Purification and biochemical characterization of an extracellular fructosyltransferase enzyme from *Aspergillus niger* sp. XOBP48: implication in fructooligosaccharide production

Jeff Ojwach¹ · Ajit Kumar¹ · Samson Mukaratirwa^{1,3} · Taurai Mutanda²

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Abstract

An extracellular fructosyltransferase (Ftase) enzyme with a molar mass of ≈ 70 kDa from a newly isolated indigenous coprophilous fungus *Aspergillus niger* sp. XOBP48 is purified to homogeneity and characterized in this study. The enzyme was purified to 4.66-fold with a total yield of 15.53% and specific activity of 1219.17 U mg⁻¹ of protein after a three-step procedure involving (NH₄)₂SO₄ fractionation, dialysis and anion exchange chromatography. Ftase showed optimum activity at pH 6.0 and temperature 50 °C. Ftase exhibited over 80% residual activity at pH range of 4.0–10.0 and $\approx 90\%$ residual activity at temperature range of 40–60 °C for 6 h. Metal ion inhibitors Hg²⁺ and Ag⁺ significantly inhibited Ftase activity at 1 mmol concentration. Ftase showed K_m , v_{max} and k_{cat} values of 79.51 mmol, 45.04 $\mu\text{mol min}^{-1}$ and 31.5 min⁻¹, respectively, with a catalytic efficiency (k_{cat}/K_m) of 396 $\mu\text{mol}^{-1} \text{min}^{-1}$ for the substrate sucrose. HPLC-RI experiments identified the end products of fructosyltransferase activity as monomeric glucose, 1-kestose (GF₂), and 1,1-kestotetraose (GF₃). This study evaluates the feasibility of using this purified extracellular Ftase for the enzymatic synthesis of biofunctional fructooligosaccharides.

Keywords Fructooligosaccharides · Fructosyltransferase · *Aspergillus niger* · 1-Kestose · 1,1-Kestotetraose · Zymography

Introduction

The biofunctional food products which are oligosaccharide in nature such as fructooligosaccharides (FOS), inulooligosaccharides (IOS), galactooligosaccharides (GOS),

xylooligosaccharides (XOS), isomaltooligosaccharides (IMOS) and mannan oligosaccharides (MOS) confer both nutritional and health benefits have received greater attention over the last decade (Pearson 2018). These foods with nutraceutical health properties are termed prebiotics and novel to study (do Prado et al. 2018). Among these prebiotics, FOS have received much attention and interest due to their excellent biological and functional properties as food additives, dietary fibers and high market value (Mutanda et al. 2014; Adhikari et al. 2018). The low-calorie FOS, besides their prebiotic effects, help in regulation of lipid metabolism, reduction of cholesterol level, inhibit the growth of harmful bacteria and improve mineral absorption in the gut (Singh et al. 2017).

FOS are oligofructose in nature containing a single glucose moiety mainly 1-kestose (GF₂), nystose (GF₃) and 1^F-fructofuranosyl nystose (GF₄), where fructosyl units (F) are bound at the β -(2 $\rightarrow$ 1) linkage of sucrose molecule (Hayashi et al. 2000; Chen et al. 2011; Perna et al. 2018; Rodríguez et al. 2011). FOS are produced by the action of enzymes fructosyl transferases (Ftases) found in some plants mainly *Helianthus tuberosus*, and *Cichorium intybus* (Apolinário et al. 2014; Koops and Jonker 1996;

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✉ Jeff Ojwach
ojwachj@gmail.com

¹ Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Private Bag X54001, Durban 4000, South Africa

² Department of Nature Conservation, Faculty of Natural Sciences, Centre for Algal Biotechnology, Mangosuthu University of Technology, P.O. Box 12363, Jacobs 4026, Durban, South Africa

³ Present Address: One Health Center for Zoonoses and Tropical Veterinary Medicine, Ross University School of Veterinary Medicine, Basseterre, West Indies, Saint Kitts and Nevis

Maicaurkaew et al. 2017; Ur Rehman et al. 2016; Vijn et al. 1997) as well as by bacterial enzymes (Picazo et al. 2019; Taştan et al. 2019; Toledo et al. 2019). Ftases act on sucrose by cleaving the β -(2 $\rightarrow$ 1) linkage, releasing glucose, and then transferring the fructosyl group to an acceptor molecule. These enzymes can produce prebiotic fructooligosaccharides FOSs that are of industrial interest. A computational study (QM/MM) for understanding the FOS synthesis process by the action of Ftase from *A. japonicus*, it was elucidated that the fructosyl ring bound in the -1 position was observed to have a 4E conformation in the oxocarbenium ion-like transition state. Based on the SCC-DFTB/MM simulations of sucrose complexes of wildtype and D191A mutant Ftase, Asp191 was shown to be responsible for the productive sugar conformation (at subsite -1) required for catalysis. A key interaction, Asp119 $\cdots$ nucleophile $\cdots$ 1-OH (substrate), was proposed to facilitate the formation of fructosyl-enzyme intermediate (Jitonnorn et al. 2018).

