Yeast cultures expressing the Ffase from *Schwanniomyces occidentalis*, a simple system to produce prebiotic sugars

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ABSTRACT The β-fructofuranosidase Ffase from the yeast *Schwanniomyces occidentalis* produces prebiotic fructooligosaccharides with health promoting properties, making it of biotechnological interest. Ffase is one of the highest and more selective known producers of 6-kestose by transfructosylation of sucrose. A Ser196Leu substitution enhanced transferase activity of the Ffase by ~2.6-fold. In this work, production of 6-kestose was simplified by directly using cultures of *Sw. occidentalis* and *Saccharomyces cerevisiae* expressing both the wild-type enzyme and the mutated variant Ffase-Leu196. Best results were obtained using cultures supplemented with sucrose and expressing the mutated protein variant, which after only 4 h doubled the amount of 6-kestose obtained with the corresponding purified enzyme. 6-Kestose represented ~70% of the products synthesised. In addition, a small amount of 1-kestose and the neofructoligosaccharides neokestose and blastose were also produced. The Ser196Leu substitution skewed production of 6-kestose and neofructooligosaccharides resulting in an increase of ~2.2 and 1.5-fold respectively, without affecting production of 1-kestose. Supplementing yeast cultures with glucose clearly showed that blastose originates from direct fructosylation of glucose, a property that has not been described for other similar proteins from yeasts. Modeling neokestose and blastose into the Ffase active site revealed the molecular basis explaining the peculiar specificity of this enzyme.

IMPORTANCE The β-fructofuranosidase Ffase from the yeast *Sw. occidentalis* produced prebiotic sugars by transfructosylation of sucrose and showed high fructosyl-acceptor promiscuity, making it of biotechnological interest. A simplified process to produce prebiotic sugars in flask using yeast cultures expressing this enzyme has been developed and its effectiveness compared with that of the purified protein. Best results were obtained by using *S. cerevisiae* cultures expressing a mutated protein variant, which

also skewed the production profile towards synthesis of improved prebiotic sugars containing β-(2→6)-bonds. The unveiled promiscuity of the enzyme together with the bias in production of products, demonstrated with the selected mutant, make the system a most valuable tool in generating new bioactive compounds in a fast and simple way.

INTRODUCTION

Fructooligosaccharides (FOS) are prebiotic sugars with health promoting properties. It is well known that they selectively stimulate the growth and/or activity of particular *Lactobacillus* and *Bifidobacterium* species of the intestinal microbiota (1-3), which have proven to cause numerous health benefits for the host (4, 5). In addition, FOS are of special interest to the food industry due to characteristics such as their high solubility, non-cariogenic and low-caloric value, and the capacity to improve taste, texture or even the shelf-life of food (6). Chemically, FOS are short-chains of fructosyl moieties (2 to 10 units) linked most frecuently to a terminal sucrose molecule by different types of glycosidic bonds. In nature, major FOS are inulin-type, with D-fructofuranosyl units linked by β-(2→1) bonds (^{1}F-FOS series, *e.g.* 1-kestose) and usually synthesized by plants and fungi, or levan-type containing β-(2→6) linkages connecting the fructosyl units (^{6}F-FOS series, *e.g.* 6-kestose), synthesized by bacteria. The neolevan-type of FOS (^{6}G-FOS series or neoFOS, *e.g.* neokestose), containing a fructosyl unit linked by β-(2→6) bond to the glucose moiety of the terminal sucrose, are less widespread in plant and microbial groups (7). These differences in chemical structure are important for the biological function of FOS, and those containing β-(2→6) linkages appear to have enhanced both prebiotic properties and chemical stabilities, as compared to the traditionally commercialized ^{1}F-FOS (8-11). These improved FOS can be obtained by

acid hydrolysis of levan polymers, albeit through a non-environmentally friendly process. Numerous microbial enzymes producing these compounds in a non-pollutant way have been previously described. Among others, β-fructofuranosidases from yeasts such as *Saccharomyces cerevisiae* (12, 13), *Schwanniomyces occidentalis* (14), *Rhodotorula dairenensis* (15) or *Xanthophyllomyces dendrorhous* (16) have already proved to be good producers of small sugars included in the ^{6}F- or ^{6}G-FOS series.

β-Fructofuranosidase Ffase from the non-conventional yeast *Sw. occidentalis* released fructose units from the non-reducing end of inulin but also produced 6-kestose and 1-kestose (in a ratio of 3:1) by transfructosylation of sucrose (14, 17). Ffase was proven to be one of the highest and most selective producers of 6-kestose reported (14, 18). The three-dimensional structure of this protein was previously solved as a homodimer, each subunit of 535 amino acids included in family 32 of the glycosyl hydrolases (GH32; 17), which provided insights concerning the mechanism underlying the enzyme hydrolase/transferase activity. Moreover, the Ffase transferase capability was improved using point mutation and directed molecular evolution techniques (19-21). Curiously, a single amino acid substitution, Ser196Leu, switched the Ffase enzymatic activity, reducing the hydrolase capacity but increasing transferase activity by 2.6-fold (19). In addition, Ffase showed high fructosyl-acceptor promiscuity. Among other hydroxylated compounds, it was able to fructosylate alditols such as mannitol and erythritol, and produced blastose, a prebiotic sucrose isomer, by direct fructosylation of glucose (18). All these findings confer the enzyme from *Sw. occidentalis* a great biotechnological potential and a clear utility to the food industry.

