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Preparation of High-Purity Fructo-oligosaccharides by *Aspergillus japonicus* β-Fructofuranosidase and Successive Cultivation with Yeast

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The short-chain FOSs with high purity were prepared using a two-step strategy: *Aspergillus japonicus* extracellular β-fructofuranosidase-catalyzed synthesis of FOSs followed by cultivation with *Pichia pastoris* (*P. pastoris*). The higher FOSs content was obtained after 8 h under the catalysis of β-fructofuranosidase at pH 5.5 and 55 °C. Successive *P. pastoris* cultivation exhausts almost all monosugars in 12 h at 30 °C, which increases the purity of FOSs, and also recovers β-fructofuranosidase activity by ceasing the inhibition of glucose from catalysis of the enzyme, yielding more FOSs. Finally, the FOSs purity was increased from 56.55 to 84.45% (26.47% 1-kestose and 57.98% nystose).

KEYWORDS: Fructo-oligosaccharides; *Aspergillus japonicus*; *Pichia pastoris*; β-fructofuranosidase

INTRODUCTION

The efficiency and purity of fructo-oligosaccharides (FOSs) synthesis from sucrose catalyzed by *Aspergillus oryzae* or *Aspergillus sydowi* β-fructofuranosidase is very low. Glucose, a byproduct of the reaction, inhibits the enzyme activity severely, resulting in decreases of the quality and quantity of FOSs; at least the bottleneck, a limitation of 60% of target FOS, cannot be easily overcome (*1–8*). There are some strategies that have been developed for obtaining FOSs with high purity: separating FOS from mono- and disaccharides by chromatography (*1, 8*) or converting mono- and disaccharides selectively to tolerable forms by means of glucose oxidase (*2–4*). The above strategies can improve the quality of FOS at some extent with high cost. An alternative approach is extraction from the root or tube of a crop such as chicory and Jerusalem artichoke, rich in inulin, to get a mixture with a broad degree of polymerization (DPn) from 3 to 100; similarly, its separation and purification processes are cost-dependent (*9–11*), and it is not easy to get short-chain FOS as 3 or 4 DPn as 1-kestose or nystose with special pharmaceutical and nutrition functions on humans and animals (*12, 13*).

By means of nutritional characteristics of *Pichia pastoris* (*P. pastoris*), which can ferment monosaccharides but not disaccharides and oligosaccharides (*14*), we established a "two-step strategy" to improve the purity of FOSs: First, extracellular β-fructofuranosidase of *Aspergillus japonicus* (*A. japonicus*) was used to catalyze the FOS synthesis from sucrose, and then, *P. pastoris* was cultivated to selectively use monosugars in the above synthesis mixture to remove the feedback inhibition of glucose, a byproduct of the β-fructofuranosidase reaction. The amount and purity of FOSs had the potential to significantly increase and showed the potential for industrial applications.

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MATERIALS AND METHODS

Chemicals, Strains, and Their Cultivation. The chemicals nystose and 1-kestose were from Sigma Co., Ltd. The other chemicals were analytical grade. *A. japonicus* 3.2769 was from China General Microbiological Culture Collection Center (CGMCC) (Beijing). *P. pastoris* GS115 was from Invitrogen (Beijing).

A. japonicus was cultured at 30 °C in the liquid medium (*15*). *P. pastoris* GS115 was cultured at 30 °C in YPD medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. *P. pastoris* GS115 was deficient in protease activity.

Preparation of Extracellular β-Fructofuranosidase of *A. japonicus*. One loop of mature spores of *A. japonicus* 3.2769 grown on Czpeck's slant at 30 °C for 4 days was inoculated into 100 mL of medium with shaking at 250 rpm and 30 °C. After 96 h of incubation, the cells were removed by centrifugation at 5000*g* for 5 min, and the supernatant containing crude β-fructofuranosidase was used for its catalysis synthesis and cocultivation with yeast.