The Ftase reported from the closely related species *Aspergillus niger* QU10 (related to *Aspergillus niger* sp. XOBP48 reported in this study) have shown to belong Glycoside Hydrolase Family 32 with a retaining mechanism. These Ftases have shown invertase (EC 3.2.1.26); endoinulinase (EC 3.2.1.7); β -2,6-fructan 6-levanbiohydrolase (EC 3.2.1.64); endo-levanase (EC 3.2.1.65); exo-inulinase (EC 3.2.1.80); fructan β -(2,1)-fructosidase/1-exohydrolase (EC 3.2.1.153); fructan β -(2,6)-fructosidase/6-exohydrolase (EC 3.2.1.154); sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99); fructan:fructan 1-fructosyltransferase (EC 2.4.1.100); sucrose:fructan 6-fructosyltransferase (EC 2.4.1.10); fructan:fructan 6G-fructosyltransferase (EC 2.4.1.243); levan fructosyltransferase (EC 2.4.1.-); [retaining] sucrose:sucrose 6-fructosyltransferase (6-SST) (EC 2.4.1.-) and cycloinulo-oligosaccharide fructanotransferase (EC 2.4.1.-) activities in family (Cantarel et al. 2009).

However, fungal sources of the enzymes used in production of FOS have proven more appealing due to their higher yield, either through fructosyl transfer and elongation of sucrose or hydrolysis of sucrose (de Almeida et al. 2018; Dominguez et al. 2014; Fernandes et al. 2018; Kawee-ai et al. 2018; Lincoln and More 2018). Commercial production of FOS via sucrose biotransformation with transfructosylating activity using fungal enzymes has been widely reported (Sánchez et al. 2010; Zhang et al. 2017). Fungi from diverse habitats have been isolated, identified and characterized for their ability to produce industrial enzymes that can synthesize FOS. Fungi belonging to genera of *Aspergillus* (Castillo et al. 2017; Fernandes et al. 2018), *Penicillium* (Hayashi et al. 2000; Xu et al. 2015), *Aureobasidium* (Xu et al. 2015; Sangeetha et al. 2015) and *Fusarium* (Gonçalves et al. 2016) have been isolated and characterized to produce transferases and hydrolytic enzymes.

Despite extensive studies of Ftases from diverse microorganisms, it is still necessary to identify novel fungal sources of transferase and hydrolase enzymes. Therefore, the bio-prospecting for indigenous fungi producing Ftases (EC: 2.4.1.9) from various terrestrial habitats in KwaZulu-Natal province of the Republic of South Africa was undertaken. A fungus *Aspergillus niger* sp. XOBP48 was successfully isolated from axenic culture of coprophilous ascomycete and was found capable of producing Ftase. Coprophilous fungi are dung loving fungi that grow and sporulate on dung substrates (Nguyen et al. 2017; Mata et al. 2018). Further, the purification and characterization of Ftase for its use as a biocatalyst to produce products with high specific functions was undertaken. To the best of the researcher's knowledge, this is the first report on the isolation, purification and characterization of Ftases enzyme from an indigenous coprophilous fungus in South Africa.

Materials and methods

Isolation, screening and identification of coprophilous fungus producing fructosyltransferase (Ftase)

Fresh herbivorous dung samples from various terrestrial habitats in and around KwaZulu-Natal province, Republic of South Africa (GPS coordinates: 29° 39'45.5" S 30° 24'17.9" E) were collected and incubated in laboratory conditions for fungal growth and isolation. After isolation and screening of sixty-one (61) axenic fungal cultures, the strain named XOBP48 was selected for further studies, based on its performance for highest transfructosylating ability of sucrose to fructooligosaccharides (FOS). The fungus was identified morphologically and by sequencing 18S rDNA internally transcribed spacer regions (ITS 4 and ITS 5) (Ramnath et al. 2014; White et al. 1990). The culture was identified as *Aspergillus niger* assigned with the GenBank accession number MH445969 from NCBI database and name as *Aspergillus niger* XOBP48 (*AnXOBP48*). The pure axenic isolate was preserved on potato dextrose agar (PDA), Czapek dox agar (CDA) and malt extract agar (MEA) at 4 °C (Ade-makinwa et al. 2017; Xu et al. 2015).

