



Engineering a heterologously expressed fructosyltransferase from *Aspergillus oryzae* N74 in *Komagataella phaffii* (*Pichia pastoris*) for kestose production

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ABSTRACT

Fructo-oligosaccharides (FOS) are one of the most well-studied and commercialized prebiotics. FOS can be obtained either by controlled hydrolysis of inulin or by sucrose transfructosylation. FOS produced from sucrose are typically classified as short-chain FOS (scFOS), of which the best known are 1-kestotriose (GF₂), 1,1-kestotetraose (GF₃), and 1,1,1-kestopentaose (GF₄), produced by fructosyltransferases (FTases) or β-fructofuranosidases. In previous work, FOS production was studied using the *Aspergillus oryzae* N74 strain, its *ftase* gene was heterologously expressed in *Komagataella phaffii* (*Pichia pastoris*), and the enzyme's tertiary structure modeled. More recently, residues that may be involved in protein–substrate interactions were predicted. In this study, the aim was to experimentally validate previous *in silico* results by independently producing recombinant wild-type *A. oryzae* N74 FTase and three single-point mutations in *Komagataella phaffii* (*Pichia pastoris*). The R163A mutation virtually abolished the transfructosylating activity, indicating a requirement for the positively charged arginine residue in the catalytic domain D. In contrast, transfructosylating activity was improved by introducing the mutations V242E or F254H, with V242E resulting in higher production of GF₂ without affecting that of GF₃. Interestingly, initial sucrose concentration, reaction temperature and the presence of metal cofactors did not affect the enhanced activity of mutant V242E. Overall, these results shed light on the mechanism of transfructosylation of the FTase from *A. oryzae* and expand considerations regarding the design of biotechnological processes for specific FOS production.

Introduction

Prebiotics are defined as “substrates that are selectively utilized by host microorganisms conferring a health benefit” [1]. Fructo-oligosaccharides (FOS) are the most well studied and commercialized prebiotics [2]. Nevertheless, even after decades of research on FOS, there is still a need for research related to improving current production processes and prebiotic alternatives. Industrial production of FOS can be achieved either by controlled hydrolysis of inulin or by sucrose transfructosylation [3,4]. FOS produced from sucrose have a lower

degree of polymerization than those produced by inulin hydrolysis, and are therefore described as short-chain FOS (scFOS) and long-chain FOS (lcFOS), respectively [3,4]. Among scFOS, those most studied are 1-kestotriose (GF₂), 1,1-kestotetraose (GF₃), and 1,1,1-kestopentaose (GF₄), which are produced either by fructosyltransferases (FTases, E.C. 2.4.1.9) or β-fructofuranosidases (FFases, E.C. 3.2.1.26) present in many plants, fungi, and bacteria [5,6]. For instance, fungal FTase acts on sucrose as a donor, cleaving the β2-α1 linkage, releasing glucose, and then transferring the fructosyl group to an acceptor molecule, sucrose or an scFOS such as GF₂, GF₃, etc [7]. scFOS have a strong bifidogenic effect on

Abbreviations: FOS, fructo-oligosaccharides; scFOS, short-chain FOS; lcFOS, long-chain FOS; GF₂, 1-kestotriose; GF₃, 1,1-kestotetraose; GF₄, 1,1,1-kestopentaose; FTase, fructosyltransferase; FFase, fructofuranosidase; GH, glycoside hydrolase; 1-SST, 1-fructosyltransferases; UT, transfructosylating activity; ST, specific transfructosylating activity.

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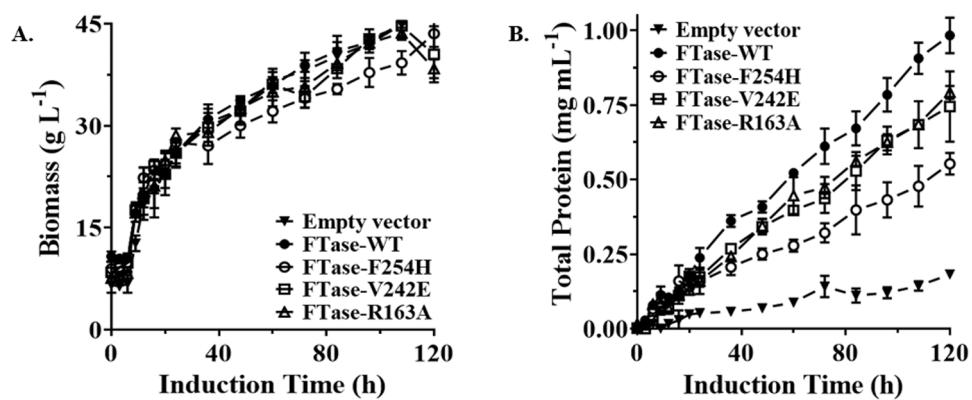


Fig. 1. Biomass and total protein of *K. phaffi* transformants. Timelapse monitoring of biomass production (A) and secreted protein (B) during the induction time of FTases, WT and variants, by selected *K. phaffi* transformants.