The enzyme activity was assayed in a mixture consisting of 0.9 mL of 55.6% sucrose dissolved in 0.05 mol/L sodium acetate buffer (pH 5.5) as the substrate and 0.1 mL of crude enzyme 100-fold diluted in the same buffer. The reaction was run at 55 °C for 1 h and stopped by adding 3,5-dinitrosalicylic acid solution (DNS), boiling at 100 °C for 5 min, and then cooling down to room temperature. The reducing sugar was determined at 540 nm. One unit of β-fructosyltransferase activity was defined as the enzyme amount producing one micromole of reducing sugar per minute (*16, 17*).

Cultivation and Collection of *P. pastoris* Cells. One loop of *P. pastoris* cells was inoculated on YPD agar (15 g/L) medium at 30 °C for 3–5 days and was inoculated into 100 mL of YPD medium and

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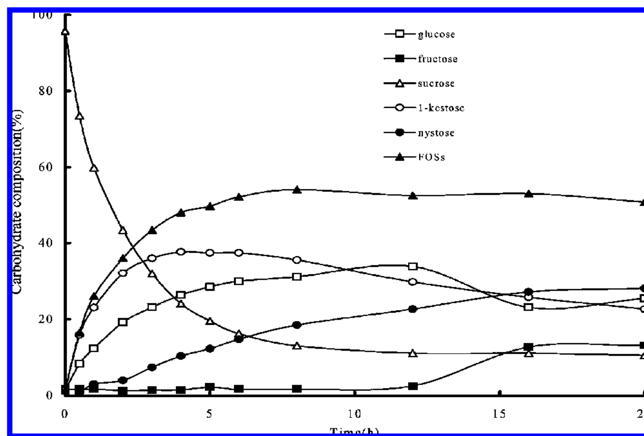

Figure 1. Content of FOSs and other carbohydrates in a catalysis reaction mixture by *A. japonicus* β-fructofuranosidase during biosynthesis.

incubated with shaking at 250 rpm and 30 °C for 24 h. A 1 mL culture was centrifugated at 10000*g* at 4 °C for 10 min. The fresh weight (FW) of cells was measured and used for successive cocultivation.

FOS Synthesis Catalyzed by β-Fructofuranosidase. The extracellular β-fructofuranosidase was added into 100 mL of 50% sucrose (W/V) dissolved in 0.05 mol/L sodium acetate buffer (pH 5.5) at the ratio 5:1 of activity:sucrose (U:g) and incubated at 55 °C for 20 h. The content of FOSs and other sugars synthesized in above solution was determined by high-performance anion-exchange chromatography (HPAEC) (*18*) when the reaction was run for 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16, and 20 h, respectively.

Biopurification of FOSs by Successive Cocultivation with *P. pastoris*. FOS products from 10 h of enzymatic synthesis were diluted to the concentration of 25% (w/v) with sterilized water and mixed with *P. pastoris* cells at the ratio of 1:0.45 (DW:FW). A 100 mL amount of such a mixture was incubated at 30 °C with agitation at 250 rpm for 24 h. The content of FOSs and other sugars was determined when cocultivation was for 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h, respectively (*18*).

HPAEC Assay of Carbohydrate. One milliliter of cocultivation mixture was centrifuged, and the supernatant was diluted with deionized water and was analyzed with HPAEC assay (*18*) using a BioLC Chromatography System. A 250 mm × 4 mm CarboPac PA100 anion-exchange column and a 25 mm × 3 mm CarboPac PA guard column (Dionex, Breda, The Netherlands) with a linear gradient ratio of 100 mmol/L NaOH and 500 mmol/L NaAc to 100 mmol/L NaOH in deionized water from 99:1 to 80:20 (v:v) were used. The running rate was 1 mL/min, and the running time was 1 h. The concentration gradients of 1, 20, 40, 60, 80, and 100 μg/mL for standard glycerol, glucose, fructose, sucrose, 1-kestose, and nystose were prepared, respectively. The total FOSs were the sum of 1-kestose and nystose. Higher FOSs (DPn, $n > 4$) less than 3% were ignored (*3*). All samples with HPAEC assays were sent to the Analysis Center of our institute for determination.