Despite the undoubted usefulness of working with FOS-producing enzymes, the development of protocols based on the use of microbial whole-cells to produce FOS is an interesting biotechnological alternative that may simplify the process by eliminating the

need to purify enzymes and consequently reducing time and production costs (22, 23). In this context, cultures of the fungus *Gliocladium virens* and the yeast *X. dendrorhous* were both successfully used to produce ^{6}F-FOS and neoFOS respectively (24, 25). In addition, a two-stage process for the production of 6-kestose using yeast cultures expressing an engineered *S. cerevisiae* invertase has also been reported (26). In this work we have tried to simplify and enhance the process of FOS production with pure Ffase by directly using yeasts cultures expressing the improved variant Ffase-Leu196. In addition, the effectiveness of the enzyme to produce FOS with β-(2→6) linkages has been evaluated and inspected from a structural point of view.

RESULTS

Potential utility of *Sw. occidentalis* cultures to produce FOS. Ffase activity from *Sw. occidentalis* was previously studied, protein was purified and its effectiveness to produce FOS demonstrated (14). This enzyme showed maximum transferase activity at 65% sucrose, where 98.9 g l^{-1} of FOS was obtained (of which 52.2, 17.4, 8.7 and 20.6 g l^{-1} were 6-kestose, 1-kestose, neokestose and blastose, respectively), which represented ~15% (w/w) of the total sugars in the mixture (18). In this work, the possibility of producing FOS directly in the *Sw. occidentalis* cultures was evaluated. Accordingly, yeasts were initially inoculated in YES medium containing sucrose in the 2-60% range, but were only able to grow at sucrose concentrations ≤ 30%. Ffase was expressed in a constitutive manner and maxima activity levels of ~400, 500 and 590 U ml^{-1} were obtained in the stationary growth phase of cultures including 2, 10 and 30% sucrose respectively (Fig. 1A). However, no FOS were detected in any of the culture filtrates analysed. Considering that pure Ffase showed maximum transferase activity at 65%

sucrose and 50ºC (18), both sucrose concentration and temperature were consequently increased in cultures showing maxima Ffase activity. In this case, production of FOS was detected just one day after sucrose addition, reaching maxima values 4 days later (Fig. 1B). The best production of FOS was 81.6 g l^{-1} (using 10% sucrose), which represented ~12 % (w/w) of total sugars in the flask mixture. As expected, the main sugar synthesized was 6-kestose, followed by 1-kestose, which represented ~63% and ~18% of the total FOS, respectively. In addition, small amounts of blastose and neokestose were also detected (Table 1). Although the level of FOS previously reached with pure Ffase was not improved by directly using *Sw. occidentalis* cultures, production of these oligosaccharides was clearly simplified without substantial effect on the composition of sugars in the mixture.

Ffase hydrolytic activity and FOS production in *S. cerevisiae* cultures. Both the wild-type Ffase (Ffase-Ser196) and a mutated variant containing Leu at position 196 (Ffase-Leu196) were previously expressed in *S. cerevisiae*, proteins were purified and kinetically analysed (19). Replacing serine by leucine drastically reduced the enzyme's apparent hydrolytic catalytic efficiency (k_{cat}/K_m ~1000 times lower) but enhanced its transferase activity by almost 2.6-times. Thus by using 34% sucrose, 28 and 75 g l^{-1} of FOS (~7 and ~21% of total sugars in mixtures respectively) were obtained with enzyme containing Ser196 and Leu196, respectively. Interestingly, the Ser196Leu substitution skewed the transferase activity of the enzyme towards the synthesis of 6-kestose since the 6-kestose/1-kestose ratio increased from 3 to 7 (19). In this work, hydrolytic activity of the *S. cerevisiae* cultures expressing the two previously referred Ffase variants was analysed and their effectiveness to produce FOS directly evaluated in flasks. Maxima levels of hydrolytic activity (~700 U ml^{-1}) were detected in cell-associated fractions of yeasts expressing the wild-type Ffase (Fig. 2A). As expected, activity of those expressing

142 the Ffase-Leu196 variant was clearly reduced (≤19 U ml^{-1}; Fig. 2B), whereas only
143 residual activity (≤ 2 U ml^{-1}) was found in any of the culture filtrates analysed and no
144 activity when using the non-transformed yeast host strain as a control. Accordingly, the
145 two *S. cerevisiae* cultures showing maxima levels of hydrolytic activity were
146 supplemented with sucrose and the temperature increased to 50ºC. Figure 3A and 3B
147 show the evolution of FOS detected in flasks and two of the most representative HPLC
148 chromatographic profiles obtained, respectively. At the maxima FOS production points,
149 98.9 g l^{-1} and 166.3 g l^{-1} of total FOS (~15 and ~26 % of total sugars in flasks
150 respectively) were obtained in cultures expressing variants Ffase-Ser196 (after 6 h) and
151 Ffase-Leu196 (after 4 h), respectively. Thus production of total FOS by simply using
152 yeast cultures expressing the mutated enzyme variant represented an increment of at least
153 2-fold the amount previously obtained by using the purified mutant enzyme (19) or the
154 *Sw. occidentalis* cultures (this work; Table 1). As expected, the FOS increase was largely
155 due to 6-kestose (^{6}F-FOS series), which in the best of the analysed cases reached 116.1 g
156 l^{-1} (with variant Ffase-Leu196) and represented ~70% of the total FOS synthetized (Table
157 1). Moreover, a considerable amount of neokestose and blastose were obtained in all
158 cultures, whereas nystose was only detected in that expressing the variant Ffase-Leu196.
159 A schematic view of the reactions occurring in flasks is represented in Figure 4.

