Screening β -Fructofuranosidases Mutant Libraries to Enhance the Transglycosylation Rates of β -(2→6) Fructooligosaccharides

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ABSTRACT

β -Fructofuranosidases can divert their hydrolytic activity towards transglycosylation for the synthesis of high value-added products, including prebiotic fructooligosaccharides (FOS). A directed evolution strategy has been employed to enhance the transferase rate of the β -fructofuranosidase (SoINV) from the *Schwanniomyces occidentalis* yeast for the production of β -(2 $\rightarrow$ 6)-linked FOS. To screen for transferase activity of the SoINV functionally expressed in *Saccharomyces cerevisiae*, a high-throughput screening protocol based on two colorimetric assays was validated (with coefficient of variance below 11 %). Mutagenic libraries were constructed by error-prone PCR and clones showing higher glucose:fructose ratio with respect to the parental type were identified. Further analysis by anion-exchange chromatography coupled with pulsed amperometric detection helped to identify mutants with improved yields for the synthesis of β -(2 $\rightarrow$ 6) fructooligosaccharides. Selected mutants displayed transferase initial rates enhanced ~2-fold over parent type, reaching production levels up to 47 g/L after 48 h of reaction for the formation of 6-kestose.

Keywords: β -(2 $\rightarrow$ 6) fructooligosaccharides, transglycosylation, *Schwanniomyces occidentalis*, prebiotics, directed evolution.

INTRODUCTION

In nature, β -fructofuranosidases (invertases; EC 3.2.1.26) are involved in the hydrolysis of sucrose (Fig. 1), but also participate in the release of fructose from various β -D-fructofuranoside substrates. This capacity can be further exploited in order to transfer such residues to other molecules rather than water, favouring the synthesis of a broad array of glyco-compounds [1,2]. In particular, β -fructofuranosidases are capable to synthesize short-chain fructooligosaccharides (FOS), in which one to three fructosyl residues are linked to sucrose (which acts both as sugar donor and acceptor) by different types of glycosidic bonds depending on the producer organism [3-5]. The transferase:hydrolase ratio widely varies from one transglycosidase to another and its modulation represents a major challenge for protein engineers [6-9]. In this scenario, different attempts to transform a glycosidase into a transglycosidase have been carried out. Site-specifically mutated glycosidases called glycosynthases were tailored by replacing the catalytic carboxyl nucleophile with a non-nucleophilic residue [10]. However, activated glycosyl fluoride donors having the opposite anomeric configurations to that of the normal glycosidase substrate are required. Approaches to define and improve the transferase activity in some plant invertases have also been performed. Thus, onion [11,12] and wheat [13] invertases were mutated according to their similarities to fructosyltransferases, localising the *hot-spots* for transferase activity on a defined area of the active site related to the bound of the fructose molecule. In a laboratory evolution approach, the hydrolytic activity of a β -glycosidase from *Thermus thermophilus* diminished whereas significantly increased the transglycosylation activity [9]. With a combination of mutagenic strategies, the transgalactosylation activity of *Geobacillus stearothermophilus* β -galactosidase has also considerably improved [14].

FOS have been recognized as functional food ingredients [15,16] since they selectively stimulate the growth/or activity of potentially health-promoting Lactobacilli and Bifidobacteria in the digestive tract [17,18]. The products generated during prebiotic metabolism are associated to further beneficial effects on the stimulation of the immune response with the concomitant potential reduction of several diseases (cancer, osteoporosis, cardiovascular disease or obesity, among others) [17]. Currently, the main industrial FOS producers belong to the group of β -fructofuranosidases from *Aspergillus* species [3,19], which provide a mixture of FOS with the characteristic β -(2 $\rightarrow$ 1) inulin-type scaffold (*e.g.* 1-kestose, Fig. 1). In the last few years, it has emerged an increasing interest in the development of novel FOS with improved and/or different prebiotic/physiological properties [20,21]. Unlike β -(2 $\rightarrow$ 1) linked FOS, the β -(2 $\rightarrow$ 6)-linked FOS where the

