RESEARCH ARTICLE

Biochemical characterization of a β-fructofuranosidase from *Rhodotorula dairenensis* with transfructosylating activity

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Abstract

An extracellular β-fructofuranosidase from the yeast *Rhodotorula dairenensis* was characterized biochemically. The enzyme molecular mass was estimated to be 680 kDa by analytical gel filtration and 172 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, of which the *N*-linked carbohydrate accounts for 16% of the total mass. It displays optimum activity at pH 5 and 55–60 °C. The enzyme shows broad substrate specificity, hydrolyzing sucrose, 1-kestose, nystose, leucrose, raffinose and inulin. Although the main reaction catalyzed by this enzyme is sucrose hydrolysis, it also exhibits transfructosylating activity that, unlike other microbial β-fructofuranosidases, produces a varied type of prebiotic fructooligosaccharides containing β-(2 → 1)- and β-(2 → 6)-linked fructose oligomers. The maximum concentration of fructooligosaccharides was reached at 75% sucrose conversion and it was 87.9 g L^{-1}. The 17.0% (w/w) referred to the total amount of sugars in the reaction mixture. At this point, the amounts of 6-kestose, neokestose, 1-kestose and tetrasaccharides were 68.9, 10.6, 2.6 and 12.7 g L^{-1}, respectively.

Introduction

Invertases or β-fructofuranosidases (EC 3.2.1.26) catalyze the release of β-fructose from the nonreducing termini of various β-D-fructofuranoside substrates. Microbial β-fructofuranosidases, in general, are dimeric or multimeric enzymes that may also catalyze the synthesis of short-chain fructooligosaccharides (FOS), in which one to three fructosyl moieties are linked to the sucrose skeleton by different glycosidic bonds depending on the source of the enzyme (Antosova & Polakovic, 2001; Sangeetha *et al.*, 2005). Fructooligosaccharides act as prebiotics (Rao, 1999), and they exert beneficial effects on human health, participating in the prevention of cardiovascular diseases or osteoporosis (Kaur & Gupta, 2002). Currently *Aspergillus* fructosyltransferase is the main industrial producer of fructooligosaccharides (Sangeetha *et al.*, 2005; Ghazi *et al.*, 2007), producing a mixture of fructooligosaccharides with an inulin-type structure, containing β-(2 → 1)-linked fructose-oligomers (^{1}F-FOS: 1-kestose, nystose). β-(2 → 6)-Linked fructooligosaccharides that contain this link between two fructose

units (^{6}F-FOS: 6-kestose) or between fructose and the glucosyl moiety (^{6}G-FOS: neokestose, neonystose) may exhibit enhanced prebiotic properties compared with commercial fructooligosaccharides (Marx *et al.*, 2000; Kilian *et al.*, 2002). The enzymatic synthesis of 6-kestose and other related oligomers has been reported in yeast such as *Saccharomyces cerevisiae* (Farine *et al.*, 2001) or *Schwanniomyces occidentalis* (Álvaro-Benito *et al.*, 2007), and in fungi such as *Thermoascus aurantiacus* (Katapodis & Christakopoulos, 2004). Production of fructooligosaccharides included in the ^{6}G-FOS series has been reported in *Xanthophyllomyces dendrorhous* cells (Kritzinger *et al.*, 2003) and the enzyme responsible for this reaction has been characterized (Linde *et al.*, 2009).

The genus *Rhodotorula* has a high biotechnological potential (Easterling *et al.*, 2009; Frengova & Beshkova, 2009). An invertase from *Rhodotorula glutinis* without transferase activity has been reported (Rubio *et al.*, 2002), as well as a β-fructofuranosidase from *Rhodotorula* sp. LEB-V10 that shows transferase activity, but the generated products were not characterized (Hernalsteens & Maugeri, 2008).

Here, we describe the biochemical characterization of an extracellular β-fructofuranosidase with a large molecular size from *Rhodotorula dairenensis*. Kinetic studies of its hydrolytic activity were performed using different substrates, and the fructosyltransferase activity of this enzyme was investigated in detail.