160 **Analysis of the neoFOS production.** With respect to neoFOS (^{6}G-FOS series), both
161 blastose and neokestose were previously detected in reaction mixtures containing the
162 purified Ffase, the maximum production of these sugars being not coincident with that
163 of total FOS (18). A similar situation was found in this work by using the *S. cerevisae*
164 cultures, where the largest amount of neoFOS occurred 8 h after the addition of sucrose,
165 where the production of total FOS decreased by ~3% (Fig. 3). Maximum production of
166 neoFOS was 46.1 g l^{-1} (31.7 g l^{-1} blastose and 13.4 g l^{-1} neokestose) with yeast

expressing Ffase-Leu196, whereas 31 g l^{-1} (23.5 g l^{-1} blastose and 7.5 g l^{-1} neokestose) was obtained with that expressing the wild type enzyme (Fig. 3A). Therefore, substitution Ser196Leu enhanced the production of neoFOS by ~1.5-fold. To our knowledge, Ffase was (and still is) the first reported enzyme from yeast producing blastose directly by the fructosylation of glucose (18), which acts as an alternative fructosyl-acceptor to sucrose in the transference reaction (Fig. 4). To analyse the potential of the *S. cerevisiae* cells expressing the two Ffase variants to synthesize blastose, 60% glucose and 20% sucrose (fructosyl donor) were added to these yeast cultures when they showed maxima hydrolytic activity. Again, the best result was obtained with cultures expressing Ffase-Leu196 after 8 h (Fig. 5A). The largest amount of blastose detected was 31.5 g l^{-1}, which represented only ~4% of the total sugars in the mixture, but 76% of total transfructosylated products. In this case, the maximum concentration of trisaccharides was reached after 6 h, and was 13.6 g l^{-1}, of which 11.5 g l^{-1} were 6-kestose and 2.1 g l^{-1} 1-kestose. Neokestose was not detected in any of the HPLC chromatograms analysed, which supports that blastose does not come from the neokestose hydrolysis. Figure 5B and 5C show results obtained by using the culture expressing Ffase-Ser-196 and the wild-type enzyme purified directly from *Sw. occidentalis* respectively. Very similar chromatographic profiles were obtained in the reaction mixtures by using both systems, which produced ~14 g l^{-1} of blastose, ~2% of total sugars in the flask (~68% of total FOS). Moreover, 7.5 g l^{-1} of trisaccharides were obtained, of which 4.4 g l^{-1} was 6-kestose, 2 g l^{-1} 1-kestose and 1.1 g l^{-1} neokestose. These results indicate that the substitution Ser196Leu also produced a slight bias on the Ffase activity towards the production of blastose in cultures supplemented (Fig. 5) or not with glucose (Fig. 3A).

Molecular basis of the enzyme specificity towards neoFOS production. The three-dimensional structure of dimeric Ffase unveiled essential features of its specificity, as the presence of up to five binding subsites and the involvement of the adjacent subunit in allocating distal parts of the substrate. (17, 20). Moreover, its preference for producing 6-kestose by transfructosylation was attributed to the presence of several polar residues near the EC motif that interact with the sucrose moiety of the 6-kestose molecule (19). Accordingly, Asn245 and Gln228 would be making hydrogen bonds to a putative sucrose acceptor bound at subsites +1 and +2, enhancing the yield of the β-(2→6)-linked transfructosylation product (Fig. 6A). Interestingly, this effect was markedly increased in the Ffase-Leu196 variant (19). On the other hand, the secondary product, 1-kestose, would result from the alternative binding of the donor sucrose with its terminal glucose unit stacking against the Trp76 at the Ffase subsite +2, as shown in Figure 6B (19, 20).

The results presented here revealed that neokestose was also a secondary transfructosylation product made by Ffase from sucrose. Interestingly, a modelled neokestose resulting from this 6G- transferase activity would present an interaction pattern at the Ffase active site similar to that proposed for 1-kestose, with its terminal fructose (blue in Fig. 6B) stacking against Trp76 side-chain This means that the acceptor sucrose can bind at these +1/+2 subsites in two equivalent modes that leave either glucose or fructose available for fructosyl transfer. These two sucrose-binding modes implicate a polar interaction with Gln228 at subsite +1 and hydrophobic interaction with Trp76 at subsite +2. It is feasible that an improved stacking interaction of glucose over fructose at subsite +2 leads to the observed twofold production of 1-kestose over neokestose as given in Table 1. The fact that this proportion is similar in both Ffase-196 variants reduces the implication of the polar links and the EC

environment in the ^{1}F and ^{6}G transferase activities, reinforcing the significance of the Trp76 role in this activity.

Another interesting finding of this work is the production of the disaccharide blastose, implying that glucose can be an acceptor for the fructose bound at subsite -1. A model for the product blastose in the active site is shown in Figure 6C. As observed in the figure, the β-(2→6)-linked glucose can make polar links to Gln228, as proposed for the other acceptor disaccharides, while stacking against the Trp314 side-chain. This putative glucose binding mode could allow the flexible C6-OH group to position properly for the nucleophilic attack on fructose leading to the formation of blastose. Interestingly, Trp314 is not conserved within the GH32 family and therefore is unique to Ffase, probably explaining why blastose has not been reported as a transfructosylating product from the enzymes previously studied from fungi and plants. In addition, blastose and neokestose appear to be bad substrates for hydrolysis by Ffase, as they accumulate in the medium. This is possibly explained in terms of the 6-linked glucose hindering fructose to acquire the required conformation for efficient break of the glycoside bond, an effect that is most evident in the Ffase-Leu196 variant as shown in Figure 5A. Furthermore, this variant produces the highest amount of blastose, although the reasons for this behaviour are unclear.