bond is formed between two fructose units (*e.g.* 6-kestose) or between the fructosyl and the glucosyl moieties (*e.g.* neokestose) are arousing great attention since they may have enhanced prebiotic properties compared to commercial FOS [22]. The short chain β -(2 $\rightarrow$ 6) linked FOS are typically synthesized from levans (β -(2 $\rightarrow$ 6) linked polymers containing a glucose at the non-reducing end) by acid hydrolysis, but cumbersome product purification makes this process unattractive [23]. Alternatively, β -(2 $\rightarrow$ 6) linked FOS can be synthesized by β -fructofuranosidases, with lower yields than those obtained through acid hydrolysis [4,24]. In particular, the enzymatic synthesis of neokestose and 6-kestose has already been reported for the *Xanthophyllomyces dendrorhous* and *Schwanniomyces occidentalis* yeast enzymes, respectively [5, 25] (Fig. 1). Indeed, *Schwanniomyces occidentalis* efficiently utilizes a wide variety of inexpensive carbon compounds by employing a consortium of degradative extracellular enzymes, which includes the β -fructofuranosidase SoINV [25], whose 3D structure was recently reported [26]. Although the main reaction catalysed by this enzyme is the hydrolysis of sucrose, interestingly it also produces 6-kestose and 1-kestose in a ratio 3:1. The 6-kestose yield obtained with SoINV is, to the best of our knowledge, the highest yet reported for a 6-kestose-producing enzyme [25]. The analysis of the amino acid sequence revealed that SoINV includes a Ser instead of a Leu at position 196, caused by a non-universal decoding of the unique mRNA Leu codon CUG. The substitution Ser196Leu enhanced the transferase activity of the enzyme by almost 3-fold along with the selectivity for the synthesis of 6-kestose [27].

In this work we have prepared a laboratory evolution strategy in an attempt to push farther the synthesis of β -(2 $\rightarrow$ 6) transglycosylation products by SoINV functionally expressed in *Saccharomyces cerevisiae*. A high-throughput screening assay that combined two colorimetric methods was designed and validated to estimate the transglycosylation:hydrolysis ratio and used for screening mutant libraries created by error-prone PCR. Best candidates were further re-screened by HPAEC-PAD to accurately estimate the β -(2 $\rightarrow$ 6) and β -(2 $\rightarrow$ 1) linked FOS production by the SoINV mutants.

MATERIAL AND METHODS

Materials

pYES2 shuttle vector (5.9 kb, *amp*, *GAL1p*, *2 μ ori*, URA3; Invitrogen, Spain) was used for SoINV heterologous expression. Glucose oxidase from *Aspergillus niger*, horseradish peroxidase, fructose dehydrogenase from *Gluconobacter industrius*, Yeast Transformation Kit and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid

(ABTS) were purchased from Sigma-Aldrich (Madrid, Spain). The Taq Polymerase used in the mutagenic PCR was from Promega (Madrid, Spain). All other reagents were of the highest available purity.

Organisms and growth conditions

Sw. occidentalis ATCC26077 was used for the amplification of the β -fructofuranosidase gene (CQ890277) [25]. *Saccharomyces cerevisiae* EUROSCARF Y02321 (BY4741; Mat a; *his3* Δ 1; *leu2* Δ 0; *met15* Δ 0; *ura3* Δ 0; YIL162w::kanMX4) was used as the expression host. Maltose Minimal Medium (MMM) contained 0.67 % Yeast Nitrogen Base, 2 % maltose, 20 mg/L His, 20 mg/L Met, and 60 mg/L Leu. Expression Medium (YPGal) contained 2 % galactose, 1 % yeast extract, 2 % peptone and 2 % ethanol. SC selection plates contained 0.67% Yeast Nitrogen Base, 2% glucose, 20 mg/L His, 20 mg/L Met, 60 mg/L Leu, and 2% agar. Super-competent (DH5 α) *E. coli* cells were supplied by the Fermentation Department of the CBMSO (Madrid, Spain) and were used for plasmid amplification using standard techniques [28]. Growths were monitored spectrophotometrically at 600 nm.

