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ORIGINAL PAPER

Production of fructosyltransferase by *Aureobasidium* sp. ATCC 20524 in batch and two-step batch cultures

Martín A. Salinas · Nora I. Perotti

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Abstract A comparison of fructosyltransferase (EC 2.4.1.9) production by *Aureobasidium* sp. ATCC 20524 in batch and two step batch cultures was investigated in a 1-l stirred tank reactor using a sucrose supply of 200 g/l. Results showed that the innovative cultivation in two step of *Aureobasidium* sp. produced more fructosyltransferase (FFase) than the single batch culture at the same sucrose concentration with a maximal enzyme production of 523 U/ml, which was 80.5% higher than the one obtained in the batch culture. The production of fructooligosaccharides (FOSs) was also analyzed; their concentration reached a maximum value of 160 g/l the first day in the two-step culture and 127 g/l in the single-batch mode. The use of the two-step batch culture with *Aureobasidium* sp. ATCC 20524 in allowing the microorganism to grow up prior to the induction of sucrose (second step), proved to be a powerful method for producing fructosyltransferase and FOSs.

Keywords *Aureobasidium* sp · Fructosyltransferase · Enzymatic production · Fructooligosaccharides

M. A. Salinas (✉) · N. I. Perotti
PROIMI-CONICET (Planta Piloto de Procesos Industriales Microbiológicos, Consejo Nacional de Investigaciones Científicas y Tecnológicas), Av. Belgrano y Pje. Caseros, San Miguel de Tucumán, CP 4000, Argentina
e-mail: msalinas@proimi.org.ar

M. A. Salinas · N. I. Perotti
Cátedra de Microbiología General e Industrial, Facultad de Ciencias Exactas y Tecnología, Universidad Nacional de Tucumán, Tucumán, Argentina

Introduction

It is an accepted opinion that fructooligosaccharides (FOSs) are a common name only for fructose oligomers that are mainly composed of 1-kestose (GF_2), nystose (GF_3) and fructosylnystose (GF_4), in which two and three fructosyl units are bound at the β-2,1 position of sucrose (GF) respectively [13]. They are considered both alimentary additives and nutraceutics. Also FOSs are not susceptible to decomposition by human or animal digestive enzymes. Several studies have demonstrated their prebiotic properties which favor an adequate function of the human digestive system, through a stimulating effect on the growth of the beneficial *Bifidobacteria* in the human colon by suppression of putrefactive pathogens and by reduction of serum cholesterol concentrations [4, 7]. They are also considered as alternative sweeteners and its sweet taste is much similar to that of sucrose [15].

Aureobasidium sp. ATCC 20524 was previously reported as a potent strain for obtaining high activities of fructo-oligosaccharide producing enzymes. When sucrose is used as substrate, FOSs were formed as a structure of 1^F-(1-β-fructofuranosyl)$_n$-sucrose, i.e., $n = 1$–3, such as 1-kestose, nystose and fructofuranosyl nystose [5, 6, 9]. These oligosaccharides are produced from sucrose by the action of fructosyltransferase (FFase) at high osmotic stress which also produces inhibition of cell growth [14].

Although it was described in *Aspergillus niger* AS0023 that the fructosyltransferase and hydrolytic activity that causes synthesis and hydrolysis of FOSs respectively, are due to different enzymes [8, 11], studies on *Aureobasidium* sp. suggests that both activities are due to the same enzyme [15].

The aim of this work was to produce fructosyltransferase by *Aureobasidium* sp. ATCC 20524 in a two step submerged

culture bioreactor using sucrose as carbon source. To that end, kinetic parameters of growth, FFase and hydrolytic enzymatic activities and synthesis of FOSs were analyzed.

Materials and methods

Microorganism

Aureobasidium sp. ATCC20524 was used in this work. The strain was maintained at −20°C in 1.5 ml vials containing 0.5 ml glycerol 40% and 0.5 ml of cell cultured grown on seed medium containing: glucose 20 g/l, peptone 10 g/l yeast extract 5 g/l.