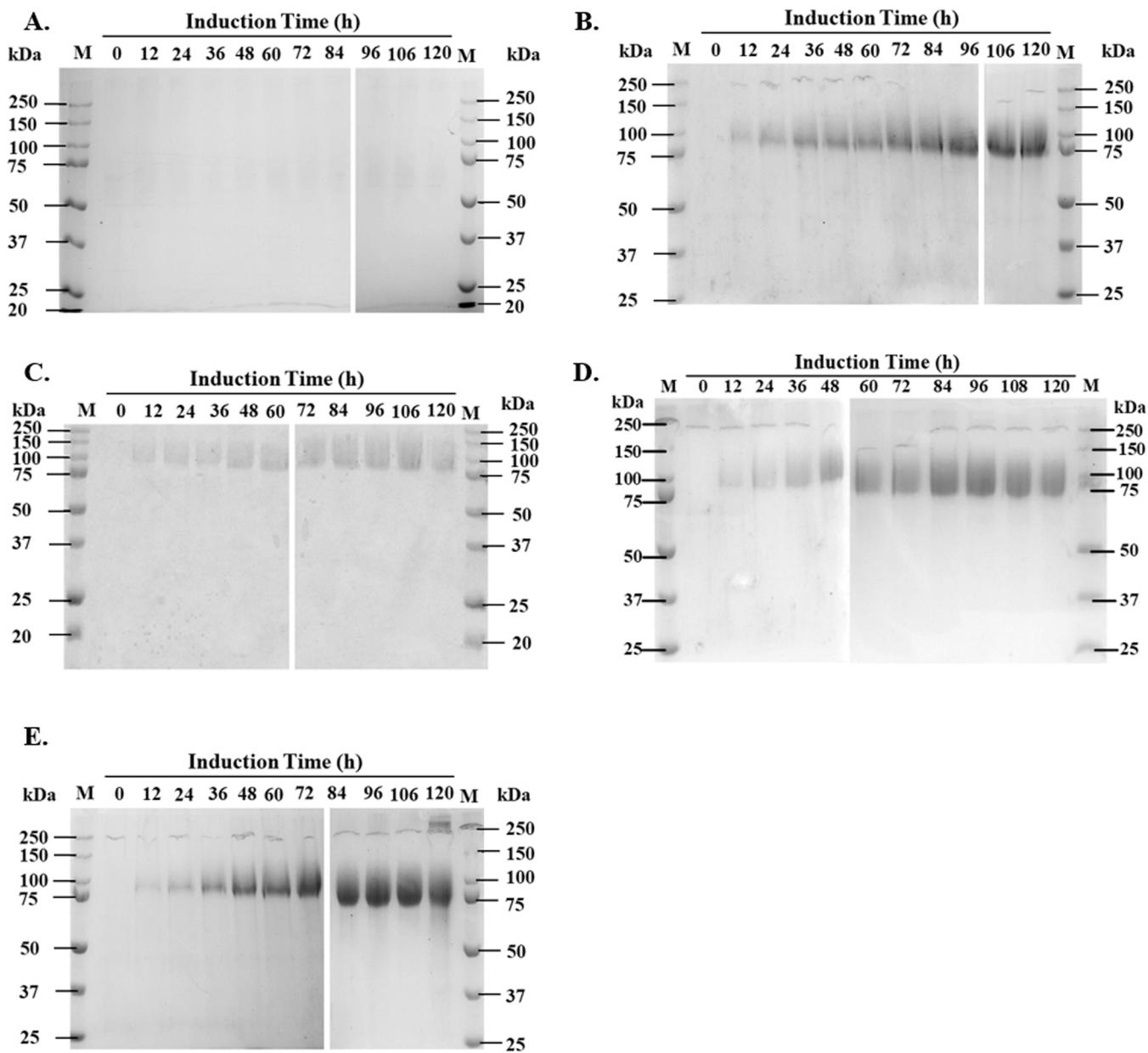


Fig. 2. Monitoring of secreted FTase wild-type and variants by selected *K. phaffi* transformants via SDS-PAGE analysis. Main protein band at ~85 kDa corresponds to secreted FTases. A. Negative control (empty pPIC9K vector), B. FTase-WT, C. FTase-F254H, D. FTase-V242E, and E. FTase-R163A.

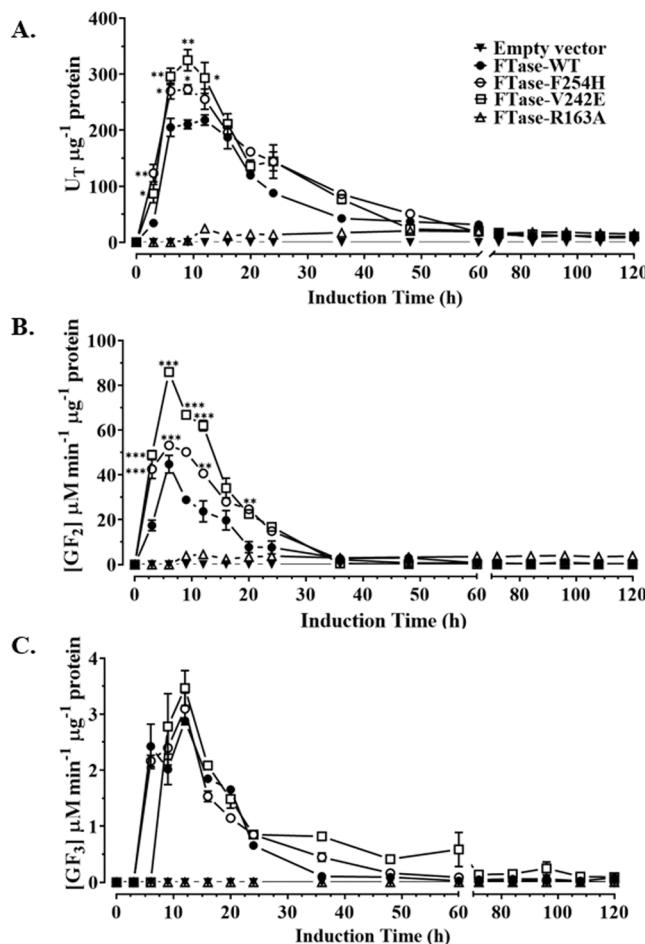


Fig. 3. Enzyme activity of FTase wild-type and variants. Specific transfructosylating activity (A) and GF₂ (B) and GF₃ (C) productivity of FTase, wild-type and mutants, at different protein induction times. Withdrawn samples were used in a 4-h reaction with sucrose 60% w/v. Data is presented as mean \pm SEM, n = 3. *, p < 0.05; **, p < 0.01; ***, p < 0.005.

intestinal health, with a large production of short-chain fatty acids [8,9]. Specifically, GF₂ showed superior selective stimulating activity of *Bifidobacterium*, compared to GF₃ and the commercial mixture of scFOS Meioligo® [9], when studied in vitro as well as in a murine animal model. In addition, GF₂ is an excellent alternative to stimulate the indigenous growth of *Faecalibacterium prausnitzii* [10], a bacterium used as a biomarker to assist in gut disease diagnostics [11] and the new generation of probiotics [12]. These findings highlight the importance of developing production systems to obtain only GF₂ or a scFOS mixture where GF₂ is the main component.