Cultivation and preparation of extracellular enzyme extract

The pure *AnXOBP48* isolate maintained on PDA at 28 °C was inoculated in a sterilized 100 mL pre-culture fermentation medium comprising of (g L⁻¹): sucrose 30, yeast extract 10, NaNO₃ 10, KCl 0.5, K₂HPO₄ 0.5, MgSO₄·7H₂O 0.5, NH₄Cl 0.05, pH 6.5. Further, 10 mL of pre-culture medium was inoculated in fresh 100 mL of the medium in a 250 mL

flask and incubated at 28 °C for 72 h at 200 rpm. Uninoculated medium served as control and was incubated under the same condition as the culture (Sánchez et al. 2010). For the preparation of extracellular enzyme extracts, the fungal mycelia were harvested by filtration using a mutton cloth after 72 h of cultivation. The remaining cell debris was collected by using a Whatman No.1 filter paper and washed twice with 100 mmol citrate–phosphate buffer, pH 6.5. All the filtrate broths were pooled together and homogenized by centrifugation at 10,000×*g* for 15 min, and the supernatant was used as the source of extracellular crude enzyme (Nguyen et al. 2005).

Purification of Ftase from coprophilous fungal isolate and zymography

All the enzyme purification steps were carried out at 4 °C. The crude enzyme extract (100 mL) was first treated with (NH₄)₂SO₄ (30–80% saturation) with gentle agitation for 4 h. The protein pellets were harvested by centrifugation at 10,000×*g* for 20 min and were re-suspended in 50 mL citrate–phosphate buffer (Na₂HPO₄·2H₂O–citric acid, 100 mmol, pH 6.5) and dialyzed using a 10 kDa cut off dialysis tubing (SnakeSkin, Rockford, USA). The dialyzed sample was filtered using a 0.45 µm filter in order to prevent clogging of the column in further purification steps. The protein concentration and enzyme activity were measured using concentrated sample before application on the AKTA Purifier 100 system (GE Healthcare, IL, USA). For purification of enzyme, 25 mL of sample was loaded on a 5 mL HiTrap QFF anion exchange column (GE Healthcare, IL, USA) pre-equilibrated with 100 mmol Na₂HPO₄–NaH₂PO₄ buffer pH 6.5. The unbound proteins were washed from the column and the 5 mL fractions of bound proteins were eluted stepwise with a 0–3 M NaCl (prepared in citrate–phosphate buffer, 100 mmol, pH 6.5) linear gradient at a flow rate of 1 mL min^{−1}. The eluted fractions were used for protein assay and fructosyltransferase activity assays (Mutanda et al. 2008). At every purification step, for the protein bands were monitored on SDS-PAGE (Laemmli 1970) and protein concentration was determined by using Bradford reagent (Bradford 1976). The zymographic analysis of the purified enzyme was done as described previously (Batista et al. 2020; Choukade and Kango 2019). Further, the purified enzyme was characterized for optimum pH and temperature, pH and temperature stability. The effect of metal ions on the enzyme activity was also studied (Fernandes et al. 2018; Oyediji et al. 2017; Sharma and Gill 2007).

Fructosyltransferase (Ftase) assay

The Ftase activity was determined by DNS method with some modifications as described previously. Briefly, 1 mL

enzyme extract was incubated with 2 mL 5% (w/v) sucrose (prepared in 100 mmol in citrate–phosphate buffer, pH 6.5) at 50 °C for 20 min. The reaction in the test tube was terminated by boiling for 10 min. The test tubes were cooled to room temperature and 2 mL DNS was added to the reaction mixture (Miller 1959; Nemukula et al. 2009). The absorbance was measured at wavelength 540 nm using Cary 60 UV–Vis spectrophotometer (Agilent, CA, USA). The reducing sugar liberated was estimated by using glucose standard curve (0–50 mmol) (Miller 1959). One unit of enzyme activity was defined as being equivalent to one µmol of reducing sugar liberated per min under the standard assay conditions (Álvaro-Benito et al. 2007; Zeng et al. 2016).

Triphenyltetrazolium chloride (TTC) plate assay

The Ftase activity of the purified protein was confirmed by performing the TTC plate assays (Xu et al. 2018). The zones of hydrolysis by the pure protein (100 µL, 200 µg) in the agar plates containing Czapek dox agar with 5% (w/v) (sucrose as sole carbon source, pH 7.0) subsequently stained with triphenyltetrazolium chloride (TTC) reagent (0.1% w/v) in 0.5 M NaOH after overnight incubation at 25 °C. In the presence of reducing sugars, the TTC is reduced to a red water-insoluble compound, triphenylformazan. The appearance of zones of hydrolysis around the well and color intensity indicated the Ftase activity of the purified protein.

Determination of optimum pH and pH stability

To determine the optimum pH for the Ftase activity, 500 µL purified enzyme (100 µg) was incubated at 50 °C for 30 min, with 2.5 mL sucrose (5% w/v) prepared in various ranges of 100 mmol buffers, pH 2–5 (sodium acetate–acetic acid), pH 6–7 (citrate–phosphate buffer), pH 8–9 (Tris–HCl), pH 10–12 (glycine–NaOH). To determine the functional stability of purified Ftase, the enzyme (100 µg) was incubated in buffers ranging from pH 3–12 for 6 h and the residual activity was determined as described above. The activity obtained at optimum pH was considered as 100% residual activity (Chen et al. 2009; Nemukula et al. 2009).