RESULTS AND DISCUSSION

Enzymatic Synthesis of FOSs. As shown in **Figure 1**, the substrate sucrose is rapidly converted into glucose and 1-kestose upon the initiation of reaction, and a part of 1-kestose starts to be converted into nystose a few hours later. The concentration of the sucrose decreases rapidly from over 80% down to 20–30% in the first 4 h. Then, the FOSs synthesis reaction rate drops down after 4 h. After 8 h, the sucrose is always kept at a stable level of about 11%. This is because the severe feedback inhibition of glucose as a byproduct on β-fructofuranosidase reduces and ceases the conversion of sucrose and finally results in the low purity of FOSs (*1–4*).

The content of 1-kestose increases steadily up over 30% in the first 3 h, reaches the highest level of 37.67% at the fourth hour, and decreases gradually later. This is because the conversion of 1-kestose is higher than its formation (**Figure 1**). The content of nystose increases slowly and linearly during the whole period (**Figure 1**). The equal amounts of 25% 1-kestose and nystose were observed at about 15 h, and the highest content of 54.09% FOSs was reached after 8 h. Actually, a desirable content of about 47% FOSs was reached at the fourth hour in terms of economics (**Figure 1**). The content of total FOSs remains almost at a stable level of 53–55% after 8 h. The content of glucose increases from zero to 30% in the first 6 h, up to 35% at 12 h, and then decreases down back to a level of below 30% later, while fructose is always about at 2.5% or less in the first 12 h and increases up to a level of 12–13% later. In fact, the glucose decreases significantly with the increase of fructose during the late period from 16 to 20 h; this change magnitude of glucose and fructose seems to be equal mole (**Figure 1**). It might be deduced that glucose isomerase coexisting in crude enzyme is activated when glucose accumulates up to some extent and the function of fructosyltransferase is weakened (*20, 21*). As result, a part of glucose is transferred into fructose.

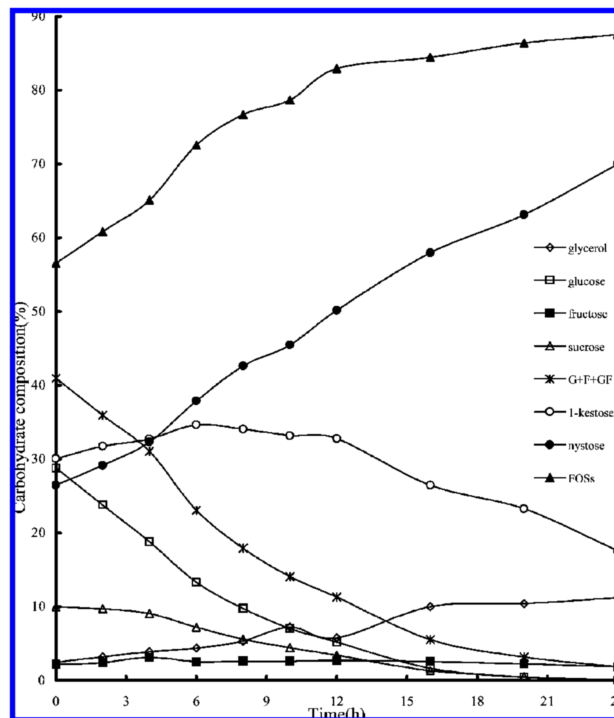

Figure 2. Content of FOSs and other carbohydrates in the successive cultivation product with *P. pastoris*.

According to the FOSs synthesis described above (**Figure 1**), a product of target sugars with a desirable composition and concentration can be obtained at different periods. For example, 4 h is enough to get the high content of 37.67% 1-kestose, while 16–20 h is needed to get the high content of near 30% nystose. Fifteen hours is suitable to get a mixture with equal amounts of 25% 1-kestose and nystose. To obtain a high content of 50−55% of total FOSs, 6−8 h can be chosen. We suggest that 4 h is a more reasonable reaction time to get the higher content of 47.5% FOSs (**Figure 1**). It is very interesting that almost only two types of short-chain FOSs, 1-kestose and nystose, were detected in the synthesis mixture (**Figures 1** and **2**); unlike in the similar process where there is a broad distribution of DPn from 3 to 12 (*8*), it is very helpful to get high-purity short-chain FOS without additional

purification processes to separate other long-chain or higher FOSs, and these short-chain FOSs have very important pharmaceutical and nutrition values (*12, 13*).