Cloning and functional expression of *Sw. occidentalis* β -fructofuranosidase in *S. cerevisiae*

Briefly, genomic DNA was isolated from *Sw. occidentalis* yeast using standard techniques. The β -fructofuranosidase gene including its own signal peptide was obtained by PCR using genomic DNA, the Expand High Fidelity Taq Polymerase (Roche) and the primers: SoBa (+1 +25) 5'-TAGGATCCAACATGGTACAAGTTTTAAGTGTATTAG-3' and SoXb (+1591 +1614) 5'-CATCTCTAGACTAGCCCTACTTATTTAGTTCTCT-3', as recommended by the supplier, but using 7.5 mM MgCl₂ and the following conditions of amplification (i) 94°C for 120 s; (ii) 35 cycles of 94°C for 10 s, 55°C for 30 s, and 72°C for 60 s, and finally (iii) 72°C for 240 s. The amplified PCR fragment (1.608 bp) was purified by agarose gel electrophoresis using the QiaExII gel extraction kit (Qiagen, West Sussex, UK). Restriction sites for BamHI and XbaI (shown in bold) were included in the primers for cloning the PCR product into the pYES2 shuttle vector, under the control of the GAL1 promoter, giving rise to the construction Ffase-pYES [27]. Plasmids were purified with the Wizard Plus SV Minipreps kit (Promega, Spain) according to the manufacturer's protocol, and sequenced (SIDI, Universidad Autónoma de Madrid, Spain).

S. cerevisiae Y02321 was transformed with Ffase-pYES (Yeast Transformation Kit) and clones were selected in SC plates. A single colony was grown in 5 mL of MMM for 24 h at 30 °C and 210 rpm (UDO₆₀₀ ~1.0). The cells were collected at 5000 rpm (Eppendorf 5810R centrifuge, Germany), 50 mL of YPGal was added and the cells were grown for 24 h at 30 °C and 210 rpm (UDO₆₀₀ ~2.5). To assay β -fructofuranosidase activity, cells were

eliminated by centrifugation, as described above, and 100 μL of supernatant was mixed and incubated with 900 μL of 5 % (w/v) sucrose in 50 mM sodium phosphate pH 5.5 at 45 °C for 1 h. The activity was determined measuring the glucose liberated by the glucose oxidase-peroxidase assay (Sigma Technical Bulletin 510). A calibration curve was established with a 2 mg/mL glucose solution. One unit of activity was defined as that corresponding to the release of 1 μmol of glucose per min under the conditions described above. The β -fructofuranosidase activity level measured in the *S. cerevisiae* cultures was 3 mU/mL, which was lower than that obtained in *Sw. occidentalis* as previously reported [26].

Random mutagenesis

Mutant libraries of SoINV gene were created by error-prone PCR testing different concentration of MnCl_2 (from 0 to 0.2 mM) to provoke different degrees of mistakes during amplification. The library was built using SoINV as DNA template and the primers A-forward (5'-GCTGTAATACGACTCACTATAGGGAATA-3' that binds to bp 5'-471-498-3' of Ffase-pYES) and B-reverse (5'-GGGCGTGAATGTAAGCATATACATAAC-3' that binds to bp 5'-794-821-3' of Ffase-pYES). PCR reactions were performed in a final volume of 50 μL containing 0.3 mM dNTPs, 3 % dimethylsulfoxide, 0.05 U/ μL of Taq polymerase, 1x Taq Buffer, 1.5 mM MgCl_2 , (0-0.2 mM) MnCl_2 , 90 nM of each primer and 0.1 ng/ μL of DNA-template mixture. The thermal cycling parameters were as follows: 94 °C for 2 min (1 cycle), 94 °C for 15s, 50 °C for 30s and 72 °C 1 min 30s (30 cycles). Purified PCR products were transformed with the *Bam*HI and *Xba*I-linearized pYES2 vector (ratio PCR products:vector, 1:1) into *S. cerevisiae*.