Inoculum culture conditions

About 0.5 ml of growth on the seed medium was used as inoculum and transferred by duplicate to 250-ml flask containing 100 ml of production broth composed by sucrose 10 g/l, $NaNO_3$ 10 g/l, yeast extract 10 g/l, KH_2PO_4 5 g/l and $MgSO_4{\cdot}7H_2O$ 0.5 g/l. Flasks were cultured at 30°C for 1 day with orbital shaking at 200 rpm.

Batch culture

The 100 ml of growth obtained as described were transferred to a 1.5-l stirred tank reactor (New Brunswick, Discovery Series 100), containing the production broth modified with an initial sucrose concentration of 200 g/l in a total volume of 1 l. The medium was adjusted to pH 6.5 and controlled during fermentation using NaOH and HCl at a concentration of 0.5 N. Dissolved oxygen was provided by passing dry air generated with a compressor at 8 kg/cm^2 and a flow of 2.5 vvm. The temperature was kept at 30°C and the agitation at 500 rpm. Experiments were carried out in duplicate.

Two step batch culture

The 100 ml inoculum was transferred to a 1.5-l stirred tank reactor (New Brunswick, Discovery Serie 100) containing 700 ml of the production broth with a sucrose concentration of 10 g/l. After 8 h of cultivation (residual sucrose: 1 g/l), 200 ml of a sucrose solution was added to reach a final concentration of 200 g/l and a final volume of 1 l. Using automatic control the temperature, pH, air flow and agitation conditions were kept at 30°C, 6.5, 2.5 vvm (8 kg/cm^2) and 500 rpm, respectively.

Enzymatic assays

FFase enzyme activity was determined by a method previously described [10]. One unit (1 U) of transfructosylating activity is defined as "the amount of enzyme activity required to transfer 1 μmol of fructose per min". While one unit of hydrolytic activity is defined as the amount of enzyme required to produce 1 μmol of fructose per minute. The reaction mixture to quantify the enzyme activities was composed by 0.75 ml of sucrose 800 g/l as substrate, 0.23 ml of 0.1 M citrate buffer (pH 5.5) and 0.02 ml of enzyme sample (extracellular enzyme solution). Reaction conditions were: pH 5.5, 55°C during 15 min, and was stopped by heating at 100°C for 15 min. Enzymatic reactions were carried out by triplicate. Results represent the average of at least three independent assays. For the sake of simplicity, standard deviation bars were excluded from graphics. Standard deviation was never more than 2.9.

Transfructosylating activity and hydrolytic activity were determined by measuring both, the released reducing glucose (G) by the glucose-oxidase method (Kit Glicemia Wiener Lab) and the released reducing sugars (S) with the DNS method respectively [12].

Concentrations of free fructose (F) and transferred fructose (TF) were calculated according to [2, 3] with the following equations:

$$F = S - G,$$

$$TF = G - F = 2G - S.$$

Analytical methods

Growth was determined by measuring optical density at 620 nm.

Proteins were determined by the method of Bradford using bovine serum albumin as protein standard measuring protein concentration at an OD of 595 nm [1].

High performance liquid chromatography (HPLC)

Sugars (sucrose, glucose, 1-kestose and nystose) were determined and quantified during cultivation using HPLC. A Rezex (Phenomenex) Oligosaccharide column was utilized at 50°C with water as the mobile phase at a flow rate of 0.3 ml/min. Detection was performed by refractive index measurement using a Differential Refractometer 2142 LKB (Bromma, Sweden).

Results and discussion

Fructosyltransferase activity and cell growth in batch culture and two step batch culture

The time course of FFase production for the two modes is shown in Fig. 1. In both types of cultivation the enzyme production increases with time and follows growth,