Successive Cultivation of *P. pastoris* in FOS Mixture Synthesized by the Enzyme. *Change of Glucose and Fructose.* The FOS mixture from enzymatic synthesis at the 10th hour was diluted to 25% (DW/V). As shown in **Figure 2**, when 100 mL of 25% FOSs mixture was incubated together with *P. pastoris* cells at 30 °C and at the ratio of 0.45:1 (yeast cells: FOSs solution, FW/DW), glucose is rapidly exhausted from 28.78% at the beginning to 1.65% after 16 h and to almost zero at 24 h. At the same time, the content of fructose changes between 1.8 and 3.1%. This is probably related to glucose isomerase mentioned above (*20, 21*).

*Change of Sucrose Content and Total Content of G + F + GF (***Figure 2***).* As the content of glucose decreases gradually and its feedback effect disappears quickly, the activity of β-fructofuranosidase is recovered; thus, sucrose is again converted into FOSs (**Figure 2**), which is similar to previous reports (*2–4*). As a result, the content of sucrose decreases gradually from 9.99% at the beginning to 1.32% at 16 h and to almost zero at 20 h. What is more interesting is that the content of both mono- and disaccharides decreases significantly from 40.98% at the beginning to 5.54% at 16 h and to 1.84% at 24 h; this huge and quick utilization especially for mono sugars is much more helpful to remove the feedback effect of glucose and to recover the activity of β-fructofuranosidase (*5–8, 19*).

Change of 1-Kestose, Nystose, and Total FOS Content. In the first 6 h, the content of 1-kestose increases slightly from about 30 to 35% due to its synthesis from sucrose but then decreases slowly and gradually down to less than 20% until the end because its synthesis is less than its conversation into sucrose and nystose. In comparison, the content of nystose is significantly and linearly increased from about 27 to 70% during the whole cultivation; obviously, this increase of nystose content is not closely related to the change of 1-kestose but highly related to the decreases of content of G + F + GF from about 40 to 2% and glucose from 30% to zero, respectively (**Figure 2**). It is exciting that 70% nystose is the highest level without regular physical and chemical separation as previously reported (*18*); so far as we know, the comparison advantage of this biopurification is obvious in terms of safety, economics, and efficiency.

In the first 12 h, the total amount of FOSs increases obviously and linearly from about 57 to 83% (at the same time, mono- and disaccharides are almost exhausted). To achieve both higher yield and higher purity of 80% FOSs, the cultivation time of 12 h is enough with only trace amounts of sucrose and glucose remaining (**Figure 2**), despite the fact that the content of 84.45% FOSs can be reached later after 12 h of cultivation with a higher cost. By fermenting FOSs syrup and the enzyme-synthesized product, using *P. pastoris*, the content of FOSs is elevated from 56.55 to 84.45% (26.47% 1-kestose and 57.98% nystose) (**Table 1**). HPAEC chromatograms of mixture from *P. pastoris* cultivation at 0 and 16 h were assayed and compared as shown in **Figure 3**; the peaks of glucose, fructose, and sucrose are significantly lowered after 16 h of cultivation (**Figure 3a**,**b**) because monosaccharide is fermented and the disaccharide is converted greatly. In contrast, the higher peak of nystose and the lower peak of 1-kestose peak imply that a trace of 1-kestose was converted into nystose.

Change of Glycerol Content. Glycerol will certainly accumulate in yeast cells to balance the high osmotic pressure outside (*15, 22*) caused by a high concentration of sugars. As expected, about 10% glycerol is produced in the cultivation (**Figure 3**). The glycerol content increases significantly in the successive cultures by three times from 0 to 16 h (**Figure 3**).

Table 1. Content of FOSs and Other Carbohydrates from Catalysis with β-Fructofuranosidase and Successive 16 h Cultivation with Yeast

sugar	product from biosynthesis by enzyme	product after cultivation with yeast
glycerol	2.47[a]	10.01
glucose	28.78	1.65
fructose	2.21	2.56
sucrose	9.99	1.33
total mono- and disaccharides[b]	40.98	5.54
1-kestose	30.06	26.47
nystose	26.49	57.98
total FOS[c]	56.55	84.45
total	100.00	100.00

[a] Percentage content (w/w). [b] The total amount of mono- and disaccharides is assumed to be the sum of glucose, fructose, and sucrose. [c] The total FOS was assumed to be the sum of 1-kestose and nystose.