High-throughput screening (HTS) assay

Transformed cells were plated in SC selection plates and incubated for 2 days at 30°C. Colonies containing the whole autonomously replicating vector were manually picked and cultured in 96-well plates (Sero-well, Staffordshire, UK) containing 50 μL of minimal medium per well. In each plate, column number 6 was inoculated with the parental type, and one well (H1-control) was not inoculated. Plates were capped and sealed to prevent evaporation and incubated at 30 °C, 225 rpm and 80 % relative humidity in a humid shaker (Minitron-INFORS, Biogen, Spain). After 48 h, 160 μL of expression medium was added to each well and the plates were incubated for 24 h. The plates (master plates) were centrifuged (Eppendorf 5810R centrifuge, Germany) at 4000 rpm for 5 min. 20 μL of supernatants were removed with the help of the liquid handler (Quadra 96-320, Tomtec, Hamden, CT) and transferred to new plates (reaction plates). 180 μL of 200 g/L sucrose in 50 mM sodium phosphate pH 5.5 was added, and the reaction plates were sealed with a thermoresistant film (Deltalab, Spain) and incubated for 1 h at 45 °C and 150 rpm. After that, reaction plates

were duplicated with the help of the liquid handler by transferring 40 μL of the reaction mixture to the two enzymatic assay plates. The first enzymatic assay plate (EAP-1) was used to assess the glucose content by the glucose oxidase/peroxidase coupled-assay (GOP-assay). The second enzymatic assay plate (EAP-2) was used to estimate the fructose content by the fructose dehydrogenase coupled-assay (FD-assay), (Fig. 2). Both enzymatic assays were adapted to a 96-well microplate format. 100 μL of glucose oxidase/peroxidase coupled-assay mixture (1.6 U/mL glucose oxidase, 2.5 U/mL horseradish peroxidase and 1 mg/mL ABTS in 50 mM sodium phosphate buffer pH 5.5) was added to EAP-1. The plates were stirred at 25 $^{\circ}\text{C}$ and the colour development was recorded at 418 nm ($\epsilon_{\text{ABTS}}^{\bullet+}=36,000 \text{ M}^{-1} \text{ cm}^{-1}$) (Plate reader SPECTRAMax Plus 384, Molecular Devices, Sunnyvale, CA). To the EAP-2, 10 μL of 100 U/mL fructose dehydrogenase in 50 mM sodium phosphate buffer pH 5.0 and 10 μL of 100 mM potassium ferricyanide (III) (Sigma) in the same buffer was added, the plate was sealed with thermoresistant film and incubated at 37 $^{\circ}\text{C}$ for 30 min. Then, 50 μL of a solution containing 1.5% of ferric sulphate (Sigma) and 8 % phosphoric acid was added to the EAP-2 plate to form the Prussian blue product [29]. The plates were stirred and the colour development was measured at 660 nm ($\epsilon=10,000 \text{ M}^{-1} \text{ cm}^{-1}$).

Re-screening

To rule out false positives, two consecutive re-screenings were carried out according to a previously reported protocol with some modifications [30].

First re-screening: Aliquots of 5 μL of the best 50 clones (those showing the highest glucose:fructose ratio) were removed from the master plates to inoculate 50 μL of minimal media in new 96-well plates. Columns 1 and 12 (rows A and H) were not used to prevent the appearance of false positives. After a 24 h incubation at 30 $^{\circ}\text{C}$ and 225 rpm, 5 μL was transferred to the adjacent wells and further incubated for 24 h. Finally, 160 μL of expression medium was added and the plates were incubated for 24 h; accordingly, each mutant was grown in 4 wells. The parental type was subjected to the same procedure (lane D, wells 7-11) and the plates were assessed using the same protocols for the screenings described above.

Second re-screening: An aliquot from the wells with the best 10 clones of the first rescreening was inoculated in 3 mL of MMM and incubated at 30 $^{\circ}\text{C}$ and 225 rpm for 24 h. The plasmids from these cultures were extracted (Zymoprep yeast plasmid miniprep kit, Zymo Research). As the product of the zymoprep was very impure and the concentration of DNA extracted was very low, the shuttle vectors were transformed into *E. coli* cells and plated onto LB-amp plates. Single colonies were picked and used to inoculate 5 mL LB-amp media and they were grown overnight at 37 $^{\circ}\text{C}$ and 200 rpm. The plasmids were then extracted (QIAprep spin miniprep kit, QIAGEN, Spain) and *S. cerevisiae* was transformed with plasmids from the best mutants as well as with the

parental type. For each variant (including parent type), five individual colonies were inoculated -by lanes- in 50 μ L of minimal media in 96-well plates (columns 1,12 and rows A, H, were not used). Plates were capped, sealed and incubated at 30 °C, 225 rpm and 80 % relative humidity. After 48 h, 160 μ L of expression medium was added to each well and the plates were incubated for 24 h. Plates were subjected to the same screening protocol described above.