Materials and methods

Materials, organisms and culture conditions

1-Kestose, nystose (TCI Europe; Zwijndrecht, Belgium), leucrose (Sigma-Aldrich) and palatinose (BENEO-Palatinit GmbH; Mannheim, Germany) were used in this work. *Rhodotorula dairenensis* (also *R. glutinis* var. *dairenensis*) CECT 1416 was isolated by the Instituto de Fermentaciones Industriales (CSIC, Madrid); it was grown at 30 °C on MM medium (0.7% yeast nitrogen base; Difco) supplemented with 2% (w/v) maltose (MMM).

Protein purification and quantification

The invertase activity secreted (0.83 U mL^{-1}) by *R. dairenensis* (1 L of MMM, for 60 h, $A_{660\,nm} = 4$) was concentrated through a 30000MWCO PES using a Viva Flow 50 system (Vivascience). The active fraction (45 mL; 14 U mL^{-1}) was dialyzed in 20 mM sodium phosphate pH 7, and applied to a DEAE-Sephacel column (30 mL). The protein was eluted with a 0–0.2 M NaCl gradient at 1.25 mL min^{-1}. The fractions showing invertase activity were eluted at 0.1 M NaCl, pooled, dialyzed in water (17.5 mL; 8.4 U mL^{-1}) and stored at − 70 °C. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% poly-acrylamide) of the samples confirmed the purity of the invertase. Broad range protein markers (Bio-Rad) were used as a control. The samples were concentrated using the Microcon YM-10 (Amicon). Peptide-*N*-glycosidase F (PNGase F; New England BioLabs) treatment was performed according to the manufacturer's protocol. Invertase activity was detected from native preparations by electro-phoresis on 6.5% polyacrylamide gels as described pre-viously (Álvaro-Benito *et al.*, 2007). Invertase activity from *S. cerevisiae* (Novozymes) was used. The protein concentra-tion was determined using the Bio-Rad microprotein deter-mination assay.

The native molecular weight of the purified enzyme was estimated by size exclusion chromatography using a BioSep-SEC-S 2000 (Phenomenex) HPLC system equilibrated with 50 mM sodium phosphate pH 6.8, at 1 mL min^{-1}. Ferritin (450 kDa), aldolase (158 kDa), ovoalbumine (44 kDa) and cytochrome *c* (12.5 kDa) were used for column calibration, with elution volumes of 5.4, 6.3, 7.2 and 8.4 mL, respectively.

Enzyme and kinetic analysis

Unless otherwise indicated, the β-fructofuranosidase activ-ity was determined by measuring the amount of glucose liberated from different substrates (0.5% w/v) in 50 mM sodium phosphate buffer, pH 5, during 20 min at 55 °C as described previously (Linde *et al.*, 2009). One unit of activity (U) was defined as that corresponding to the release of 1 μmol glucose min^{-1}.

The Michaelis–Menten kinetic constants were deter-mined using 4.5 mU of pure enzyme and sucrose (0–50 mM) or 1-kestose (0–100 mM). The plotting and analysis of the curves was carried out using the SIGMAPLOT program (version 7.101). Kinetic parameters were calculated by fitting the initial rate values to the Michaelis–Menten equation $v = V_{max}[S]/K_m + [S]$. The catalytic constant, $k_{cat} = V_{max}/[E]$ represents the number of reaction processes that each active site catalyzes per unit time.

The estimation of hydrolase activity at different pH and temperature values was carried out under the aforemen-tioned conditions using sucrose as the substrate and the buffers indicated previously (Linde *et al.*, 2009). Tempera-ture stability was determined by incubating 1.4 mU of the pure enzyme at different temperatures, removing the sam-ples at regular intervals and estimating the residual activity as aforementioned.

Production of fructooligosaccharides

The invertase was added to a sucrose solution (515 g L^{-1}) in 0.2 M sodium acetate buffer (pH 5.6) and in a total reaction volume of 2 mL. The final activity was adjusted to 0.5 U mL^{-1} and the mixture was incubated at 40 °C in an orbital shaker at 150 r.p.m. At different times, 40-μL aliquots were withdrawn, diluted with 160 μL of water, and incu-bated for 10 min at 90 °C to inactivate the enzyme. The samples were analyzed by HPLC with a quaternary pump (Delta 600, Waters) coupled to a 5-μm Luna-NH2 100A column (4.6 × 250 mm; Phenomenex) as indicated pre-viously (Álvaro-Benito *et al.*, 2007).