Determination of optimal temperature and temperature stability

To determine the optimum temperature for Ftase activity, 500 µL purified enzyme (100 µg) was incubated at temperature ranges between 30 and 80 °C at the optimal pH (citrate–phosphate buffer, pH 6.0, 100 mmol) with 2.5 mL sucrose (5%, w/v) for 30 min and the activity was measured as described above. The enzyme thermostability was investigated by incubating the enzyme (100 µg) at different temperatures (40–80 °C at 10 °C interval) for 6 h and assayed

at optimum conditions (pH 6 and temp 50 °C) as described above (Mutanda et al. 2009). The residual activity obtained at optimum conditions was considered as 100% residual activity (Lincoln and More 2018).

Determination of kinetic parameters

To determine the K_m and v_{max} values, the purified Ftase (100 µg) was incubated with varying sucrose concentrations (0, 5, 10, 20, 50, 100, 150, 200, 400 and 500 mmol) and the activity was determined at optimum conditions as described above. The enzyme final concentration in the reaction mixture was set as 1.43 µmol considering the molecular mass equal to 70 kDa. K_m and v_{max} values were obtained by applying Michaelis–Menten equation in Lineweaver–Burk plot and the amount of product liberated at the endpoint (Ghazi et al. 2007). The catalytic constant of the enzyme substrate reaction (k_{cat}), also referred to as the turnover number, represents the number of reactions catalysed per unit time by each active site; was determined using the equation, $k_{cat} = v_{max}/[E]t$, where v_{max} is the maximum velocity and $[E]t$, is the total enzyme concentration. Catalytic efficiency was calculated by the equation, k_{cat}/K_m .

Effects of metal ions on Ftase activity

To determine the effect of different concentrations of metal ions (1 mmol and 10 mmol of $MgSO_4$, $MgCl_2$, $NaCl$, $CaCl_2$, $AgNO_3$, $FeSO_4$, $CuSO_4$, $ZnCl_2$, $HgCl_2$ and KCl), 100 µg of purified Ftase was incubated with the metal ions for 30 min and assays were performed as described above. The enzyme activity determined without addition of any metal ion was considered as 100% residual activity (Gonçalves et al. 2016).

Determination of carbohydrate content of the reaction

To determine the carbohydrate content of the reaction mixture catalysed by the purified enzyme, 1 mL of 50% (w/v) phenol was added to the 1 mL of reaction mixture obtained as described above. Concentrated sulphuric acid (5 mL) then was rapidly added to the mixture and homogenized by vortexing (Masuko et al. 2005). The tubes were incubated in a water bath for 10 min at 25 °C for colour development and the absorbance was measured at wavelength 485 nm. The blank sample included substituting FOS end products with 100 mmol citrate–phosphate buffer, pH 6. The carbohydrate content was extrapolated from a glucose standard curve (0–5 mg mL⁻¹) (Nielsen 2010). All the experiments were carried out in triplicate.

Determination and identification of FOS end products by thin-layer chromatography (TLC) and HPLC-refractive index detector (HPLC-RI)

The end products of the purified Ftase activity on sucrose were analyzed by TLC and HPLC-RI as describes previously (Han et al. 2020; Tihomirova et al. 2016; Correia et al. 2014; Manosroi et al. 2014; Reiffová and Nemcová 2006). Briefly, syringe filtered 5 µl reaction mixture samples (FOS products) were spotted on TLC Silica gel 60 F254 (Merck, MA, USA) and then compared with standard solutions of glucose, fructose, sucrose and oligosaccharide standards with a short-chain degree of polymerization (Dp3, Dp4 and Dp5). For HPLC-RI (YL 9100 system, TechnoLab System, Dubai, UAE), Pinnacle II Amino (3 µm, 150 × 4.6 mm) column was used with a flow rate of 1.0 mL min⁻¹ and the mobile phase used was 70% acetonitrile with 30% water and 0.04% (w/v) ammonium hydroxide. The retention times of FOS produced by the reaction of the partially purified Ftase were compared with the pure standards of 1-kestose, 1,1-kestopentaose, and 1,1,1-kestotetraose (Mussatto et al. 2009).

Statistical analysis

One-way ANOVA at 95% confidence level ($\alpha=0.05$) was used to analyze generated data from all the experiments (evaluation v8, Origin pro, OriginLab, MA, USA). All the experiments were performed in triplicate and expressed as mean $\pm$ standard deviations.