HPAEC-PAD analysis of the selected mutants

Product analysis by high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) was incorporated to accurately estimate the progress of FOS production. Reaction conditions were as follows: 0.1 U of parental type or selected mutants was added to 1 mL of 600 g/L sucrose in 50 mM sodium phosphate buffer pH 5.5, and the mixture was incubated at 45 °C and 225 rpm. Aliquots were removed at different times and filtered using a 0.45 μ m Durapore[®] membrane coupled to an eppendorf tube. The concentration of the different products was measured on a ICS3000 Dionex system consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. A pellicular anion-exchange 4x250 mm Carbo-Pack PA-1 column (Dionex, USA) was used at 30 °C. A mobile phase gradient from water to a solution containing 150 mM NaOH and 100 mM sodium acetate was performed at 1.0 mL/min in 30 min. The peaks were analyzed using Chromeleon software. Identification of the different carbohydrates was done based on glucose, fructose, sucrose and 1-kestose standards. Neokestose and 6-kestose standards were obtained by preparative HPLC.

DNA sequencing

Plasmid-containing SoINV variants were sequenced by using a BigDye Terminator v 3.1 Cycle Sequencing Kit (SIDI, Universidad Autónoma de Madrid, Spain), and the primers A-forward and B-reverse previously described.

Protein modelling

Protein model of the β -fructofuranosidases from *Sw. occidentalis* was obtained from the protein Data Bank; PDB code 3KF5 and 3KF3 [26] and analyzed with Deep-View/Swiss-Pdb Viewer.

RESULTS AND DISCUSSION

HTS Assay: design and validation

Ideally, screening assays that detect enzymatic activity are based on the recognition of reaction products from natural or surrogate substrates (such as chromophoric or fluorophoric compounds) that can be

easily measured by direct or indirect enzymatic-coupled assays. Transglycosidase (transferase) activities are not so straightforwardly assessed in a high-throughput context since the release of detectable compounds upon the formation of the glycosidic bond has not been successfully accomplished yet [31]. Recently, a screening strategy to assay evolved β -galactosidases for galacto-oligosaccharide production was described [14]. In particular, the assay was based on the hydrolysis of a potential transglycosylation product (lactosucrose); however, a decrease of the hydrolytic activity on the trisaccharide does not necessarily implies a higher transglycosylation activity, in such a manner that an additional TLC assay using nitrophenyl-galactoside as donor and lactose as acceptor had to be implemented in the screening.

To circumvent some of these shortcomings, in our laboratory evolution program we have coupled two colorimetric methods in the frame of one screening assay, which gave a direct estimation of the proportion of transglycosylation to hydrolysis, (Fig. 2). This method has been tested during the screening of mutant libraries constructed in yeast to identify variants of a β -fructofuranosidase with improved transglycosylation activity.

As Fig. 1 summarizes, every transglycosylation step yields a glucose molecule as by-product, whereas the hydrolysis produces an equimolar mixture of glucose and fructose. The main parameter used to discriminate between mutants was consequently the glucose:fructose ratio, which provided a rough estimation about the transferase activity of every clone. In principle, only those variants that produce a higher amount of glucose than the parent type but with a lower or similar fructose production would be considered suitable candidates for further characterization. For the estimation of glucose in the reaction mixture, a glucose oxidase/peroxidase coupled-assay (GOP-assay) [32] was employed whereas the fructose was quantified by the fructose dehydrogenase coupled-assay (FD-assay) [29] (Fig. 2 and Material and Methods Section for further details).