FTases and FFases belong to the glycoside hydrolase (GH) family, specifically the GH-J clan that merges GH32 and GH68 families [13]. Although FTases and FFases can be found in plants and bacteria, fungi remain their primary source for industrial production of scFOS [14]. In nature, most plant invertases [15,16] and sucrose:sucrose:1-fructosyltransferases (1-SST) [17,18] produce GF₂ as the major FOS. Recently, the production was reported of GF₂ and GF₃ in a ratio of 8:2 by the heterologously expressed 1-SST from *Schedonorus arundinaceus* in *Pichia pastoris* PGFT6x-308 [19]. To achieve the production of GF₂ as the major product using a fungus Ftase, it is essential to reduce the enzyme's affinity for GF₂ to reduce its acceptor role and the production of GF₃.

During the last decade, FOS production has been studied using the *Aspergillus oryzae* N74 strain [20–23] and the enzyme's tertiary structure modelled [24,25]. The crystal structure of *Aspergillus japonicus* FTase has provided insight into the active site of fungal-FTases. Thus, residues

D60, D191, and E292 were identified as the catalytic triad in the active site of *A. japonicus* FTase [7]. In a previous *in silico* study, docking analysis identified important residues for protein–substrate interactions from *A. oryzae* N74 FTase homologous to those in *A. japonicus* FTase (PDB ID 3LF7) and mutations of the identified active site residues in *A. oryzae* N74 FTase were simulated *in silico* [25]. The docking analysis of FTase variants predicted an equal affinity for GF and GF₂, while for GF₃ and GF₄ the affinity was reduced [25]. The aim of the present study was to validate experimentally the *in-silico* results by producing recombinant *A. oryzae* N74 FTase and three active mutants in *Komagataella phaffii* in order to obtain a productive system favoring 1-kestotriose production over other scFOS.

Materials and methods

Generation of FTase constructs in pPIC9K

DNA encoding the sequence of *A. oryzae* N74 FTase (GenBank: GU145136.1) was codon-optimized for expression in *K. phaffii* GS115 and custom-synthesized (GeneArt, Thermo Fisher Scientific, CA, USA). The secretion signal from *Saccharomyces cerevisiae*, known as α-factor, was fused to the N-terminus of the FTase. The gene fragment was digested with flanking restriction enzymes *Xba*I and *Eco*RI and ligated with the *Bam*HI- and *Eco*RI-digested pPIC9K (Invitrogen, Thermo Fisher Scientific, CA, USA), an expression vector used in *K. phaffii* GS115. A detailed amino acid sequence of the engineered FTase is presented in Supplementary Table S1. The size and sequence of *ftases* in pPIC9K were verified by diagnostic digestion and DNA sequencing. All procedures were carried out using standard molecular biology methods [26].

FTase site-directed mutagenesis

In *A. japonicus*, it was determined that residues D60, D191, and E292 form the catalytic triad and their mutation causes the complete loss of enzymatic activity with sucrose as substrate [7]. Furthermore, residues D119, H144, R190, E318, H332, L78, F118, Y369, A1370, W398, I143, and Y404 form a negatively charged active-site pocket [7]. In addition, R190, E292, E318, H332, and I143 are also involved in the stability of the fructosyl or glucosyl moiety at the + 1 subsite (following the nomenclature of [27]) [7]. Thus, the homologous residues to those of interest were previously identified due to their role as catalytic nucleophile, transition-state stabilizer, general acid/base catalyst residues, or stabilizer of the fructosyl or glucosyl moieties. Residues D39, D164, E216, I124, R163, V242, and F254 in *A. oryzae* N74 FTase are homologous to D60, D191, E292 I143, R190, E318, and H332 in *A. japonicus* FTase [25].

The effect of individual mutations in the *A. oryzae* N74 FTase on the dissociation constant (K_D) towards sucrose and main scFOS, was assessed via alanine scanning [28], namely D39A, D164A, E216A, I124A, R163A, V242A and F254A, as well as V242E and F254H, which were included to match the residues in *A. japonicus* [25]. Among the *in silico* assessed mutations, R163A, V242E, and F254H were selected due to their effect on K_D values, specifically those that resulted in a high K_D between the FTase and GF₃ and GF₄. Site-directed mutagenesis of *ftase* was performed by Genscript (Piscataway, NJ, USA) to generate pPIC9K plasmids carrying individual mutations R163A, V242E, and F254H.

K. phaffii GS115 transformation

All constructs were linearized and used independently to transform *K. phaffii* GS115 cells by electroporation in a 0.2 cm cuvette at 1200 volts and 200 amps. The constructs used included pPIC9K containing the wild type FTase (FTase-WT) as well as the mutated FTases mentioned above (FTase-F254H, FTase-V242E, and FTase-R163A). Additionally, the empty pPIC9K backbone was used as a negative control. *K. phaffii* GS115 transformants of Mut-phenotype were screened by culturing them in