Results and discussion

Biochemical characterization of a β-fructofuranosidase from *R. dairenensis*

To purify the invertase activity from *R. dairenensis*, the stationary culture was processed as described in Materials and methods. The overall yield of the purification was 50%, and only one band of 172 kDa was evident on SDS-PAGE (Fig. 1a). Treatment with PNGase F resulted in a shift in the apparent molecular mass of this protein to 144 kDa (Fig. 1b). Thus, *N*-linked oligosaccharides appeared to represent only about 16% of the total protein mass.
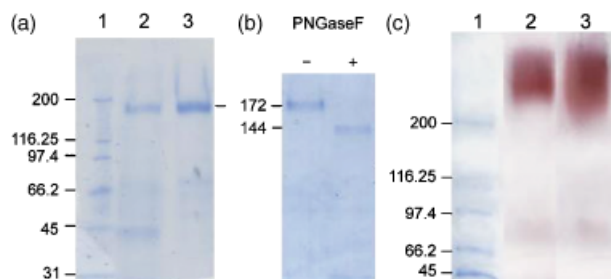
Fig. 1. SDS-PAGE analysis of the purified enzyme and PNGase F treatment. (a) Purification: the concentrated culture filtrate from *Rhodotorula dairenensis* was subjected to SDS-PAGE before (lane 2) or after DEAE-Sephacel chromatography (lane 3). Lane 1, protein standards. (b) Purified invertase not digested (10 µg, lane −) or digested (10 µg lane +) with 0.2 U of PNGase F for 10 min at 37 °C. (c) Purified invertase activity was revealed *in situ* (lane 2) and the *Saccharomyces cerevisiae* enzyme was used as a control (lane 3). Lane 1, protein standards. The positions of the molecular mass markers are indicated (in kDa) at the left of (a) and (c).
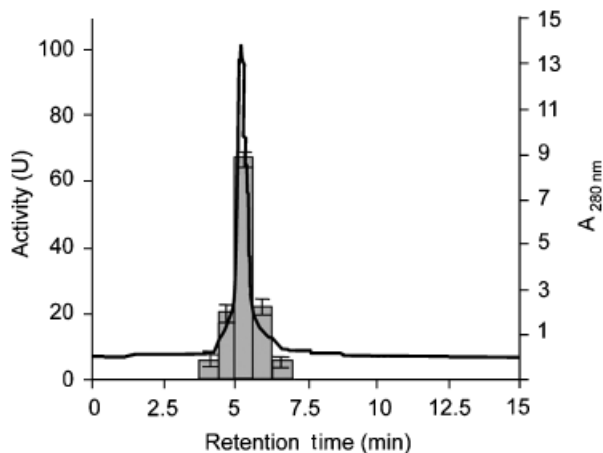

Fig. 2. Estimation of native enzyme size. Purified invertase (20 µL, 70 U) was analyzed by size exclusion chromatography. Chromatogram indicates UV absorbance of the eluate collected (line). Peak elute at 5.12 min. Invertase activity (bars) was assayed in 0.3-mL fractions; data represent the mean of three independent measurements.

The purified enzyme yielded a smeared band of up to 200 kDa in activity-staining gels (Fig. 1c). Invertase from *S. cerevisiae* was used in this test as a control, and it also produced a smeared band, which probably corresponded to the 270-kDa glycosylated, functionally active homodimer described previously (Gascón *et al.*, 1968). The purified enzyme from *R. dairenensis* yielded a single homogenous peak by analytical size exclusion chromatography, which corresponds to an estimated molecular mass of 680 kDa (Fig. 2). Therefore, the active protein from *R. dairenensis* probably occurs as a homotetramer of 172-kDa subunits.

Invertases previously described in yeasts such as *S. cerevisiae* (Taussing & Carlson, 1983), *Schizosaccharomyces pombe* (Moreno *et al.*, 1990), *Pichia anomala*

(Rodríguez *et al.*, 1995), *Candida utilis* (Belcarz *et al.*, 2002) and *X. dendrorhous* (Linde *et al.*, 2009) are dimeric or multimeric enzymes, which have an average molecular weight of 60–65 kDa for the nonglycosylated-monomeric form. Several atypical enzymes have been described such as that from *R. glutinis* (around 47 kDa; Rubio *et al.*, 2002) and *Arxula adeninivorans* (about 100 kDa; Boer *et al.*, 2004). The active forms of these two enzymes exist as a dimer (100 kDa), and a hexamer (600 kDa), respectively.