DISCUSSION

The β-fructofuranosidase from *Sw. occidentalis* is an excellent producer of prebiotic sugars, mainly 6-kestose, a β-(2→6) linked containing FOS with enhanced properties and chemical stability (10, 11). Initially, the biotechnological interest of this sugar led us to

analyse the hydrolase/transferase activity of the Ffase in detail and determine some of the structural aspects involved in the enzyme specificity. Concerning the highest yield of total FOS produced in this work, ~166 g l^{-1} exceeds the levels produced by other microbial cultures previously used to form sugars mostly included in the traditionally commercialized ^{1}F-FOS series. Indeed, only ~12.5 g l^{-1} of ^{1}F-FOS were produced by growing the thermophilic fungus *Sporotrichum thermophile* on a high sucrose concentration medium (27) and ~130 g l^{-1} with *Aureobasidium pullulans* via sucrose fermentation (28). Very different results were also obtained in relation to the production of 6-kestose. Whereas the highest yield of 6-kestose obtained in this work, ~116 g l^{-1}, was reached after 6 h at 50ºC (Table 1), just 3 g l^{-1} and a remarkable 200 g l^{-1} of this trisaccharide were achieved by using cultures of the fungus *Gliocladium virens* after 4-5 days at 28ºC (24) and yeast expressing an engineered version of the *S. cerevisiae* invertase after nearly a week at 50ºC (26), respectively.

In general, most probiotic *Bifidobacterium* species grow better on low rather than on high molecular weight FOS (29) with kestoses being the best bacterial stimulators (30). Specifically, FOS in the ^{6}F-FOS series showed resistance to degradation in the upper intestinal tract and efficiently induced synthesis of short-chain fatty acids (10, 11), supporting its prebiotic capacity. Thus far, prebiotic oligosaccharides have almost exclusively targeted bifidobacteria, with kestoses being additionally able to strongly activate the intestinal bacterium *Faecalibacterium prausnitzii*, which helps to promote health in infants (31). Together with the evident biotechnological interest of short-chain FOS, these sugars also show better sweetening power than those with higher degrees of polymerization (32), making our results of clear interest to the food industry.

Compared with commercial FOS, generally included in the ^{1}F-FOS series, neo-FOS showed an improved prebiotic effect (8) and better chemical stability against pH and

264 temperature changes (9), which a priori would facilitate the processing and storage of
265 food containing this FOS series. In addition, and more recently, different studies have
266 even suggested that neokestose may have an antineoplastic effect as well as a potential
267 use as a therapeutic agent for the treatment of melanoma (33, 34). Moreover and
268 concerning the disaccharide blastose, it also showed prebiotic properties by stimulating
269 the growth of different *Lactobacillus* species (35). Despite the clear biotechnological
270 potential of blastose for the food industry, this sugar was previously obtained only as a
271 secondary product of enzymatic reactions that use sucrose as substrate. Thus the
272 mycelium-bound transfructosylating activity of the fungus *Clasdosporium*
273 *clasdosporiodies* and the β-fructofuranosidase from the yeast *Xanthophyllomyces*
274 *dendrorhous* produced 34 g/L (36) and 22 g/L (37) of blastose, respectively. In both
275 cases, it was produced by the hydrolysis of the trisaccharide neokestose. More recently,
276 1.3 g/L of blastose was also obtained using trehalose as substrate through a sequential
277 transfructosylation-hydrolysis reaction involving a *Bacillus subtilis* levansucrase and a
278 commercial prokaryotic trehalase (38). To our knowledge, Ffase from *Sw. occidentalis*
279 was (and still is) the first reported enzyme from yeast producing blastose directly by the
280 fructosylation of glucose, which acts as a fructosyl-acceptor alternative to sucrose in the
281 transference reaction (18).

282 As previously commented, the change in the Ffase hydrolase- transferase activity
283 observed upon Ser196Leu replacement would indicate that this position (or the region
284 where it is located) might influence the retention time of sucrose in the enzyme catalytic
285 pocket with a subsequent higher probability of including a sucrose acceptor instead of a
286 water molecule, leading to transfructosylation (19). The results obtained in this work by
287 supplementing yeasts cultures with glucose (as a fructosyl-acceptor) follow the same
288 pattern with glucose being more efficiently retained in the catalytic pocket of the Ffase-

Leu196 variant, as production of blastose with yeast expressing this variant doubles that of the corresponding wild-type enzyme (Fig. 5). In addition, Ffase has shown high fructosyl-acceptor promiscuity, being able to fructosylate different hydroxylated compounds such as some monosaccharides (glucose, galactose), oligosaccharides (raffinose, nystose), alditols (mannitol, erytritol) and other glycosides (acarbose, α-methyl-glucoside; 18). Fructosylation of some of these compounds could increase their solubility and functional properties as well as producing potential sweeteners for the food and beverage industries. In this context, Ffase could be an excellent tool for the single fructosylation of a variety of acceptors forming new hetero-oligosaccharides and fructo-conjugates. The use of yeast cultures expressing mutated variants of this enzyme (such as the variant Ffase-Leu196) with modified transferase activity and the addition of new fructosyl-acceptors, provides a simplified platform to produce compounds with potential application in food and pharmaceutical industries, a future goal to be addressed.

MATERIALS AND METHODS

Microorganisms, growth and expression conditions. *Schwanniomyces occidentalis* ATCC26077 was maintained and cultured on YEI [20 g l^{-1} yeast extract (Difco, USA), 15 g l^{-1} inulin (Orafti GR, Beneo Ibérica S.L., Spain)] and YES [20 g l^{-1} yeast extract, 2-10-30-40-60% sucrose (Acor, Mercadona, Spain)] media respectively. *Saccharomyces cerevisiae* EUROSCRAF Y02321 (MY4741; *Mat a*, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0, *YIL162w(SUC2)::kanMX4*) transformants expressing the wild-type β-fructofuranosidase from *Sw. occidentalis* (wild-type Ffase containing Ser196; also variant Ffase-Ser196) and the Ffase-Leu196 variant (containing Leu196) were obtained as previously referred (19).