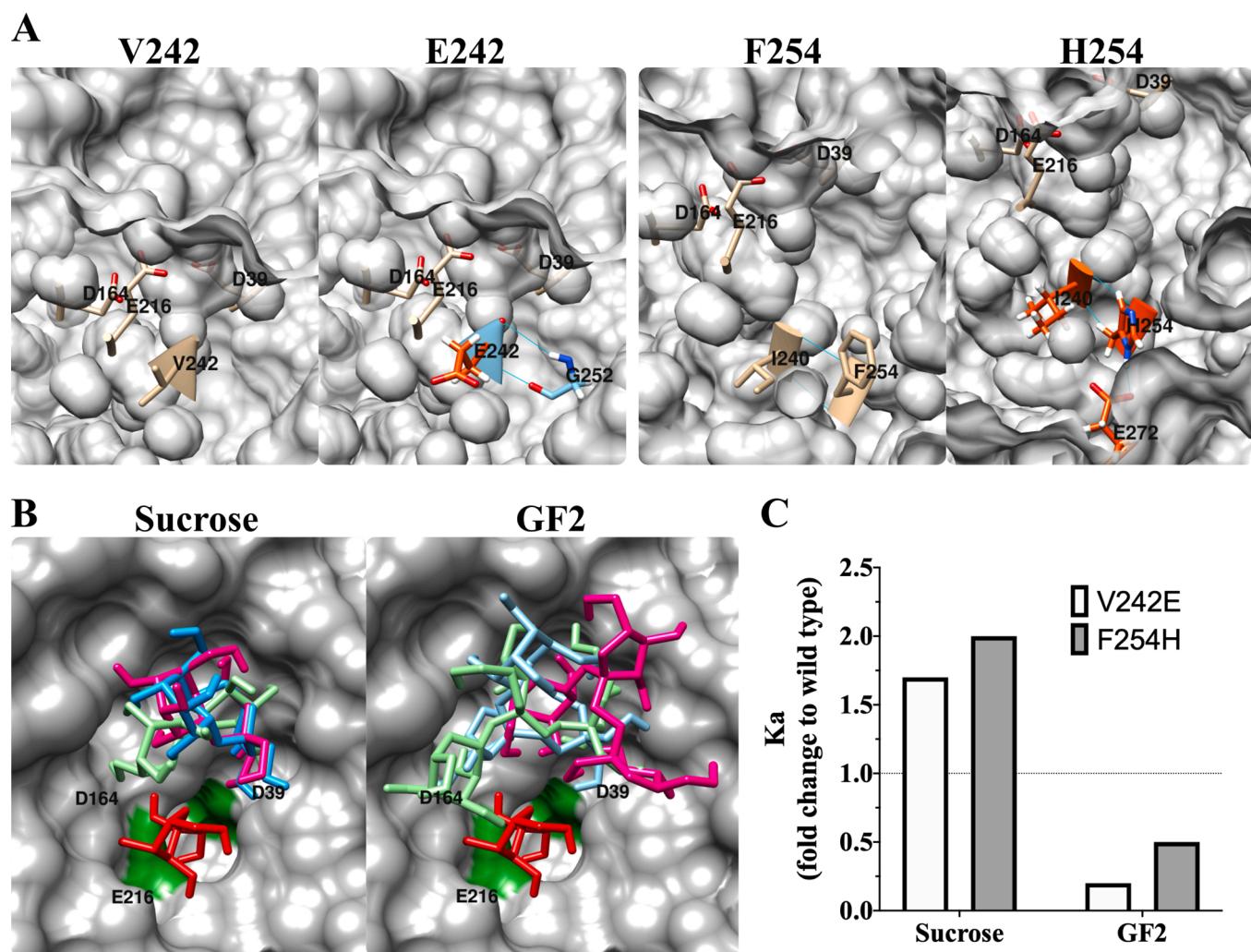


Fig. 4. Structural analysis and molecular docking of wild-type and mutant FTases.

(A) Amino acids V242 and F254 from *A. oryzae* N74 FTase were mutated to E242 and H254. Mutations V242E and F254H induced novel H-bonds (blue lines) with G254 and E272, respectively. (B) To analyze the effect of V242E and F254H mutations on the scFOS synthesis mechanism, dockings against sucrose and GF₂ were performed with the WT (blue), V242E (green) and F254H (pink) *A. oryzae* N74 FTase, previously docked with fructose (red) within the active cavity. (C) *K_a* fold change to wild type FTase (dotted line) for sucrose and GF₂ docked with V242E and F254H FTases previously docked with fructose within the active cavity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MM plates [1.34% (w/v) yeast nitrogen base, 4 × 10⁻⁵% (w/v) biotin, and 0.5% (v/v) methanol] following the manufacturer's instructions (Thermo Fisher Scientific, CA, US). Insertion of the *ftase* gene, wild-type (WT) and mutants, was confirmed by colony PCR using the primers FW: 5'-GGG TCA CGC TAC TTC TCC AG-3' and RW: 5'-GCA ATG TGC TGA GTC TCC AA – 3' which amplify a 201 bp fragment of the *ftase* cDNA (*Supplementary Fig. S1A*). The phenotype was also confirmed by PCR using the primers FW: 5'-GAC TGG TTC CAA TTG ACA AGC-3' and RW: 5'-GCA ATG TGC TGA GTC TCC AA – 3' which amplified a 720 bp fragment, containing part of the AOX1 promoter, the α-factor secretion signal and part of the FTase N-terminus (*Supplementary Fig. S1B*).

Shake flask cultures

Screening of the *K. phaffii* GS115 transformants obtained for each FTase construct was performed at 10 mL scale. Transformants that showed the highest transfructosylating activity were grown at a 100 mL scale. Cell culture was conducted in YPD medium [yeast extract 1% (w/v); peptone 2% (w/v); dextrose 2% (w/v)] for 48 h at 28 °C and 250 rpm. Cells were harvested by centrifugation, re-suspended in BMG medium [potassium phosphate 100 mM pH 6.0 ± 0.2; yeast nitrogen base

1.34% (w/v); biotin 4 × 10⁻⁵% (w/v); glycerol 1% (w/v)] and cultured for 24 h at 28 °C and 250 rpm. Cells were subsequently harvested and re-suspended in the BMM medium [potassium phosphate 100 mM pH 6.0 ± 0.2; yeast nitrogen base 1.34% (w/v); biotin 4 × 10⁻⁵% (w/v); glycerol 1% (w/v); methanol 0.5% (v/v)] and cultured for 120 h at 30 °C and 120 rpm. Methanol was added under controlled conditions to maintain a final concentration of 0.5% (v/v). Samples were withdrawn at selected times and stored at –20 °C until use. The transformants with the highest enzyme activity were selected to further characterize FTase activity. Sample crude extracts were used to verify recombinant FTases secretion by sodium dodecyl sulfate/polyacrylamide gel (SDS-PAGE) under reducing conditions. Coomassie blue staining was used to reveal the protein bands.

Enzyme activity

Samples from cultures of *K. phaffii* GS115 transformants with the different pPIC9K-FTase constructs were filtered through a 0.2-μm-pore-size membrane (Advantech, CA, USA). A first approach was based on quantifying the released glucose due to sucrose hydrolysis to identify transformants potentially having FTase activity. For this, 900 μL of 66%

Table 1

Residues interacting with sucrose and 1-kestotriose (GF_2) within the active cavity of wild-type and mutant FTases previously docked with fructose. Residue interactions through hydrogen bonds are underlined. All other amino acids are involved in hydrophobic interactions.