Results and discussion

Isolation and identification of the strain

During the primary screening of the fungal cultures, 61 pure fungal strains were inoculated in the basal medium with sucrose as a sole carbon source. Only 30 strains were considered for secondary screening as these strains showed considerable characteristic of sucrose hydrolysis. During the second screening, cultivated in Czapek dox broth with sucrose as a sole carbon source under submerged fermentation, 21 filamentous fungal strains showed high Fructosyltransferase (Ftase) activity. Isolate from buffalo dung named as XOBP48 showed very high Ftase activity of 529.5 U mL and characterized further (Unpublished data). The 18S rDNA gene sequence submitted to NCBI nucleotide BLAST (Altschul et al. 1990) search identified the strain as *Aspergillus niger* and named as *Aspergillus niger* strain XOBP48 (AnXOBP48). The partial rDNA sequence was submitted to NCBI GeneBank was assigned accession number MH445969. A rooted phylogenetic tree (unpublished data) constructed using the neighbor-joining method based on maximum

composite likelihood of 18S rDNA for Ftase producers revealed, *Aspergillus sydowii* (accession no: AJ289046.1), *Aspergillus niger* (accession no: AB046383.1), *Penicillium oxalicum* GXU20 KP178534.1 and *Aureobasidium pullulans* NAC8 KX023301.1 were found as the close homologous to AnXOBP48.

Purification of extracellular Ftase

AnXOBP48 inoculated in liquid media containing sucrose as sole carbon under the submerged fermentation conditions resulted in the production of total 1229.43 U (12.29 U mL^{-1} and specific activity of 261.48 U mg^{-1} of protein) of extracellular crude Ftase. The culture supernatant precipitated with 30–80% at 10% interval (showed maximum activity at 70% optimum) of $(\text{NH}_4)_2\text{SO}_4$ showed 10.1 U mL^{-1} of Ftase activity. After dialysis, a considerable jump in Ftase specific activity was observed as calculated to be 439.13 U mg^{-1} of protein (Table 1). Similar results were reported by previous studies where using ethanol precipitation for the purification of an extracellular β -D-fructofuranosidase from *Fusarium graminearum*, showed a threefold increase and relatively lower total activity (Gonçalves et al. 2016). Previously, various studies have been reported for the purification of Ftase via $(\text{NH}_4)_2\text{SO}_4$ precipitation (Park et al. 2001; Wei et al. 2014). The extracellular crude enzyme lost considerable activity after initial saturation at 70%, resulting in about 6.6% yield as it proved difficult to precipitate the enzyme at a low salt concentration. A similar observation was observed during a partial purification of Ftase from *Aspergillus foetidus* where $(\text{NH}_4)_2\text{SO}_4$ precipitation was optimal at 75% salt concentration (Wang and Rakshit 2000). In a similar report, purification of Ftase from *Asparagus* yielded 5.6% protein after 75% $(\text{NH}_4)_2\text{SO}_4$ saturation (Shiomi and Izawa 1980). Furthermore, the reconstituted pellets applied to anion exchange chromatography (HiTrap QFF column) and fractions eluted with 0–3 M NaCl resulted in the removal of large contaminating proteins. The fractions showed two broad peaks for both unbound proteins (A_1 – A_{10}) and bound protein (B_3 – B_7) (Figure S1). The anion exchange fractions resulted in the increase in specific activity of $1219.17 \text{ U mg}^{-1}$ of protein with a total recovery of 15.53% (Table 1). Finally, 4.66-fold purification of the enzyme was achieved (Table 1).

There are numerous reports on the production and purification of Ftase from *Aspergillus* sp. However, the purification yield of Ftase obtained in the present study was higher compared to previous reports. A novel Ftase from *Lactobacillus reuteri* was purified with two steps involving Nickel resin chromatography and anion exchange column chromatography (Resource-Q), with 5.8 purification fold and a 7% recovery (Van Hijum et al. 2002). This outcome is corroborated by studies carried out on a partially purified Ftase (EC 2.4.1.9) from *Rhodotorula* sp. LEB-V10 in free and immobilized form.

The pooled fractions from anion exchange chromatography indicated the purification of the enzyme. A 12% denaturing SDS-PAGE showed some contamination with other proteins in cell lysate and dialyzed $(\text{NH}_4)_2\text{SO}_4$ fractions (Fig. 1a) but a single band after purification (Fig. 1b). The purified Ftase was resolved around molecular mass $\approx 70 \text{ kDa}$. The single protein band excised from the unstained gel corresponding to the Coomassie stained gel and reconstituted in 100 mmol citrate–phosphate buffer pH 6.0 showed $9.34 \pm 0.12 \text{ U}$ of Ftase activity confirming the correct location of Ftase enzyme in the SDS-PAGE. The previous studies reported the molecular weight between 35 and 120 kDa (Antosova and Polakovic 2001; Hernalsteens and Maugeri 2010) for Ftase from fungal sources. Consequently, the zymogram studies also confirmed the prominent extracellular Ftase activity of the purified protein in the gel (Fig. 2a). A similar zymography approach was used to analysis of Ftase activity of *Aspergillus tamarii* NKRC 1229 (Heyer and Wendenburg 2001), *Aspergillus tamarii* Kita (Batista et al. 2020), *Aspergillus oryzae* S719 (Han et al. 2020) and *Aspergillus tamarii* NKRC 1229 (Choukade and Kongo 2019). In *A. tamarii* UCP1279 mycelia exhibited a total 36 units of Ftase/g mycelia. The m-Ftase produced by the strain NKRC 1229 was also 75 kDa protein. The Ftase nature of the purified protein was also confirmed by TTC plate assay which showed the appearance of a red zones of hydrolysis around the well and color intensity indicated the transfructosylating activity of the purified protein (Fig. 2b).

