosylation activity (unfilled bars) and mycelium hydrolytic activity (gray bars). Experimental conditions: activities were performed in rotary shaker at 190 rpm agitation for 60 min using 0.2 mol.L^{-1}tris-acetate buffer (pH 5.0) and 636 g.L^{-1} sucrose as substrate.

In Fig. 6, a great influence of pH values on extracellular FTase transfructosylation activity and hydrolytic activity is observed, showing, respectively, maximum values of 146 $U.mL^{-1}$ at pH 6.0 and 35 $U.mL^{-1}$ at pH 3.5. The pH range from 4.5 to 5.5 showed the best ratios (At/Ah), having a maximum of 38 at pH 5.0. On the other hand, Fig. 7 shows an almost constant mycelial FTase hydrolytic activity as a function of pH, and a maximum mycelial FTase transfructosylation activity of 3265 $U.g^{-1}$ dry cell at pH 5.0, resulting in (At/Ah) of about 13. Some studies stated the optimum pH for these enzyme activities from 4.5 to 6.5 [50].

The difference between activity behavior of mycelial and extracellular FTase may occur because mycelial enzymes are affected by the cell. Enzymes inside the cells require that substrates and products cross the membrane; additionally, the cellular environment affects enzymatic activities. In addition, enzymes on the cell surface are stabilized by the cell membrane and are less accessible to degradation and adsorption of materials from the reaction medium [51].

IV. CONCLUSION

FOS production using *Aspergillus oryzae* IPT 301 by submerged fermentation results in a 45.8% yield after 24 h of fermentation, and maximum extracellular FTase activity of 36 $U.mL^{-1}$ at 40 h. The temperature used in fermentation (30 °C) is very different from the one 55 °C resulting in the maximum transfructosylation activity and (At/Ah) ratio. The optimum temperature and pH for transfructosylation activity, ideal for FOS production, are different for the extracellular and mycelial enzymes. Using a proper pH value and temperature, it is possible to maximize the transfructosylation activity and minimize the hydrolytic activity for FOS production using the FTase from the microorganism studied.

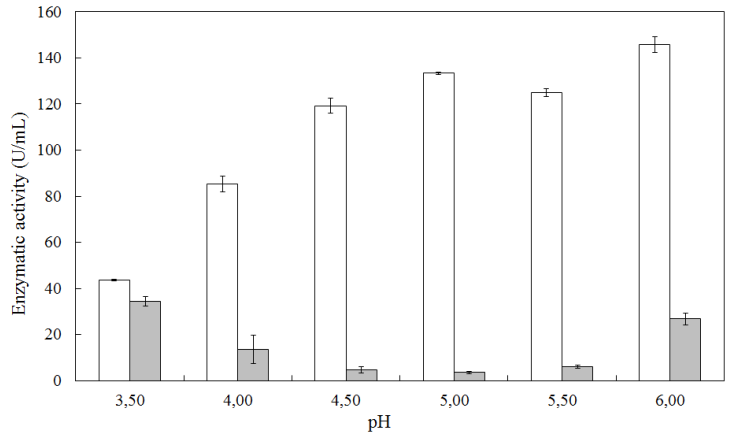

FIGURE 6: Effects of pH on extracellular transfructosylation activity (unfilled bars) and extracellular hydrolytic activity (gray bars). Experimental conditions: activities were performed in rotary shaker at 55 ˚C, 190 rpm agitation for 60 min using 0.2 $mol.L^{-1}$tris-acetate buffer and 636 $g.L^{-1}$ sucrose as substrate.

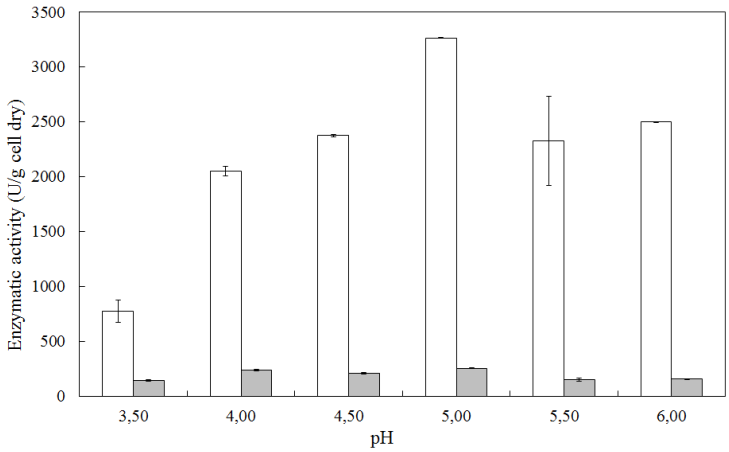

FIGURE 7: Effects of pH on mycelium transfructosylation activity (unfilled bars) and mycelium hydrolytic activity (gray bars). Experimental conditions: activities were performed in rotary shaker at 55 ˚C, 190 rpm agitation for 60 min using 0.2 $mol.L^{-1}$tris-acetate buffer and 636 $g.L^{-1}$ sucrose as substrate.

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