The enzyme from *R. dairenensis* displayed maximum activity at pH 5 (Fig. 3a) and a temperature of 55–60 °C (Fig. 3b). It was preincubated for different periods of time before substrate addition at temperatures in the range
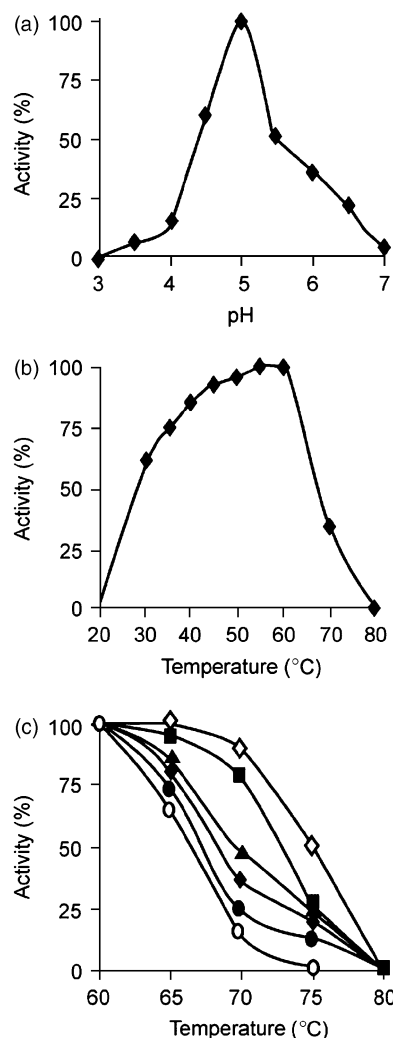

Fig. 3. Temperature, pH dependence and thermostability profiles. The effect of pH (a) and temperature (b) on the *Rhodotorula dairenensis* invertase activity was evaluated at 55 °C and pH 5, respectively. (c) Purified enzyme was incubated for 5 (open diamond), 10 (square), 20 (triangle), 30 (closed diamond), 60 (closed circle) and 120 min (open circle), before the addition of the substrate. The remaining activity was determined at 55 °C. Each point represents the mean of four independent measurements, with an SD of ± 7%.

40–80 °C. No inactivation was detected after 24 h at 40–60 °C, whereas incubation for 4 days at 50 and 60 °C decreased its activity by 50% and 80%, respectively. It was completely inactivated within 5 min at 80 °C (data not shown). The enzyme was then preincubated at 60–80 °C for 5–120 min, and a 50% loss of activity (T_{50}) was produced in the 67–75 °C range (Fig. 3c).

Substrate specificity of the enzyme and kinetic properties

The enzyme from *R. dairenensis* was able to liberate glucose from fructosyl-β-(2 → 1)-linked nonreducing carbohydrates such as sucrose [α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranose], 1-kestose [α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranose] or nystose [α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranose], as well as from raffinose [α-D-galactopyranosyl-(1 → 6)-D-glucopyranosyl-(1 → 2)-β-D-fructofuranose], leucrose [α-D-glucopyranosyl-(1 → 5)-D-fructofuranose] and inulin [α-D-glucopyranosyl-(1 → 2)-[β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranose]$_n$]. However, while a specific activity of approximately 36 ± 2.8 U mg^{-1} was quantified for sucrose, 19.4 ± 0.8 U mg^{-1} was measured for 1-kestose, and a very weak activity was observed for nystose (0.63 ± 0.06 U mg^{-1}), raffinose (0.21 ± 0.018 U mg^{-1}), leucrose (0.18 ± 0.009 U mg^{-1}) and inulin (0.08 ± 0.009 U mg^{-1}). It was not active on maltose [α-D-glucopyranosyl-(1 → 4)-D-glucopyranose], lactose [β-D-galactopyranosyl-(1 → 4)-D-glucopyranose] or palatinose [α-D-glucopyranosyl-(1 → 6)-D-fructofuranose]. This enzyme displayed Michaelis kinetics toward sucrose and 1-kestose (results not shown), and the kinetic parameters obtained are presented in Table 1. The K_{m} value of 1.2 mM for sucrose was similar to that obtained with the *C. utilis* enzyme (1–2 mM; Belcarz *et al.*, 2002), and less than that measured for the enzyme from yeasts such as *X. dendrorhous* (4 mM; Linde *et al.*, 2009), *S. occidentalis* (4.9 mM; Álvaro-Benito *et al.*, 2007) or *R. glutinis* (227 mM; Rubio *et al.*, 2002). In addition, the catalytic efficiency defined by the k_{cat}/K_{m} ratio showed that the *R. dairenensis* fructofuranosidase hydrolyzes sucrose approximately 34 times more efficiently than 1-kestose (Table 1).