Optimum pH and pH stability of Ftase

The partially purified Ftase showed optimum activity at pH 6.0. The relative activity declined below pH 4.0 and

Table 1 Summary of purification procedure of Ftase from An XOBP48

Purification step	Protein (U mL^{-1})	Enzyme activity (U mL^{-1})	Specific activity (U mg^{-1})	Fold purification	Yield (%)
Crude	0.047 ± 0.002	12.29 ± 0.6	261.48 ± 13	1	100
$(\text{NH}_4)_2\text{SO}_4$ (70%)	0.023 ± 0.001	10.1 ± 0.5	439.13 ± 22	1.68	48.93
HiTrap QFF	0.0073 ± 0.0	8.9 ± 0.45	1219.17 ± 61	4.66	15.53

Results represent the mean $\pm$ SD of experiment conducted in triplicate

Fig. 1 SDS-PAGE of purified Ftase. **a** A 12% SDS-PAGE showing: Pre-stained protein marker (Lane M), $\approx 200 \mu\text{g}$ total protein from crude enzyme preparation (Lane 1), $\approx 200 \mu\text{g}$ total protein from dissolved precipitates from 70% $(\text{NH}_4)_2\text{SO}_4$ saturation (Lane 2), $\approx 200 \mu\text{g}$ total protein from dialyzed fraction of 70% $(\text{NH}_4)_2\text{SO}_4$ saturation (Lane 3); **b** Pre-stained protein marker (Lane M), $\approx 200 \mu\text{g}$ total protein from concentrated pooled fractions from HiTrap QFF anion exchange chromatography (Lane 1)

Fig. 2 Zymography and TTC activity assay. **a** Zymogram showing the in-gel activity of Ftase (Lane 1) and Molecular weight marker (Lane M); **b** TTC activity assay showing the appearance of red zone indicating FOS production during purified Ftase and sucrose reaction

above pH 7.5 (Fig. 3a). The optimal pH for Ftase from *A. aculeatus* was reported to be between 5.0 and 7.0 (Ghazi et al. 2007; Huang et al. 2016) with no significant activity recorded below pH 3.5 and above 9.5. Ftases from other fungal species have been reported to have optimal pH range of 5.5–7.0 similar to the present study (Fernández et al. 2004; Magadam and Yadav 2018; Sánchez et al. 2010). The enzyme was stable between pH 4.0 and 10.0 for 6 h retaining about 98% of its residual activity (Fig. 3b). This characteristic of stability in the neutral pH range was also comparable to an extracellular Ftase from *A. oryzae*, (Sangeetha et al. 2004; Wei et al. 2014). The extracellular fructosyltransferase from *A. pullulans* showed stability between pH 4.5 and 7.0 and optimum activity was at pH 5.5 (Yun et al. 1997a, b). Interestingly, these findings differ with that of *A. phoenicis* with transfructosylating and hydrolytic activity that exhibited an optimum pH value of 8.0 (Fernández et al. 2004).

Optimum temperature and temperature stability of Ftase

Ftase showed optimum temperature at 50 °C. However, a slight decline in activity was observed above 60 °C (Fig. 4a). These results are like the previous study reporting the sucrose biotransformation to FOS by *Aspergillus* sp. N74 where maximum activity was found between 50 and 60 °C (Sánchez et al. 2008, 2010). Another fungal Ftase produced by *A. oryzae* and *A. niger* have been reported to show optimum temperature in the same range (Hidaka et al. 1988; Sangeetha et al. 2005a, b). The purified Ftase showed thermal stability over a wide range of temperature between 40 and 60 °C, retaining almost 90% of its residual activity after 6 h of incubation (Fig. 4b). Thermal stability of *A. pullulans* fungus exhibiting Ftase activity was monitored over 8 h and the biocatalyst was inactivated at temperatures above 60 °C even at high saccharide concentration (Madlová et al.