Protein model	Binding molecule	
	Sucrose	1-kestotriose (GF_2)
Wild type + Fructose	Fructose <u>Gln57</u> Gly66 Gln68 Phe98 Tyr96 Lys160 Trp305	Fructose <u>Gln57</u> Gln68 Ala65 <u>Tyr96</u> Phe98 Lys160 <u>Asp127</u> Arg163 Glu132 Trp305
p.V242E + Fructose	Fructose <u>Gln57</u> Ala65 <u>Gly66</u> <u>Gln68</u> Tyr96 Phe98 <u>Lys160</u> Arg163 <u>Asp127</u> Trp305	Fructose Gly66 Gln68 Ala65 <u>Tyr96</u> Lys160 <u>Asp127</u>
p.F254H + Fructose	Fructose <u>Gln57</u> Gly66 Gln68 Tyr96 Phe98 <u>Lys160</u> <u>Asp127</u> Trp305	Fructose <u>Gln57</u> Val64 Ala65 Gly66 Gln68 <u>Tyr96</u> Phe98 Lys160 <u>Asp127</u> Trp305 Glu309

(w/v) sucrose in 500 mM sodium phosphate buffer, pH 5.5, were mixed with 100 μ L of the cell-free extract and incubated at 60 °C for 4 h. The reaction was stopped by incubating the sample in boiling water for 10 min, followed by incubation in an ice-bath until analysis. The glucose released into the supernatant was measured using a glucose-oxidase kit (BioSystems, Barcelona, Spain), following the manufacturer's instructions. The cell-free extract from *K. phaffii* GS115 containing the empty vector was used as a negative control. Transformants that showed the largest release of glucose were selected to assess the transfructosylating activity.

Selected transformants for each of the FTases studied were evaluated at 100 mL scale cultures. Transfructosylating activity was assayed from samples taken during the induction phase. The reaction was conducted as described above and analyzed via high-performance liquid chromatography (HPLC) to further characterize the carbohydrate composition. The HPLC system (Shimadzu, Tokyo, Japan) was equipped with a refractor index and an Aminex HPX-87 C column (300 mm × 7.8 mm, Bio-rad, California, USA). Ultrapure water was used as eluting solvent at a flow rate of 0.6 mL min⁻¹, at 84 °C. The concentrations of saccharides, sucrose (GF), glucose (G), fructose (F), 1-kestotriose (GF_2), 1,1-kestotetraose (GF_3), and 1,1,1-kestopentaose (GF_4), were determined by interpolation using external standards. HPLC standards of GF_2 , GF_3 , and GF_4 were from Wako Pure Chemical Industries (Osaka, Japan); other analytical standards were from Sigma-Aldrich (St Louis, MO, USA). A representative chromatogram of sample analysis via HPLC is presented in [Supplementary Fig. S2](#). Transfructosylating activity was defined as the amount of enzyme transferring 1 μ M of fructose per minute (U_T), while the specific transfructosylating activity (U_S) was expressed as U_T

μ g⁻¹ of total protein). The total-transferred fructose is defined as glucose released minus free fructose.

Biomass and total protein quantification

Cultured samples from the different FTases (WT and mutants) were analyzed for biomass and total protein concentration following standard procedures [29,30]. Biomass concentration was quantified by measuring the optical density at 610 nm (OD_{610}) and its correlation with dry-weight biomass from a calibration curve [31]. Total protein concentration in cell-free extracts was determined using the micro BCA kit (Pierce, IL, USA) following the manufacturer's instructions.

Bioinformatics simulations

The tertiary structure of FTase from *A. oryzae* N74 was previously modeled by using I-TASSER server [25]. Site-directed mutagenesis was performed with UCSF Chimera, version 1.14 (build 42091) [32]. Topological similarities between protein structures were assessed by using TM-score server [33]. To analyze the effect of mutations on the FOS synthesis mechanism, dockings against sucrose and GF_2 were performed for the WT and mutant FTases previously docked with fructose within the active cavity. Molecular dockings were performed as previously described [25] by using AutoDock Vina [34]. Docking for each substrate was run 20 times and constrained to the active cavity. Results of protein–substrate interactions are reported as association constant (K_a). Protein–substrate interactions were evaluated by using LigPlot⁺ v.2.2.4 [35]. Molecular graphics were generated with UCSF Chimera, version 1.14 (build 42091) [32].

Statistical analysis

Differences between groups were tested for statistical significance using Student's t-test or one-way ANOVA. An error level of 5% ($p < 0.05$) was considered significant. All analyses were performed using SPSS v21.0 (SPSS Inc., Chicago, IL, USA). All results are shown as means ± standard deviation (SD).

Results and discussion

Cloning FTases in *K. phaffii* GS115

Mutations in *A. oryzae* N74 FTase, namely R163A, V242E, and F254H [25], were selected to study their effect on the production of GF_2 as the main scFOS. Compared to FTase-WT, modelling indicated that mutation R163A has the highest impact on FTase affinity for sucrose and scFOS by increasing the calculated dissociation constant (K_D) between 1.4- and 2.3-fold. In contrast, mutations V242E and F254H essentially did not affect the predicted K_D between the FTase and sucrose or GF_2 , but did affect affinity for GF_3 and GF_4 . While both mutations increased the K_D between the FTase and GF_3 1.2-fold, the K_D between the FTase and GF_4 increased 3.3- and 2.1-fold for V242E and F254A, respectively [25]. In this sense, it was expected that the activity of FTase-R163A would be lower than that of FTase-WT due to the loss of affinity for all substrates. On the other hand, it is likely that FTase-V242E and FTase-F254A would present unaltered sucrose transformation to GF_2 , but a significant reduction of GF_3 and GF_4 production. As expected, *K. phaffii* GS115 transformants were obtained for WT and mutant FTase. The presence of *ftase* was confirmed via colony PCR of zeocin-resistant colonies. [Supplementary Fig. S1](#) shows representative images of agarose gels of colony PCR results.