Strains were maintained on SCD agar plates [6.7 g l^{-1} yeast nitrogen base (Difco, USA), 20 g l^{-1} glucose, 0.1 g l^{-1} leucine, 0.05 g l^{-1} histidine, 0.05 g l^{-1} methionine, 15 g l^{-1} agar] and grown on YP-Gal [10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 20 g l^{-1} galactose] to express the Ffase activity. YP-Glu [YP-Gal but including glucose instead of galactose] was used as a negative expression control. Cultures were incubated at 30ºC using a rotary shaker (230 rpm) and analysed spectrophotometrically at 660 nm (OD_{660}).

β-Fructofuranosidase hydrolytic activity assay. Unless otherwise indicated, standard β-fructofuranosidase hydrolytic activity was assayed at 50ºC for 20 min by measuring the amount of reducing sugars released from sucrose (20 g l^{-1} in sodium acetate 100 mM pH 5.5) using the dinitrosalicylic acid (DNS) method adapted to 96-well microplates as described previously (14). Glucose 0.2-3.0 g l^{-1} was used for the calibration curve. Reaction mixtures without enzyme or sucrose were used as negative controls. One unit of activity (U) was defined as that catalysing the formation of 1 µmol of reducing sugar per minute under the above described conditions. Each reaction was performed at least in triplicate.

β-Fructofuranosidase activity and FOS production in flasks. *Sw. occidentalis* and the *S. cerevisiae* transformants expressing Ffase-Leu196 and -Ser196 variants were cultivated in 50 ml-flasks containing 5 ml of YES medium including sucrose in the 2-60% range (*Sw. occidentalis*) or YP-Gal media (*S. cerevisiae*). As negative controls, the *S. cerevisiae* host strain was also cultivated in YP-Gal and the transformants in YP-Glu. Samples from the cultures were taken at different times to determine both cell growth (OD_{660}) and β-fructofuranosidase activity. For enzymatic determinations 5 µl of culture filtrates was used. For wall-associated protein analysis, yeast cultures (80-100 µl) were lysed with YeastBuster™ Protein Extraction Reagent according to the manufacturer's

337 instructions (Novagen Merk, Germany) and β-fructofuranosidase hydrolytic activity
338 assayed as commented above.

339 For standard FOS production analysis, the 50 ml-flasks including 5 ml of the yeasts
340 inocula were cultivated to the point showing maxima β-fructofuranosidase hydrolytic
341 activity (*Sw. occidentalis* for ~ 40-60 h, ~7-8 UOD_{660}; *S. cerevisiae* transformants for
342 ~20-22 h, ~7-8 UOD_{660}). Then 20 ml of 81% sucrose (Acor, Mercadona, Spain) in
343 sodium acetate 100 mM pH 5.5 was added (final sucrose concentration 65%) and cultures
344 were incubated at 50ºC. Samples were taken at different times and boiled for 10 min. The
345 composition of sugars in the mixture was evaluated by HPLC-ELSD. To analyse the
346 production of blastose from glucose, similar assays were performed but 20 ml of a
347 solution containing 25% sucrose and 75% glucose instead of just sucrose was added
348 (final sucrose and glucose concentration 20% and 60%, respectively) to the 50 ml-flasks
349 containing the *S. cerevisiae* cultures.

350 **HPLC-ELSD analysis.** Sugars in the reaction mixtures were identified and quantified by
351 HPLC with a quaternary pump (mod. 600E, Waters Corp.) coupled to a 5-µm Purple-NH_2
352 column (4.6 mm x 250 mm) from Análisis Vínicos S.L. (Tomelloso, Spain). An
353 evaporative light-scattering (ELS) detector (mod. 1000, Polymer Laboratories, Ltd.;
354 Church Stretton, UK) equilibrated at 90ºC and an automatic injector (mod. 717 Plus,
355 Waters Corp.; Milford, USA) were used. Acetonitrile/water 80:20 (v/v), degassed with an
356 in-line vacuum generator (ser. 200, Perkin-Elmer Corp.; Eden Prairie, USA), was used as
357 a mobile phase at 1.0 ml/min for a total analysis time of 45 min. The temperature of
358 column was kept constant at 25ºC. The data obtained were analysed using the Empower®
359 software (v. 1.0; Waters Corp.). Before injection, each sample was diluted with ultrapure
360 water and passed through nylon-fiber syringe filters of 0.45-µm-pore size (Scharlau, S.L.;
361 Sentmenat, Spain). All compounds were quantified on the basis of peak areas. Each

362 analysis was conducted at least in duplicate. Fructose, glucose and sucrose used as
363 standards were from Sigma-Aldrich Corp. (St. Louis, USA); 1-kestose and nystose were
364 from TCI-Europe N.V. (Zwijndrecht, Belgium); 6-kestose, blastose and neokestose were
365 synthesized from sucrose using the extracellular β-fructofuranosidases from *Rhodotorula*
366 *dairenensis* (15), *Cladosporium cladosporioides* (36) and *Xanthophyllomyces*
367 *dendrorhous* (16), respectively.