Fig. 3 Optimum pH and pH stability of purified Ftase. **a** Ftase activity in U mL⁻¹ determined by performing the enzyme assays at pH 2–12. ≈ 100 μ g total protein from pooled fractions was incubated with 5% (w/v) sucrose dissolved in different pH buffers in a total 3 mL reaction mixture and incubated as described in “Fructosyltransferase (Ftase) assay”. **b** the stability of Ftase at pH 2–12. ≈ 100 μ g total protein

from pooled fractions was incubated in pH 2–12 buffers for 6 h and the assays were performed by adding 5% (w/v) sucrose dissolved in optimum pH buffer and activity was measured at optimum pH and temperature as described above. The % residual activity was calculated as compared to the activity shown at optimum conditions. The error bars indicate the standard deviations of three replicates

Fig. 4 Optimum temperature and temperature stability of purified Ftase. **a** Ftase activity in U mL⁻¹ determined by conducting the enzyme assays at temperature from 30 to 80 °C. ≈ 100 μ g total protein from pooled fractions was incubated with 5% (w/v) sucrose dissolved in pH 6.5 buffer in a total 3 mL reaction mixture and incubated at temperature from 30 to 80 °C as described in “Fructosyltransferase (Ftase) assay”. **b** the stability of Ftase at temperature 40–80 °C at 10 °C interval. ≈ 200 μ g total protein from pooled fractions was incu-

bated at temperature 40–80 °C at 10 °C interval for 6 h in pH 6.5 buffer and cooled to room temperature after incubation period. The reaction was commenced by adding 5% (w/v) sucrose dissolved in optimum pH 6.5 buffer and optimum temperature 50 °C as described above. The % residual activity was calculated as compared to the activity shown at optimum conditions. The error bars indicate the standard deviations of three replicates

2000). These results corroborated with those obtained in the current study. Ftase with special characteristics such as high thermal and pH stability and are desirable for biotechnological applications as an average optimum temperature of 50 °C is reported. Nevertheless, a temperature of 60 °C has been recommended as effective for industrial application (Mutanda et al. 2009; Nigam 2013). The high optimum temperature and temperature stability are advantageous for industrial applications and can be used at large-scale FOS

production as they reduce the risk of microbial contamination, as well as lower the viscosity and increase substrate solubility (Virgen-Ortíz et al. 2016).

Effects of metal ions

The effect of different metal ions at concentrations of 1 mmol and 10 mmol on Ftase activity was determined. There was enhancement of Ftase activity by Ca²⁺, Mg²⁺,

K⁺ at 10 mmol whereas no significant effect on Ftase activity by Fe²⁺ (Table 2) was observed. The effect of other metal ions such as Na⁺, Zn²⁺, K⁺ and Cu²⁺ at lower concentrations was associated with stabilization of the enzyme. Metal ions such as Ca²⁺, Fe²⁺ and Mg²⁺ were used to optimize transfructosylating activity (Katapodis et al. 2003). These metal ions stimulated transferase and hydrolytic activity. There was a complete inhibition of Ftase activity by Hg²⁺ and Ag⁺ exhibiting a very low activity of 0.04 U mL⁻¹ and 0.53 U mL⁻¹, respectively, was recorded. Similar results were recorded for Ftase from *A. niger* (Guo et al. 2016). These heavy metals are reported to interact with sulfhydryl groups in Ftases and invertases, thereby leading to conformational changes and enzyme precipitation (Lincoln and More 2018). In other reports, Cu²⁺, Mn²⁺, Zn²⁺ and MgSO₄ enhanced enzyme activity of *A. niger* ATCC 20611 Ftase (Hirayama et al. 1989).

Enzyme reaction kinetics

The Lineweaver Burk plot (Figure S2) showed the K_m value of 79.51 mmol when sucrose was used as the substrate. The v_{max} value was found to be 45.04 $\mu\text{mol min}^{-1}$, while k_{cat} was 31.49 min^{-1} (considering the molecular mass 70 kDa for Ftase). The catalytic efficiency of the enzyme was found to be 396 $\mu\text{mol}^{-1} \text{min}^{-1}$. A fungal species of *A. aculeatus* M105 produced an extracellular Ftase with higher K_m and v_{max} and k_{cat} values of 2267 mmol, 1347 $\mu\text{mol min}^{-1}$ and 1550.2 s^{-1} , respectively, (Huang et al. 2016) as compared to Ftase reported in this study. Ftase from *F. graminearum* exhibited a K_m value of 31.6 mmol (Gonçalves et al.

2016). Other studies reported K_m of 35.67 mmol and v_{max} 3.98 $\mu\text{mol min}^{-1}$ (L'Hocine et al. 2000). Interestingly, the K_m values of an Ftase enzyme from *Rhodotorula* sp. using sucrose as a substrate, the enzyme displayed a rather sigmoid behavior showing a v_{max} value of 2.36 $\mu\text{mol min}^{-1}$ (Hernalsteens and Maugeri 2008).