Production of FTases by recombinant *K. phaffii* in shake flask culture

FTase transformants, WT and mutants, were studied at a 100-mL shake flask culture scale. Biomass and protein production of selected

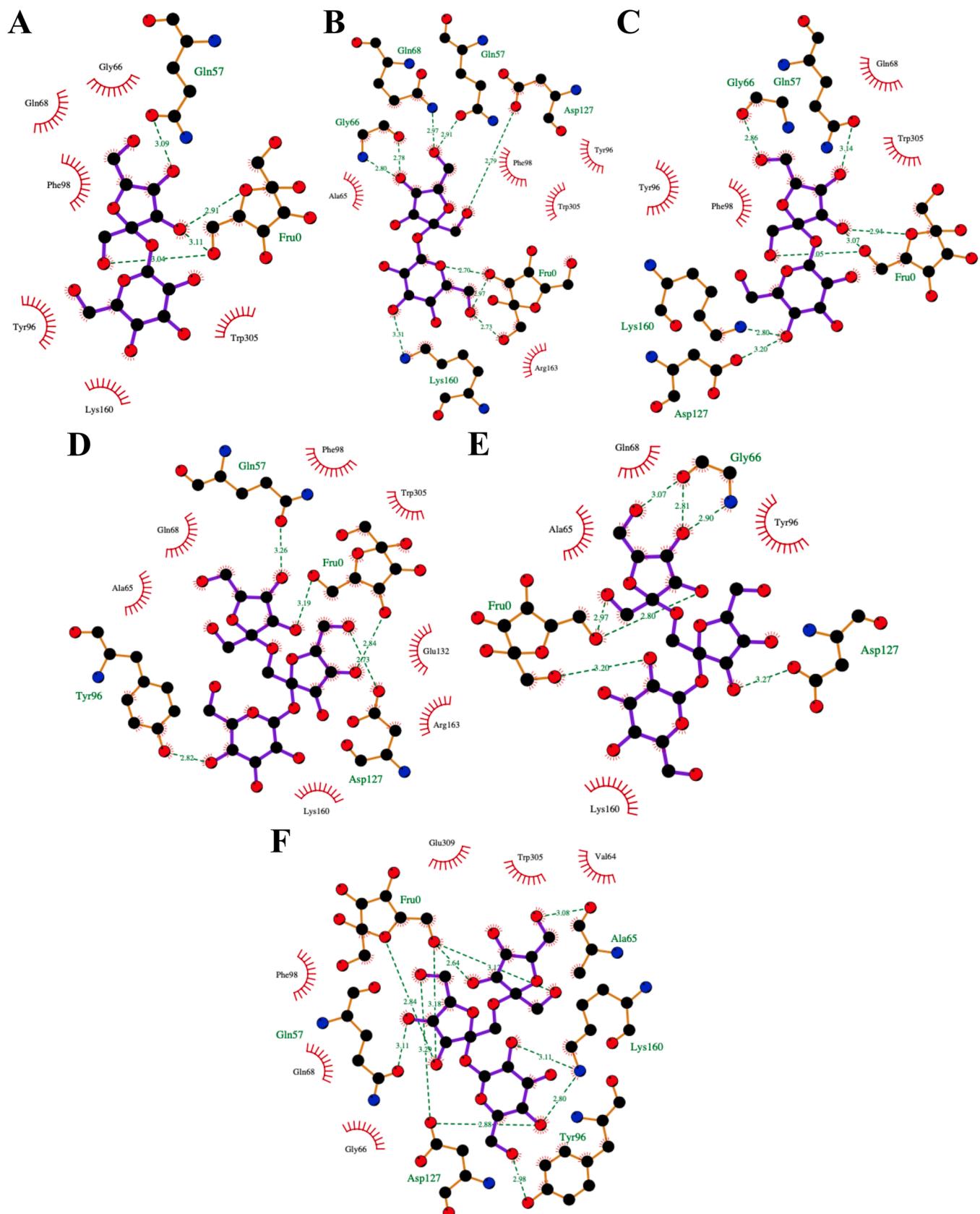


Fig. 5. Protein-substrate interactions for wild type and mutant FTases. Molecular docking of sucrose (A-C) and GF₂ (D-F) against WT (A and D), V242E (B and E), and F254H (C and D) *A. oryzae* FTases, previously docked with fructose (Fru) within the active cavity. H-bonds presented as green dotted lines, while amino acids interacting through hydrophobic interactions are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

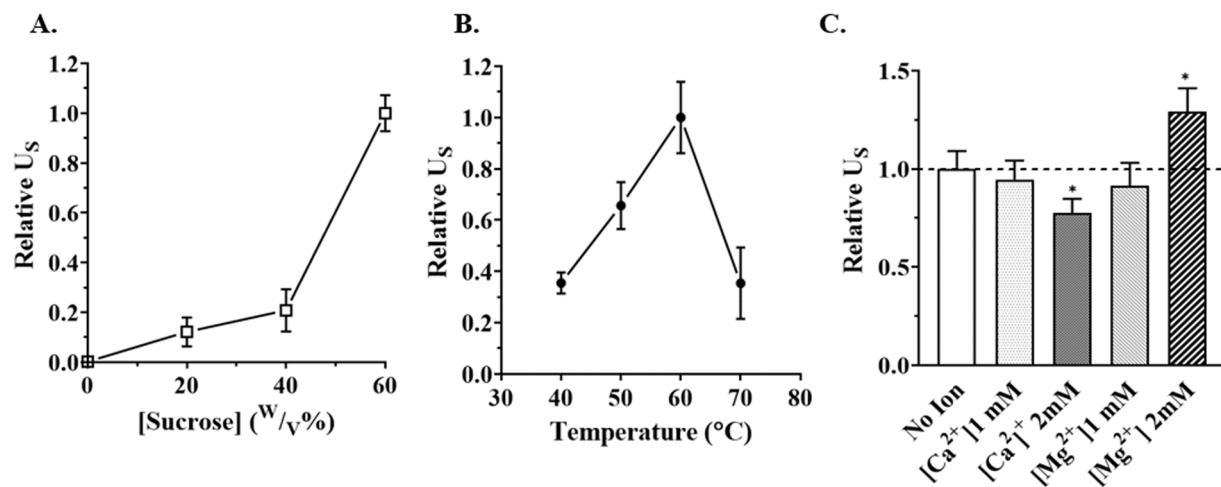


Fig. 6. Characterization of FTase-V242E. Effect of initial sucrose concentration (A), reaction temperature (B), and Ca^{2+} and Mg^{2+} ions (C) on the specific transfructosylation activity (U_S) of FTase-V242E. Data is presented as mean \pm SEM, $n = 3$. * , $p < 0.05$.

transformants was monitored for 120 h. Fig. 1 shows the time profiles of biomass and protein. Despite similar biomass profiles among selected transformants, the concentration of total secreted protein differed considerably between them. No plateau was reached for the biomass or secreted total protein within the selected induction-time window. The transformant FTase-WT produced the highest concentration of total secreted protein, followed by the transformants FTase-V242E, FTase-F254H, and FTase-R163A. To assess the quality of the secreted protein, sample aliquots taken at selected induction times were analyzed by SDS-PAGE (Fig. 2). For all FTases, one main band around ~ 85 kDa was observed, which became thicker as the induction time increased, correlating with the total protein time profile. The SDS-PAGE analysis also shows a higher molecular weight band at ~ 250 kDa that did not correspond to the FTase. Given its presence at time 0 h of protein induction and its almost invariable intensity across induction times, it was assumed to be an artifact produced during sample preparation. No other protein band was observed in the SDS gels, suggesting that FTase is the only protein secreted to the culture medium. The negative control, *P. pastoris* transformed with the empty pPIC9K vector, showed the lowest amount of protein secreted to the culture medium, with no discernable band observed via SDS-PAGE.