368 **Protein purification from *Sw. occidentalis* and FOS production.** *Sw. occidentalis* was
369 grown on YEI medium and extracellular β-fructofuranosidase (Ffase) was purified as
370 previously referred (17). In brief, yeasts were grown to stationary phase (600 ml; 12
371 UOD_{660}; ~100 U ml^{-1}; ~12 U mg^{-1}), culture filtrates were concentrated and fractionated
372 using VivaFlow 50 system (Vivascience) and then applied to a DEAE-Sephacel
373 chromatography column that was eluted using a 0-0.5 M NaCl gradient. Samples
374 containing activity eluted at 0.15 M salt and were analysed by ProtoBlue Safe Colloidal
375 Coomassie staining (National Scientific, Atlanta, GA, USA)-SDS-PAGE (8%). Only
376 those containing apparently pure proteins were pooled and concentrated (~740 U ml^{-1} and
377 99 U mg^{-1}). Protein purification was evaluated in SDS-PAGE gels using standard
378 methods and sample concentrations were determined with the Bio-Rad microprotein
379 assay (Hercules, CA, USA) in accordance with the manufacturer's specifications using
380 bovine serum albumin as standard.

381 FOS production mediated by the purified Ffase was analysed by HPLC-ELSD as
382 referred above. Reaction mixtures (100 μl) including 10 U ml^{-1} of pure enzyme, 20%
383 sucrose and 60% glucose in 0.2 M sodium acetate pH 5.5 were incubated for up to 5 h as
384 previously referred (18).

385 **Protein computer analysis and molecular modelling.** The Ffase structural analysis was
386 carried out using the COOT program (39) and the figures were obtained with PYMOL

(40). The sucrose transfructosylation product 6-kestose was modelled from the experimental 6-kestose crystal structure coordinates extracted from the Cambridge Structural Database (CSD Refcode CELGIJ). Coordinates for blastose were constructed using the GLYCAM program (41) and exported in its lowest energy state. Both, 6-kestose and blastose were modelled at Ffase active site by superimposing their terminal fructose moiety onto the fructose located at subsite -1 previously found in the Ffase structure (17; Protein Data Bank [PDB] 3KF3). The 1-kestose and neokestose molecules (also transfructosylation products) were modelled into the Ffase active site as inferred from structural superimposition of the corresponding *X. dendrorhous* β-fructofuranosidase complexes (42; PDB codes 5KFB and 5KF7) onto the Ffase-fructose complex (PDB code 3KF3). The ligands were manually adjusted by small torsion of their glycosidic bonds, to best packing to Trp76 side chain keeping polar interaction to Gln228 and/or Asn254.

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FIGURE LEGENDS

Figure 1. Analysis of FOS produced by the *Sw. occidentalis* cultures. (A) The yeast *S. occidentalis* was cultured in 5 ml of YES medium including 2 (triangles), 10 (circles), 30% (squares) of sucrose. The culture growth (dotted lines) and the extracellular hydrolytic activity (solid lines, green symbols) were measured at the times indicated using sucrose as substrate. (B) Percentage of FOS (solid lines) and sucrose (broken lines) refer to the total amount of sugars present in the flasks. Yeasts were grown in 50 ml-flasks containing 5 ml of YES medium-sucrose 2 (triangles), 10 (circles) and 30% (squares) for 45, 40 and 60 h, respectively. Then, 65% sucrose (final concentration) was added and flasks stirred at 50ºC. Sugars in the mixtures were evaluated by HPLC-ELSD at the indicated times.

Figure 2. Hydrolytic activity of *S. cerevisiae* cultures expressing Ffase variants. Inocula from *S. cerevisiae* expressing the Ffase-S196 (**A**) and Ffase-L196 (**B**) variants were grown (open circles) in 50 ml-flasks containing 5 ml of YP-Gal. Extracellular (closed triangles) and cellular (green circles) enzyme hydrolytic activity was measured at the times indicated using sucrose as substrate. Each point represents the average of three independent measurements with a standard deviation of ±5%.

Figure 3. Analysis of FOS produced by the yeasts expressing Ffase variants. (A) In the top panel, percentage of total FOS (solid lines) and sucrose (broken lines) both referring to the total amount of sugars present in reaction mixtures obtained by using Ffase-Leu196 (closed circles) and Ffase-Ser196 (closed triangles). Yeasts were cultured in 5 ml of YP-Gal to the point showing maxima Ffase hydrolytic activity (Fig. 2). Then

20 ml of sucrose 81% was added and flasks were stirred at 50ºC. Sugars in the mixtures were evaluated by HPLC-ELSD at the indicated times. In the bottom panels, the concentration of neoFOS produced at the indicated times by cultures expressing the referred Ffase variants is presented. Blastose (green bars), neokestose (beige bars). **(B)** Representative HPLC-chromatograms of the sugars mixtures obtained in the cultures expressing the indicated Ffase variants. The flasks were stirred for 6 h at 50ºC and 20 µl of the reaction mixture was analysed. Peak assignations: (1) fructose, (2) glucose, (3) sucrose, (4) blastose, (5) neokestose, (6) 1-kestose, (7) 6-kestose, (8) nystose.

Figure 4. Schematic view of the reactions based on sucrose mediated by the Ffase. Nystose was only detected in cultures expressing the Ffase-Leu196 variant. The transferred fructose unit is highlighted in red.

Figure 5. Profile of blastose and total trisaccharides produced in reactions supplemented with glucose. *S. cerevisiae* transformants expressing the Ffase-Leu196 **(A)** and Ffase-Ser196 **(B)** variants were cultivated in 5 ml of YP-Gal to the point showing maxima Ffase hydrolytic activity and then 20 ml of 20% sucrose and 60% glucose (both final concentration) were added. Flasks were stirred at 50ºC and sugars in the flasks were evaluated by HPLC-ELSD at the indicated times. **(C)** The composition of sugars in the reaction mixtures containing the pure wild-type Ffase, 20% sucrose and 60% glucose (both final concentration) was analysed. Only concentration of blastose (green bars) and total trisaccharides (grey bars) are presented in the three panels above. The trisaccharides 6-kestose, 1-kestose and neokestose were obtained with the variant Ffase-Ser196 while only 6-kestose and 1-kestose with the Ffase-Leu196.