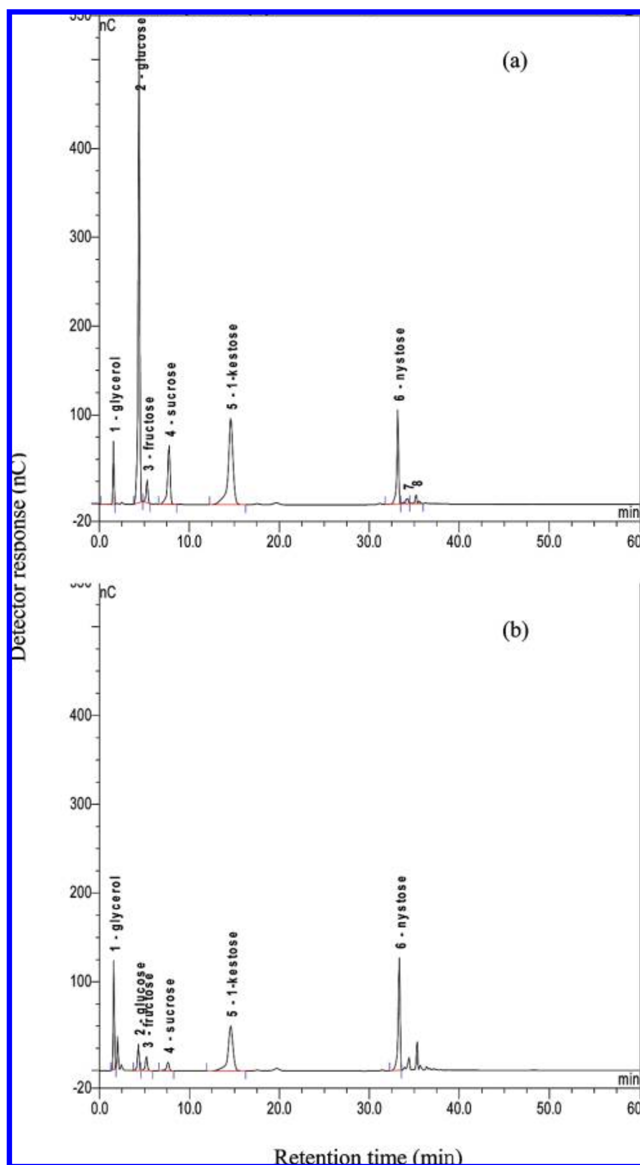

Figure 3. HPAEC chromatogram of FOSs and other sugars from catalysis of β-fructofuranosidase (**a**) and successive 16 h cultivation product with *P. pastoris* (**b**). The numbers are retention time of glycerol, glucose, fructose, sucrose, 1-kestose, and nystose; they are 1.58, 4.42, 5.33, 7.67, 14.83, and 33.42th min, respectively.