The secreted protein encoded by *ftase* consists of 508 amino acid residues with a molecular weight (MW) of ~ 55.7 kDa. However, the observed MW, on reduced SDS-PAGE, showed only one monomeric protein in the cell-free medium of ~ 85 kDa (Fig. 2). Glycan modifications of the FTase, have previously been observed in FTases and FFases from *Aspergillus* strains [36,37] and it is well known that N- and O-glycosylations are present in heterologous proteins produced in *P. pastoris* [38–40]. Potential N- and O-glycosylated sites were therefore assessed via NetNGlyc 1.0 [41] and NetOGlyc 4.0 [42]. Based on the amino acid sequence of Ftase-WT, 11 sites were predicted, 6 N-glycosylated and 5 O-glycosylated (Supplementary Table S1). Previous reports corroborate an increment of ~ 30 kDa in FTase MW [36,37]. To identify the reason for the MW difference between predicted and produced FTase, *ftase* was sub-cloned in the bacterial expression vector pET21-b(+) and transformed into *E. coli* to produce FTase-WT (Supplementary Methods). As *E. coli* does not naturally encode the protein glycosylation machinery [43,44], as was expected, the purified FTase-WT exhibited a MW of ~ 55 kDa (Supplementary Fig. S3), suggesting that the observed difference between predicted and experimental MWs of FTases is due to glycosylation in *K. phaffii*.

FTase transfructosylating activity

The specific transfructosylating activity (U_S) during protein

induction of secreted Ftase was monitored for WT and mutant. Each sample was used in a 4 h reaction with a sucrose solution, and the final reaction product was used for carbohydrate analysis, as summarized in Fig. 3. Although different U_S values were observed for FTase-WT, FTase-F254H and FTase-V242E, they have a similar trend (Fig. 3A), reaching the maximum activity between 6 and 12 h after induction, followed by a sharp decrease over the following 48 h, and an almost constant value until the end of the induction time. FTase-V242E and FTase-F254H reached a U_S of 325 ± 25 and 273 ± 15 $\text{U}_T \mu\text{g}^{-1}$ of protein, respectively, which was significantly ($p < 0.05$) higher than that obtained by FTase-WT (210 ± 13 $\text{U}_T \mu\text{g}^{-1}$ of protein). However, when the enzyme activity dropped after 12 h induction, U_S were similar for all FTases, ranging from 10 to 22 $\text{U}_T \mu\text{g}^{-1}$ of protein. In contrast, the R163A mutation virtually abolished transfructosylating activity, leading to a maximum U_S of 20 ± 10 $\text{U}_T \mu\text{g}^{-1}$ of protein throughout the time the enzyme was secreted.

This dramatic drop in U_S for FTase-WT, FTase-V242E and FTase-F254H was unexpected. A progressive increase in U_S over induction time was anticipated given the incremental production of total protein observed for all strains (Figs. 1B and 2B-E). It is well-known that fungal FTases exert a transfructosylating activity at high sucrose concentrations and that the hydrolytic activity is favored at low sucrose concentrations [45,46]. However, other reaction parameters are important in controlling the fructosylating and hydrolytic activities of fungal FTases. For example, the hydrolytic activity of *A. niger* FTase was marked in the function of the amount of enzyme and reaction time with no association with the ping-pong mechanism reported for FTases, leading to the rapid degradation of FOS [47]. These observations agree with previous reports in which a long reaction time can lead to the potential hydrolysis of synthesized fructans, resulting in a reduced transfructosylating activity [48,49]. These reports suggest that a high enzyme concentration due to accumulation over the induction time also results in marked hydrolysis of the fructans synthesized in the initial stage of the reaction, which would explain the reduced transfructosylating activity after 12 h of induction.

FTase-WT, FTase-V242E and FTase-F254H produced the scFOS GF₂ and GF₃ (Fig. 3B-C). The trends observed in scFOS production and specific transfructosylating activity were similar, showing the maximum concentration of GF₂ and GF₃ with the enzyme produced at 6 and 12 h of induction, respectively, followed by a sharp drop in the productivity of these scFOS with the protein secreted thereafter. Compared to FTase-WT, FTase-V242E and FTase-F254H produced larger amounts of GF₂ with the enzyme produced during most of the first 20 h of induction; however, no significant difference was observed in production of GF₃. The maximum GF₂ productivity was 86 ± 3 , 54 ± 2 , and 44 ± 4 $\mu\text{M min}^{-1}$

μg^{-1} of protein for FTase-V242E, FTase-F254H and FTase-WT, respectively (Fig. 3B), while the maximum GF₃ productivity was in the range of $3.3 \pm 0.5 \mu\text{M min}^{-1} \mu\text{g}^{-1}$ of protein for all enzymes. Conversely, the induced FTase-R254A led to minimum amounts of GF₂ ($5 \mu\text{M min}^{-1} \mu\text{g}^{-1}$ of protein) when it was produced, and GF₃ was not detected. This performance correlates with the observed transfructosylating activity of FTase-R254A. Production of GF₄ was not detected for any of the assessed FTases.

These results confirm previous *in silico* predictions where mutations V242E and F254H, which displayed a high transfructosylating activity *in vivo*, altered the hydrogen bonding between ligand and enzyme, potentially reducing the enzyme affinity for GF₃ and GF₄ without affecting the K_D for sucrose and GF₂ [25]. On the other hand, the R163A mutation, which affects the enzyme activity by practically abolishing the fructose transfer, was predicted to disrupt the number of hydrogen bonds and hydrophobic interactions, reducing the affinity with GF, GF₂, GF₃, and GF₄ [25]. R163 is part of the RDP motif [6], which contains a catalytic center where sucrose hydrolysis and fructan polymerization occur [50]. Thus, the reaction data for the mutant variants are consistent with predictions based on the FTase protein structure.