Figure 6. Modelling of transfructosilating products of Ffase. Close-up view of the Ffase active site (PDB code 3KF3) in surface representation complexed with fructose (shown as white sticks). Relevant residues are shown in yellow and catalytic residues highlighted in orange. The other subunit of the dimer is represented in cartoon format. The modelled transfructosylation products, obtained as described in the experimental section, are represented in (A) 6-kestose, (B) 1-kestose, green, and neokestose, blue and (C) blastose. For clarity, fructose is represented only in (A). Polar interactions are shown as dashed lines. The different positions of sucrose (+1/+2) or glucose at (+1) portions represent the potential binding modes of acceptors for the formation of the different transglycosylation products.

607 **TABLE 1** Concentration of sugars produced in flasks at the maxima FOS production point by the

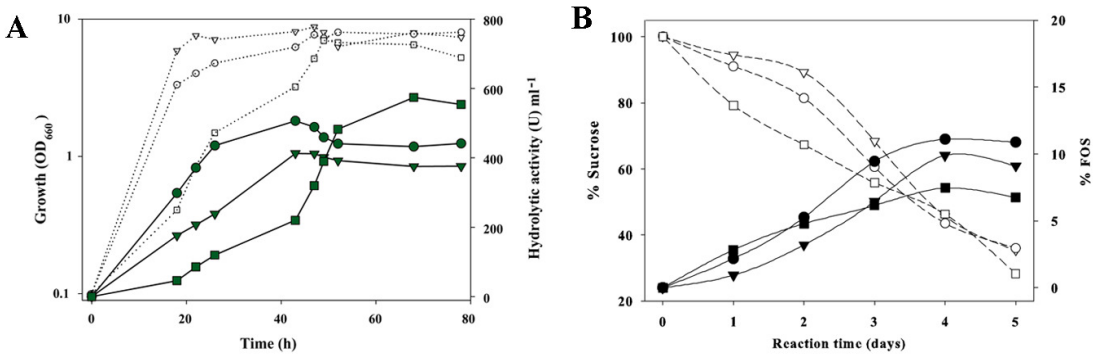
608 *Sw. occidentalis* and *S. cerevisiae* cultures expressing the indicated Ffase variants

	Sw. occidentalis	*S. cerevisiae*	
Sugar[a]	Ffase-Ser19 (wt)	Ffase-Ser196	Ffase-Leu196
6-Kestose	51.4	53.2	116.1
1-Kestose	14.5	16.8	16.7
Neokestose	5.9	8.4	9.3
Blastose	9.8	20.5	14.0
Nystose	ND	ND	10.2
Total FOS	81.6	98.9	166.3
Ratio[b]	3:1	3:1	7:1

609 [a] Concentration of sugars in g l^{-1}

610 [b] 6-Kestose:1-kestose ratio

611 ND, not detected; wt, wild-type



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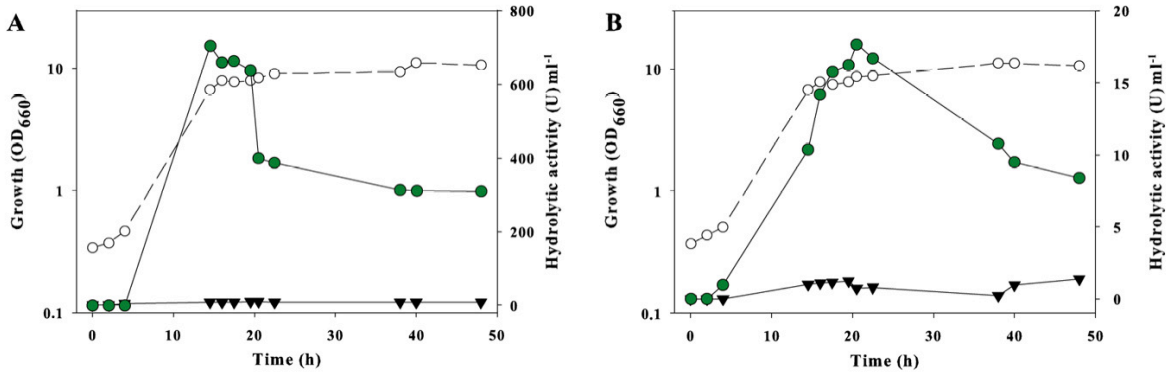

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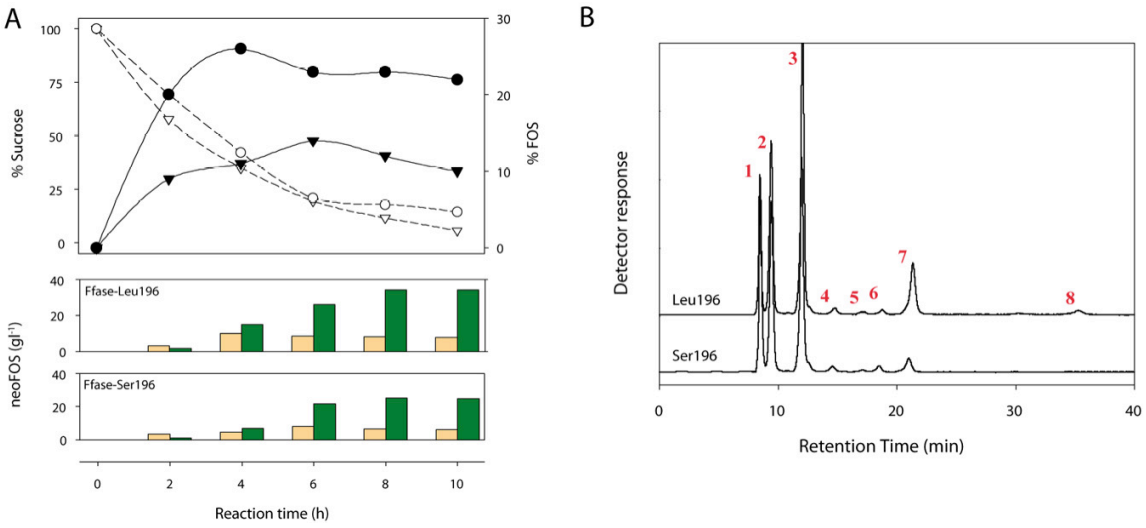