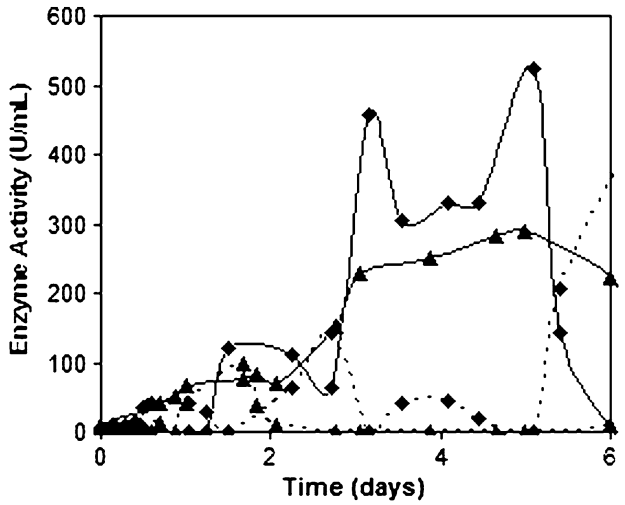

Fig. 1 Fructosyltransferase (*dashed curves*) and hydrolytic activity (*dotted curves*). Comparison between 2SBC (*filled diamonds*) and BC (*filled triangles*). During the first 8 h in the 2SBC before the induction with sucrose no transfructosylating activity was detected and only hydrolyzing activity was detected. The maximal values for transferase activity was obtained in the fifth day. A quite similar behavior was found for the specific FFase activity, with maximum values of 3.51 U/μg and 1.95 U/μg in 2SBC and BC, respectively

reaching a maximal enzyme production of 523 U/ml in the two step batch culture (2SBC) in 5 days, which was 80.5% higher than the one obtained in the batch culture (BC) (290.3 U/ml).

The second step in the 2SBC was performed 8 h after the beginning of the process when the residual sucrose was of 1 g/l. In this cultivation mode was observed a slight inhibition of cell growth due to the quickly increment of the osmotic pressure generated by the second step. At the end of the 2SBC 60 g/l of cell mass was obtained as opposed to a cell mass of 90 g/l for the BC (Fig. 2).

Transfructosylating activity (UT) and hydrolyzing activity (UH)

In order to determine the relationship between the enzymatic activity and the transformation of sucrose into FOSs in both systems, transfructosylating and hydrolyzing activities in the culture broth were assayed along the time course of cultivation and the changes in both activities were followed over the course of 7 days as is shown in Fig. 1. In the BC mode during the first 2 days, the hydrolyzing activity was very low. Then, was essentially zero while the FFase activity reached its maximum value at the fifth day and decayed thereafter.

Assays of the enzymatic activities in the 2SBC showed maximum values of FFase activity and hydrolyzing activities were 523 U/ml in the fifth day and 142.8 U/ml in the third day, respectively. Overall, in both cultivation modes FFase activity was much higher than hydrolyzing activity during all fermentation assays, decaying immediately after fifth day.

Production of FOSs

In the BC mode, FOSs reached maximum values of 51.8 and 74.6 g/l for 1-kestose and nystose, respectively, with the first day and declined thereafter. After 2 days of cultivation, sucrose was completely consumed (Fig. 3).

In the 2SBC mode, maximum values of FOSs production of 102.4 g/l for 1-kestose and 59 g/l for nystose were obtained during the first day and sucrose was completely consumed. Then, subsequently FOSs declined and fell down to zero after 48 h (Fig. 4). This represents an increase in 1-kestose production of 100% in 2SBC cultivation mode comparing the BC mode.

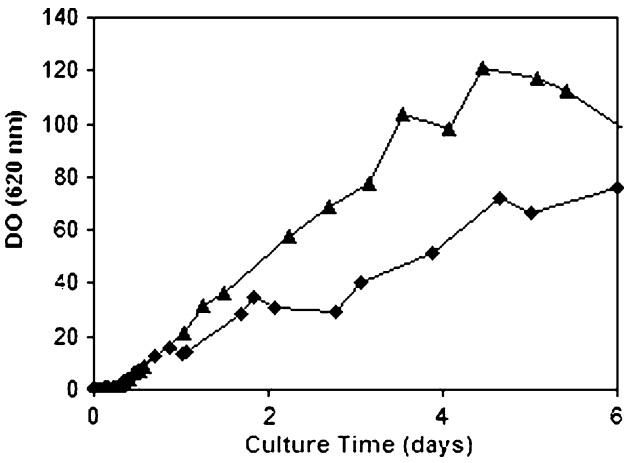

Fig. 2 Growth pattern of *Auerobasidium* sp. for the production of fructosyltransferase in 2SBC (*filled diamonds*) and BC (*filled triangles*), maximum values were 60 and 90 g/l, respectively