To shed light on the mechanism by which V242E and F254H mutations favor the production of GF₂, several bioinformatics analyses of wild-type and mutant FTases were performed. Structural analysis of mutant proteins compared with the WT FTase showed that V242E and F254H mutations did not induce significant overall changes since the Tm score and RMSD values were 1 and 0, respectively, for both mutations. Nevertheless, when the local effect of V242E and F254H mutations was modelled, it was predicted that these mutations generated novel H-bonds (Fig. 4). These may induce small structural changes in the vicinity of the active pocket, affecting the interaction with the substrates and scFOS synthesis.

As previously mentioned, fungal FTase cleaves the $\beta 2-\alpha 1$ linkage of sucrose, releasing glucose and transferring the fructosyl group to an acceptor molecule, such as sucrose GF₂, GF₃, etc [7]. To analyze the effect of V242E and F254H mutations on the scFOS synthesis mechanism, dockings against sucrose and GF₂ were performed for the WT and mutant FTases previously docked with fructose in the active cavity. Molecular dockings predicted that sucrose docked within the active cavity in a similar position for V242E and F254H mutations to that of the WT protein. At the same time, significant changes were observed for GF₂ docking for both mutations compared to WT (Fig. 4B-C). Protein-substrate interactions were predicted with LigPlot⁺ and are summarized in Table 1 and presented Fig. 5. In all the cases, sucrose and GF₂ generated H-bonds with the fructose present within the active cavity. Regarding the amino acid interactions, it was predicted that Q57, G66, Q68, Y96, F98, D127, K160, and W305 interacted, in most of the dockings, with sucrose and GF₂. Specifically, amino acid interactions with sucrose were highly conserved between the WT and mutant models, involving Q57, G66, Q68, Y96, Phe98, D127, K160, and W305 (80% of predicted interactions). Nevertheless, the number of H-bonds between sucrose and mutant proteins increased to 5 and 4 for the V242E and F254H models, respectively, which may correlate with the 1.7- and 2.0-fold increase in the K_a for V242E and F254H mutations, respectively (Fig. 4C). In contrast, docking with GF₂ showed that only 5 residues (41% of predicted interactions) were highly conserved between the evaluated models (i.e., A65, Q68, Tyr96, D127, and K160), suggesting a high variability in the interactions with this FOS. Specifically, a reduction in the number of interactions with GF₂ was observed with V242E, whereas F254H seems to induce novel interactions with this FOS. In addition, the number and amino acids involved in H-bonds were also affected by both V242E and F254H mutations. These changes in the type and number of interactions may correlate with the lower K_a predicted for GF₂ with V242E and F254H mutations, 0.2- and 0.5-fold, respectively, compared to that of the wild-type enzyme (Fig. 4C). As reported for *A. japonicus* FTase, binding of the acceptor substrate (sucrose, GF₂, GF₃, or GF₄) is mediated by the interaction with negatively charged and

hydrophobic residues [7]. Molecular docking simulations predicted that V242E and F254H mutations favor the interaction of sucrose with both negatively charged and several hydrophobic amino acids, compared with those predicted with the WT enzyme (Table 1). Taken together, these analyses suggest that V242E and F254H induce structural changes that may increase the binding affinity of sucrose, as well as a better interaction profile favoring synthesis of GF₂.

Partial characterization of FTase-V242E

Among the different FTase mutations, FTase-V242E showed the highest transfructosylating activity and the highest production of GF₂. The *K. phaffii* transformant with pPIC9K-FTase-V242E was therefore selected for further characterization of the mutated FTase. The effects were assessed of initial sucrose concentration, reaction temperature, and the presence of Ca²⁺ and Mg²⁺ on transfructosylating activity (Fig. 6). For the selected sucrose-concentration range, the relative U_S showed a monotonic increment (Fig. 6A), obtaining the highest activity at 60%^{W/V} of sucrose. Conversely, the relative U_S exhibited a progressive increment with increased reaction temperature, reaching its maximum at 60 °C, followed by a sharp drop at 70 °C (Fig. 6B). Divalent ions, specifically Ca²⁺ and Mg²⁺, did not affect the relative U_S at 1 mM, but at 2 mM, these ions showed a different and opposite effect on transfructosylating activity. While Ca²⁺ had an antagonistic effect, Mg²⁺ exhibited an agonist effect (Fig. 5C). In agreement with these results, the heterologous expression of FTase from *A. niger* produced in *K. phaffii* showed a maximum relative transfructosylating activity at 55 °C, and its activity was enhanced in the presence of Mg²⁺ (5 mM) and inhibited by 0.5 and 5 mM Ca²⁺ [51]. A similar effect of the reaction temperature and these divalent ions has been observed on purified FTase from *A. oryzae* S719 [37].

Conclusions

In this work, the FTase from *A. oryzae* N74 was expressed heterologously in *K. phaffii* GS115. Three independent single-point mutations were introduced to study their effect on the enzyme's transfructosylating activity. The mutations were selected based on their relevance to the catalytic site and affinity towards different ligands, such as sucrose and scFOS. The mutation R163A virtually abolished transfructosylating activity, demonstrating the relevance of the arginine residue in the catalytic domain D. On the other hand, the transfructosylating activity was improved by V242E or F254H mutations. Compared to FTase-WT, the maximum transfructosylating activity was achieved with FTase-V242E, leading to a higher production of 1-kestotriose (GF₂) without affecting the production of 1-1,1-kestotetraose (GF₃). These changes in the scFOS synthesis profile may result from structural changes in the vicinity of the active cavity, which increased the affinity of sucrose during GF₂ synthesis. The partial characterization of the heterologous FTase-V242E produced in *K. phaffii* showed similar behavior to endogenous FTases in *Aspergillus* strains and heterologous FTases produced in yeast systems. Overall, these results shed light on the mechanism of transfructosylation of the FTase from *A. oryzae* and may contribute valuable information towards the design of biotechnological processes to produce specific scFOS.

Conflict of interest

All authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.nbt.2022.02.005](https://doi.org/10.1016/j.nbt.2022.02.005).

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