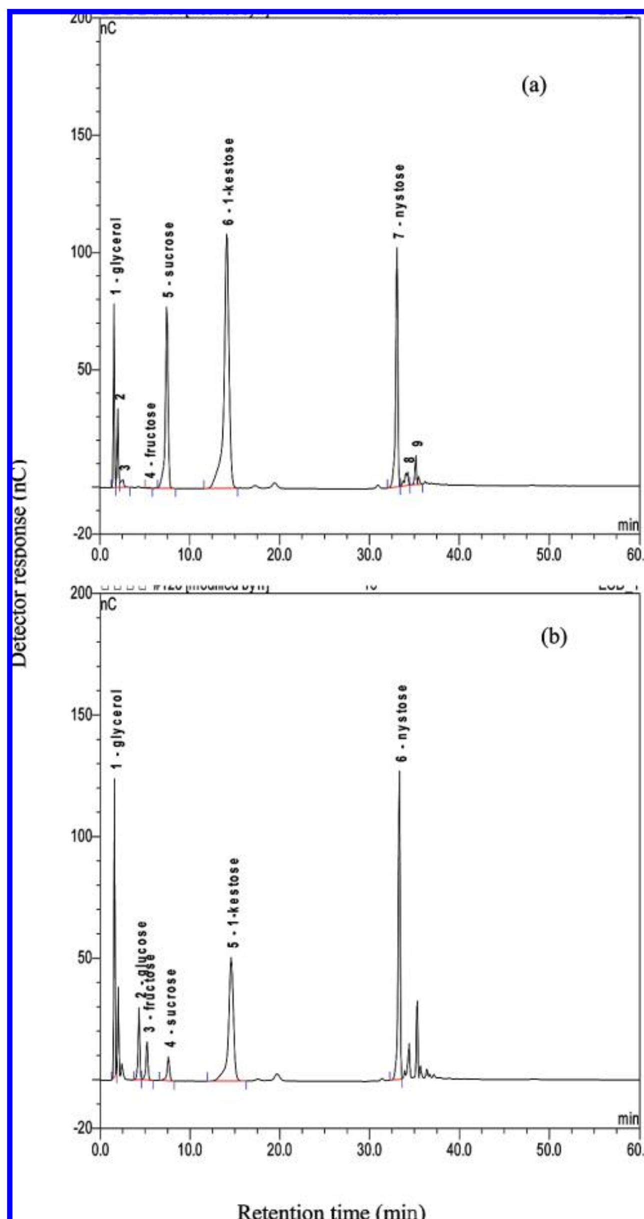

Figure 4. HPAEC chromatogram of FOSs and other sugars from successive 16 h cultivation of *P. pastoris* with inactivated β-fructofuranosidase (**a**) and the active enzyme (**b**).

Response to the high osmotic stress outside, glycerol is synthesized in yeast cells from dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp) (*22, 23*).

Interaction between β-Fructofuranosidase Activity and Yeast. To verify the recovery and contribution of extracellular β-fructofuranosidase during yeast cultivation, the FOS synthesis mixture from enzymatic synthesis was heated in boiling water for 10 min to inactivate the enzyme prior to cultivation as the control. As shown in **Figure 4a**, the distribution and content of sucrose, 1-kestose, and nystose as three major products are not changed in the control sample after 16 h of cultivation (**Figure 4b**), which is similar with the result from **Figure 3a**; on the contrary, the content of nystose is significantly increased and the contents of 1-kestose and especially for sucrose are significantly decreased in the treatment with active enzyme after 16 h of cultivation. Besides, the contents of glucose from both of the samples are kept at similar levels. It can be concluded that *P. pastoris* not only utilizes monosaccharide but also facilitates the recovery of β-fructofuranosidase activity to effectively transfer 1-kestose and sucrose into nystose.

In summary, the two-step strategy—enzymatic synthesis of FOS followed by its successive cultivation with *P. pastoris*—for the production of high-purity FOSs was established. The synthesis time for the higher conversion of sucrose into FOS is between 4 and 8 h at 55 °C, and the successive cultivation time for removing the glucose feedback effect should be 12 h or longer at 30 °C. In combination of two steps, a total of 84.45% FOSs is achieved with only 1.65% glucose, 2.56% fructose, and 1.33% sucrose remaining. Other characteristics include almost only two types of short-chain FOSs—1-ketose and nystose—and exist in FOSs synthesis, and yeast cocultivation mixtures, high contents near 70% nystose and near 90% total FOSs, can be reached after successive cocultivation with yeast. These results can contribute to novel approach developments with a potential for industrial synthesis of high-purity FOSs.

ABBREVIATIONS USED

A. japonicus, *Aspergillus japonicus*; *P. pastoris*, *Pichia pastoris*; DW, dry weight; DNS, 3,5-dinitrosalicylic acid; FOSs, fructo-oligosaccharides; FW, fresh weight; Gpd, glycerol-3-phosphate dehydrogenase; Gpp, glycerol-3-phosphatase; DPn, degree of polymerization; *n*, moiety number of sugar; HPAEC, high-performance anion-exchange chromatography.

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