The validation of the assay was carried out by checking the response of parental type supernatants vs the two colorimetric methods. Fresh transformants containing Ffase-pYES were inoculated in a 96-well plate, the SoINV gene was expressed and different volumes of supernatants were tested finding a linear behaviour between the amount of β -fructofuranosidase and the response of both colorimetric assays, (Fig. 3A, B). To calculate the coefficient of variation of both colorimetric assays, test plates containing parental type in the different wells were prepared. *S. cerevisiae* cells containing the Ffase-pYES were plated on SC selection plates. Individual clones were transferred to test plates and after inducing expression, the activities were evaluated from fresh supernatants preparations, as described in the Methods section. The coefficients of variation (CV) for the GOP- and FD-assays were 11 % and 7.2 % respectively; both values are acceptable to study libraries tailored for directed evolution experiments, (Fig 3C, D) [33]. Two consecutive re-screens were used to rule out

the presence of false positives. Finally, HPAEC-PAD chromatography was employed to confirm the formation of transglycosylation products by each selected mutant and to follow the reaction progress in 136 h.

Library Construction and Analysis

In general terms, a mutational rate of 2-5 mutations per Kb is suitable for directed evolution approaches. The mutational rate was initially calculated from landscapes of small libraries using different MnCl_2 concentrations during the PCR, (Fig. 4). The amplified PCR products were transformed into *S. cerevisiae* as described above. 90 clones from each PCR condition were selected and inoculated in 96-well plate in minimal media. The activity was measured following the HTS assay described above. In most of the cases, an error rate resulting in a library with 30-45 % of clones having less than 10 % of the parent activity is suitable for directed evolution approaches. The 0.02 mM MnCl_2 library was chosen (with ~38 % of mutants having less than 10 % of the parental type's activity). Accordingly, a 0.02 mM MnCl_2 library of ~900 clones was constructed and screened for transferase:hydrolase activity, (Fig. 5). Those mutants with an increase in the final glucose content and either a decrease or a sustain value in the final fructose content in comparison with the parental enzyme were selected (42 mutants were chosen and further re-screened). After two consecutive re-screens, NR8 and BC6 mutants were the final candidates and their transglycosylation activities were studied in deeper detail. It is worth noting that although different assays are available for the verification of improved transglycosylation activity in mutant libraries, most of them are based on rough TLC analysis of reactions involving nitrophenyl-galactoside donors and disaccharides as acceptors [9,14]. In the present work, the progress of the transglycosylation reaction catalyzed by NR8 and BC6 mutants was accurately analyzed by HPAEC-PAD. Similar chromatographic profiles for the parental and selected mutant enzymes were observed, (Fig. 6A). In all the cases 6-kestose was the main transglycosylation product, however other trisaccharides such as 1-kestose and neokestose were also formed but in a lower proportion. Trisaccharides also served as acceptors yielding minor concentrations of several tetrasaccharides. The NR8 mutant compared to the parental showed an enhancement at the initial rates of 6-kestose production (46.6 g/L vs 29.8 g/L at 48 h), which was less marked after 136 h (78.5 g/L vs 65.6 g/L), (Fig. 6 B). Similarly, BC6 mutant improved significantly the rates at the initial stages of the reaction (24.0 g/L vs 11.9 g/L at 16 h,) but with lower extent after 136 h (70.4 g/L vs 65.6 g/L). Further rounds of evolution have been carried out based on the results reported here. Interestingly, the ultimate variant of this evolution process (unpublished material) display catalytic efficiency (k_{cat}/K_m) values 7-fold better than parent type (1,4 vs 0,2 s⁻¹ mM⁻¹) for the production of 6-kestose.