Transfructosylating activity

The transfructosylating activity of the *R. dairenensis* enzyme was assayed with sucrose (Fig. 4a). A blank reaction in the absence of enzyme was also assessed and peaks 4–9 were not evident (data not shown).

The maximal fructooligosaccharides production for a particular enzyme depends on the relative rates of transfructosylation and hydrolysis (Plou *et al.*, 2007). The time course for the reaction of *R. dairenensis* β-fructofuranosidase is

Table 1. Kinetic analysis of the β-fructofuranosidase from *Rhodotorula dairenensis*

Substrate	k_{cat} (min^{-1})	K_{m} (mM)	k_{cat}/K_{m} (min^{-1} mM^{-1})
Sucrose	$6.5 \pm 0.4 \times 10^3$	1.2 ± 0.4	$5.4 \pm 1.3 \times 10^3$
1-Kestose	$3.5 \pm 0.2 \times 10^3$	22.4 ± 3.5	$1.6 \pm 0.1 \times 10^2$

Reaction rate measurements were performed in triplicate. Values of k_{cat} were calculated from the V_{max}, considering a protein molecular mass of 172 kDa. The k_{cat}/K_{m} SE were obtained by fitting the normalized Michaelis–Menten equation as $v = (k_{cat}/K_{m})[S]/(1 + [S]/K_{m})$.
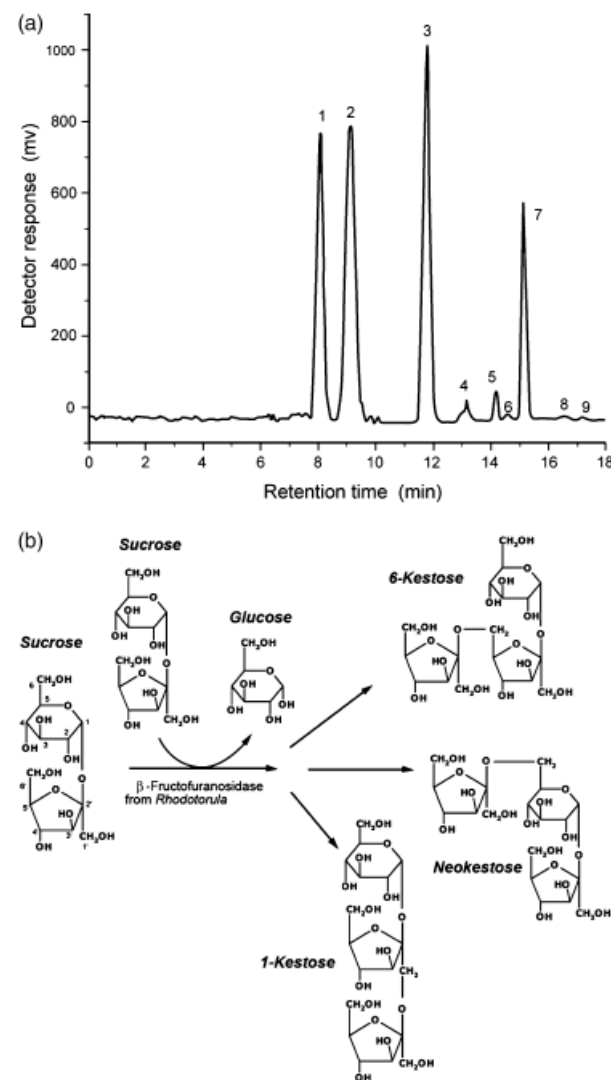