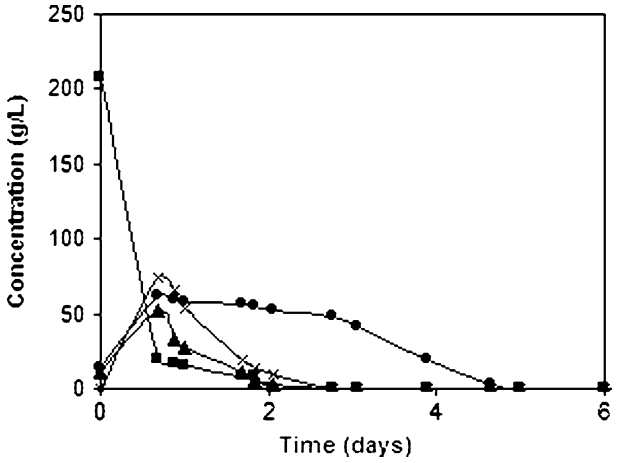

Fig. 3 BC: Concentration of carbohydrates in the bioreactor during the production of fructosyltransferase. Sucrose (*filled squares*), Glucose (*filled circles*), 1-Kestose (*filled triangles*), Nystose (*crosses*)

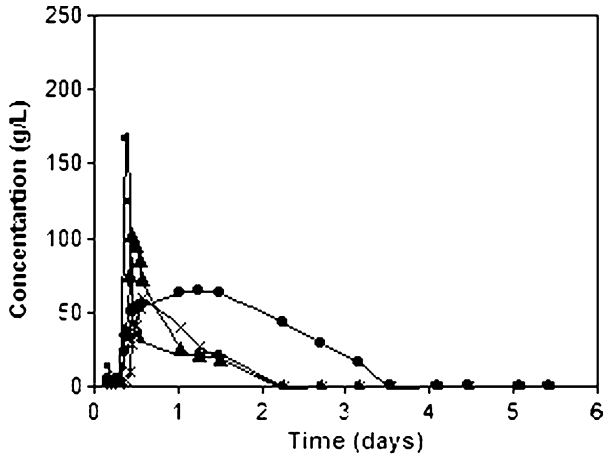

Fig. 4 2SBC: Concentration of carbohydrates in the bioreactor during the production of fructosyltransferase. Sucrose (*filled squares*), Glucose (*filled circles*), 1-Kestose (*filled triangles*), Nystose (*crosses*)

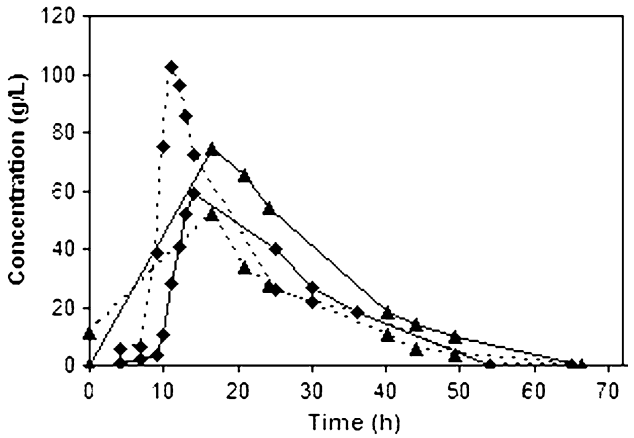

Fig. 5 1-Kestose (*dotted curves*) and Nystose (*dashed curves*) patterns. Comparison between 2SBC (*filled diamonds*) and BC cultivation modes (*filled triangles*)

The patterns of FOSs production observed in BC and 2SBC modes are shown in Fig. 5. Despite pulse addition of sucrose after 8 h in the 2SBC, the maximum value of 1-kestose occurred earlier in the 2SBC mode than in the BC mode. This behavior was also observed for nystose production, but surprisingly, in this case a much higher production of nystose was obtained in the BC cultivation mode. Summarizing, in the BC mode nystose was the FOS most produced, but the total FOSs production was much higher in the 2SBC than in the BC mode (Fig. 5).