The 3D structure of β -fructofuranosidase from *Sw. occidentalis* was recently reported [26]. Being folded into a catalytic and a β -sandwich domain, SoINV falls into the family 32 of the glycosyl-hydrolases (GH) according to the Carbohydrate Active Enzymes database [34]. Multiple-sequence alignment of the GH32 reveals the presence of some conserved motif, including Asn-**Asp**-Pro-Asn-Gly (NDPNG), and **Glu**-Cys (EC), each of them containing a key acidic residue (in bold) implicated in substrate binding and hydrolysis [35]. The reaction mechanism comprising these conserved residues proceeds by a double-displacement in which a covalent glycosyl-enzyme intermediate is formed. Thus, the catalytic mechanism proposed for the β -fructofuranosidase implies that Asp23 (NDPNG) acts as a nucleophile and Glu204 (EC) as the acid/base catalyst (according to the numbering for the invertase from *S. cerevisiae* [36]). The same kind of amino acids (Asp50 (NDPNG) and Glu230 (EC)), are also the catalytic residues in the β -Fructofuranosidase from *Sw. occidentalis* [27]. BC6 mutant contains one single mutation CAA/CTA (Q78L), which is located near the catalytic residue Asp50. The change Q78L means the replacement of a polar amino acid by a hydrophobic one, which may possibly produce a local rearrangement that would affect the overall enzyme activity. NR8 mutant contains one single mutation TTT/GTT (F523V) placed in the β -sandwich domain, which could have a direct catalytic role on the enzyme activity [27].

Transglycosylation rates are dependent on enzyme kinetics [37] but the change in the relative concentration of the reactants (water and sucrose) could imply a shift in the thermodynamic equilibrium, consequently affecting the transferase rate. All these factors are difficult to assess, especially in the high-throughput scenario where directed enzyme evolution takes place. The HTS assay described in this work constitutes a valuable tool to engineer transglycosidases for practical purposes, and it is currently being used by our group to tailor new high-efficient transglycosylases (unpublished material). Along these lines, the expression system in *S. cerevisiae* for the β -fructofuranosidases from *Sw. occidentalis* is a suitable platform to recreate diversity by *in vivo* means (*e.g. in vivo* DNA shuffling, *in vivo* assembly of mutant libraries, *in vivo* overlap extension and more [38]) with the aim of engineering novel transferase activities.

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FIGURE LEGENDS

Figure 1: Schematic representation of the hydrolysis and transglycosylation reaction. The specificity of different β -fructofuranosidases in the formation of fructooligosaccharides is also displayed.

Figure 2: HTP-screening employed to detect the transferase activity in the SoINV mutant library.

Figure 3: Validation of the colorimetric assays for the SoINV-library. Linearity of the GOP-assay (A) and the FD-assay (B). Each point corresponds to the average of 5 experiments. Activities of parental type plotted in descending order for the GOP-assay (C) and the FD-assay (D). *S. cerevisiae* cells were transformed with Ffase-pYES and plated on SC plates. Individual colonies were picked and inoculated in a 96 well-plate. The activities of the clones were evaluated from fresh supernatant preparations. Dashed lines indicate the coefficient of variation of the assays.

Figure 4: Landscapes for libraries made under different error-prone PCR conditions (different concentrations of MnCl_2). Activity of clones is plotted in descending order. Solid horizontal line shows the activity of the parental type in the assay. Dashed lines indicate the coefficient of variation of the assay. The percentages refer to the amount of mutants having less than 10 % of the parental type's activity.

Figure 5: Directed evolution landscapes. Activities of clones from 0.02 mM MnCl_2 library screened with the GOP-assay (for glucose detection, A) and with the FD-assay (for fructose detection, B). Activities of clones are plotted in descending order. C) Glucose vs fructose relative activities. Dashed lines in A and B represent the coefficient of variation of the assay and in C the parental type activity. Mutants selected for further re-screens are squared. Mutants BC6 and NR8 are highlighted inside open circles.

Figure 6: (A) HPAEC-PAD chromatograms corresponding to the reaction with sucrose after 136 h. A, NR8 mutant; B, BC6 mutant; C, SoINV parent type. The peaks correspond to sucrose (1), glucose (2), 1-kestose (3), fructose (4), 6-kestose (5), neo-kestose (6) and tetrasaccharide (7). (B) Time-course of 6-kestose production catalyzed by SoINV parent type (black square), BC6 mutant (white circle) and NR8 mutant (white triangle).

FIGURE 1

HYDROLYSIS

TRANSGLYCOSYLATION

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6