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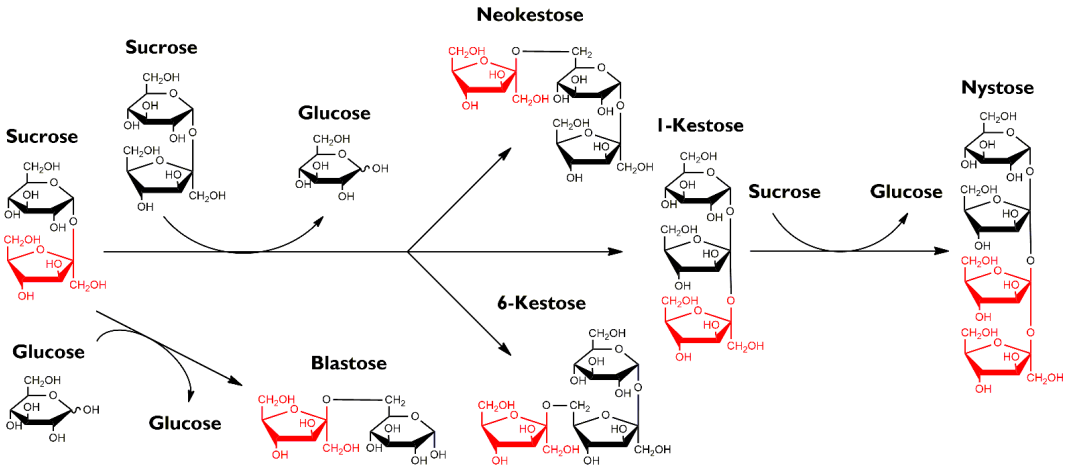

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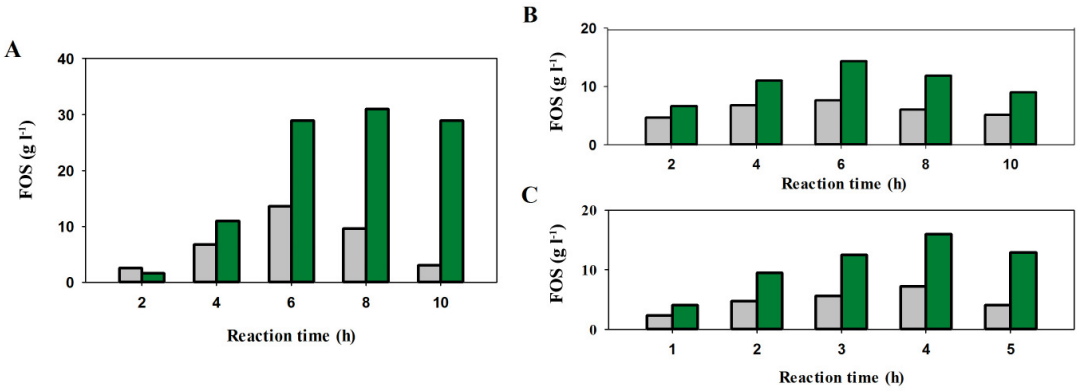

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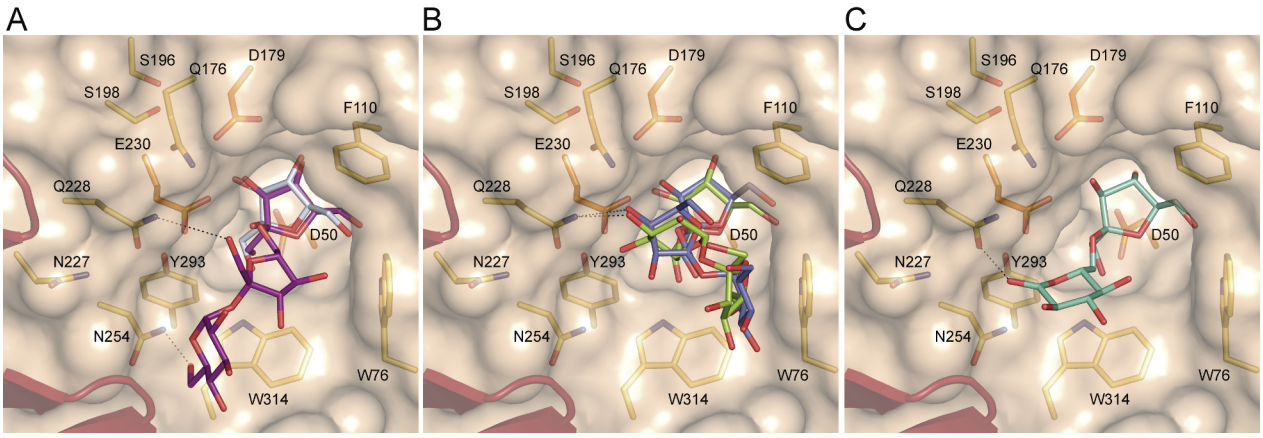

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