After 2 days of cultivation, high molecular weight polysaccharides appeared in the chromatograms in both modes of operation. Although it has been reported that a related strain *Aureobasidium pullulans* is able to produce only 1-kestose, nystose and fructosylnystose [10], the observation of high molecular weight polysaccharides, together with the fact that FOSs increased at 48 h while FFase activity increased until day 5, led us to speculate that Ffase activity could explain the production of polysaccharides of higher molecular weights from FOSs observed in HPLC chromatograms obtained (Data not shown).

Concluding remarks

By using *Aureobasidium* sp. ATCC 20524 in a two step batch culture system, an increase of more than 80% of the FFase activity and 30% of FOSs production compared with that seen in single batch culture mode was observed. Additionally, 1-kestose production showed an increase of 100% compared with that of the BC mode cultivation at the same concentration of sucrose.

From culture parameters, the kinetics of FFase production by *Aureobasidium* sp. ATCC 20524 may be classified growth associated. However the patterns obtained in FOSs production show a de-coupling from growth and from FFase synthesis. This could be attributed to the fact that the production of FOSs is related to the initial concentration of sucrose, the osmotic stress caused and the observation of high molecular weight polysaccharides.

The use of the two step batch culture with the strain studied, allowing the microorganism to grow up before the induction with sucrose (second step), showed very high enzyme productivity, and its transfructosylating activity was much higher compared to its hydrolyzing activity. Therefore this culture mode improves productivity without adversely affect the posterior purification and cost because the same conditions are used in both cultures. Finally, it is feasible to conclude that the 2SBC method is a powerful tool for the production of FFase as well as FOSs.

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References

1. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
2. Chen WC (1995) Production of B-Fructofuranosidase by *Aspergillus japonicus* in batch and fed-batch cultures. Biotechnol Lett 17:1291–1294
3. Chen WC, Liu CH (1996) Production of B-fructofuranosidase by *Aspergillus japonicus*. Enzyme Microb Technol 18:153–160
4. Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1412

5. Hayashi S, Nonokuchi M, Imada K, Ueno H (1990) Production of Fructosyl-transferring enzyme by *Auerobasidium* sp. ATCC 20524. J Ind Microbiol 5:395–400
6. Hayashi S, Shimokawa Y, Nonoguchi M, Takasaki Y, Ueno H, Imada K (1991) Nutritional status of *Aureobasidium* sp. ATCC 20524 for the production of B-fructofuranosidase. World J Microbiol Biotechnol 7:522–525
7. Hidaka H, Eida T, Takizawa T, Tokunaga T, Toshira Y (1986) Effects of fructooligosaccharides on intestinal flora and human health. Bifidobacteria Microflora 5:37–50
8. Hidaka H, Hirayama M, Sumi N (1988) A fructooligosaccharide-producing Enzyme from *Aspergillus niger* ATCC 20611. Agric Biol Chem 52:1181–1187
9. Jung KH, Lim JY, Yoo SJ, Lee JH, Yoo MY (1987) Production of Fructosyltransferase from *Aureobasidium pullulans*. Biotechnol Lett 9:703–708
10. Jung KH, Yun JW, Kang KR, Lim JY, Lee JH (1989) Mathematical model for enzymatic production of fructooligosaccharides from sucrose. Enzyme Microb Technol 11:491–494
11. L'Hocine L, Wang Z, Jiang B, Xu S (2000) Purification and partial characterization of fructosyltransferase and invertase from *Aspergillus niger* AS0023. J Biotech 81:73–84
12. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–427
13. Shin HT, Baig SY, Lee SW, Suh DS, Kwon ST, Lim YB, Lee JH (2004) Production of Fructo-oligosaccharides from molasses by *Aureobasidium pullulans* cells. Bioresour Technol 93:59–62
14. Vandáková M, Platková Z, Antosová M, Báles V, Polakovic M (2004) Optimization of cultivation conditions for production of fructosyltransferase by *Aureobasidium pullulans*. Chem Pap 58:15–22
15. Yun JW (1996) Fructooligosaccharides—occurrence, preparation, and application. Enzyme Microb Technol 19:107–117