Fig. 4. (a) HPLC chromatogram corresponding to the reaction of sucrose with the β-fructofuranosidase from *Rhodotorula dairenensis*. 1, Fructose; 2, glucose; 3, sucrose; 4, disaccharide; 5, neokestose; 6, 1-kestose; 7, 6-kestose; and 8–9, tetrasaccharides. (b) Schematic view of the transfructosylation reactions.

depicted in Fig. 5. The maximum yield of fructooligosac-charides (87.9 g L^{-1}) was reached in 71 h, and the total sucrose conversion was close to 75%. At this point, the amount of 6-kestose, neokestose, 1-kestose and tetrasac-charides was 68.9, 10.6, 2.6 and 12.7 g L^{-1}, respectively. This means a maximum fructooligosaccharides production of 17.0% (w/w) of the total carbohydrates in the reaction mixture. The other sugars were glucose (165 g L^{-1}), fructose (122 g L^{-1}) and sucrose (127 g L^{-1}). After 71 h, fructooligo-saccharides were progressively hydrolyzed (Fig. 5b), which is commonly observed in similar fructofuranosidase-catalyzed synthetic reactions (Álvaro-Benito *et al.*, 2007; Linde *et al.*, 2009).

The main industrial fructooligosaccharides producers are currently enzymes from *Aspergillus*, which generally provide a mixture of molecules of the inulin-type β-(2 → 1) struc-ture, ^{1}F-FOS, whereas the invertases from yeast usually provide fructooligosaccharides with a levan or neolevan-type β-(2 → 6) structure. The fructofuranosidase from *X. dendrorhous* (Linde *et al.*, 2009) produces mainly neokes-tose (^{6}G-FOS) and those from *S. cerevisiae* (Farine *et al.*, 2001) and *S. occidentalis* (Álvaro-Benito *et al.*, 2007) pro-duce mainly 6-kestose (^{6}F-FOS), with neokestose (^{6}G-FOS) or 1-kestose (^{1}F-FOS), respectively, being byproducts of the reaction. The enzyme from *Rhodotorula* sp. LEB-V10 also shows transferase activity but the generated products were not characterized (Hernalsteens & Maugeri, 2008), and as far as we know, no transferase activity has been detected
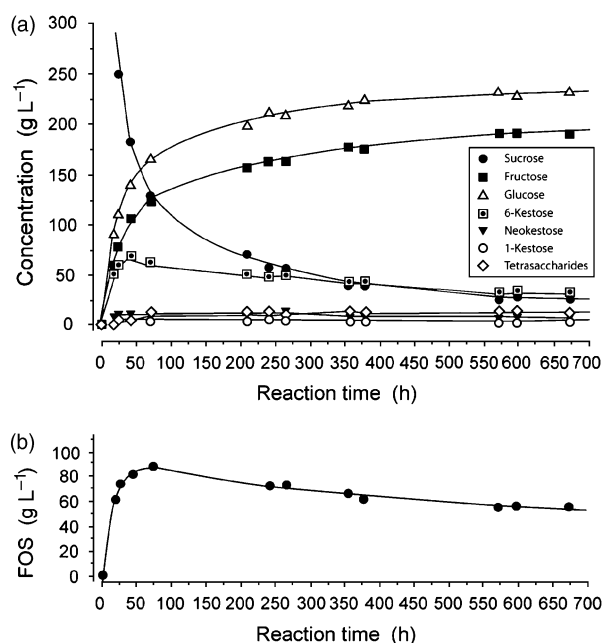
or reported for the β-fructofuranosidase from yeast such as *P. anomala* (Rodríguez *et al.*, 1995), *C. utilis* (Belcarz *et al.*, 2002), *R. glutinis* (Rubio *et al.*, 2002) and *S. pombe* (Moreno *et al.*, 1990). A comparative amino acid sequence analysis of all these proteins might help to clarify their different proper-ties, but unfortunately, some of these sequences, including that from *R. dairenensis*, are still not available and the structural motives required for transferase activity of most of these fructofuranosidases are not defined.

Acknowledgements

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Fig. 5. (a) Time-course of fructooligosaccharide (FOS) production as catalyzed by the β-fructofuranosidase from *Rhodotorula dairenensis*. (b) Formation of total fructooligosaccharides.

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