Carbohydrate content analysis of the reaction product

The product of the purified Ftase and sucrose reaction was found to contain about 27.27% (w/w) of total sugar content. The carbohydrate content of an extracellular β -fructofuranosidase of *A. japonicus* fungus was reported to be 20% (w/w) similar to β -fructofuranosidase enzyme of *A. niger* which was 17% (Nguyen et al. 2005). Additionally, the *Aureobasidium* sp. Ffase produced 30 and 53% (by transferase and invertase activity) of sugar content, respectively (Hayashi et al. 1992), as compared to the Ftase enzyme reported in present study. Moreover, several researchers have applied this method to determine the concentration of oligosaccharides complex type carbohydrates and glycoproteins in FOS synthesis (Cuesta et al. 2003; Liu et al. 1973; Lo et al. 1970; Rao and Pattabiraman 1989; Robinson et al. 2018; Saha and Brewer 1994; Zavřel et al. 2018). This analysis was necessary to establish functional carbohydrate products produced after the sucrose biotransformation by the purified Ftase.

Detection of FOS production by TLC and HPLC-RI

The purified Ftase was used for FOS production via sucrose elongation to yield FOS and monomeric glucose. The TLC plate showed the presence and preponderance of 1-kestose (GF₂) and 1,1-kestotetraose (GF₃) along with glucose and sucrose produced at the end of the reaction (Fig. 5a) confirming the transfructosylating nature of the enzymes. HPLC-RI confirmed the end products of the enzyme substrate reaction as glucose, 1-kestose (GF₂) and 1,1-kestotetraose (GF₃) (Fig. 5b) with a retention time of 4.983 min, 8.408 min and 11.767 min, respectively. Figure S3 shows the peak for the standards and their retention time. To show the industrial and biotechnological applications of purified Ftase, it is therefore imperative to establish that the oligomers produced are identified as FOS during Ftase and sucrose enzyme reaction. A similar method of HPLC qualitative analysis was employed in fortification of fruit juices containing FOS (Renuka et al. 2009). The coprophilous fungus (strain used in the present study) showed potential to synthesize FOS by producing 1-kestose (GF₂), 1,1-kestotetraose (GF₃) and monomeric glucose. There was pronounced decrease of glucose production to GF₂ which could be attributed to inhibition from glucose accumulation. The similar results were shown

Table 2 Effect of metal ions on enzyme activity of Ftase from *AnX-OBP48*

Metal ions	Relative activity (%) ^a	
	1 mmol	10 mmol
Control ^b	100 ± 0.00	100 ± 0.001
MgSO ₄	66.37 ± 0.6	107.72 ± 0.2
MgCl ₂	57.7 ± 0.38	73.75 ± 0.26
Na ⁺	80.17 ± 0.15	94.93 ± 0.83
Ca ²⁺	94.3 ± 0.36	101.7 ± 0.74
Ag ⁺	1.54 ± 0.7	0.53 ± 1.91
Fe ²⁺	119.3 ± 0.64	83 ± 0.15
Cu ²⁺	77.21 ± 0.53	64.75 ± 1.98
Zn ²⁺	85.62 ± 0.75	68.36 ± 0.87
Hg ²⁺	1.48 ± 0.44	0.04 ± 0.18
K ⁺	74.23 ± 0.33	102.21 ± 0.54

^aThe relative activity of Ftase was determined with addition of various metal ions at optimal pH and temperature (pH 6.0, 50 °C)

^bThe relative activity of Ftase (pH 6.0, 50 °C) without addition of metal ion

Fig. 5 TLC and HPLC-RI analysis of the Ftase and sucrose reaction products. **a** TLC profile showing standards of fructose (1), glucose (2), sucrose (3), 1-kestose (4), nystose (5), fructofuranosyl nystose (6) and reaction products (7); **b** The products of the reaction between

of purified Ftase and sucrose analysed by HPLC-RI. The retention time and peak area of the FOS and glucose liberated were compared to the retention time and peak area of standards for the purpose of quantification

previously where synthesis of scFOS from *Aureobasidium pullulans* showed a pronounced decrease in the FOS concentration from GF₂ to GF₄ suggesting the enzyme acceptor site could be presumptively sizeable to accommodate up to GF₄ (Yun 1996). In addition, the production of GF₃ (nystose) has been reported to produce higher biological activity than other oligomers (Zduńczyk et al. 2005).

Conclusion

In the present study, a transferase GH32 family (EC: 2.4.1.9) enzyme from an indigenous coprophilous fungus was purified and characterized. The purified enzyme exhibited a good transfructosylating activity to catalyze sucrose to produce FOS. The enzyme was purified to 4.66-fold with a specific activity of 1219.17 U mg⁻¹ and total yield of 15.53%. The enzyme showed remarkable broad pH and temperature stability. The enzyme was not adversely affected by the presence of metal ions except for Hg²⁺ and Ag⁺ causing about 95% inhibition. In addition, Fe²⁺ and Mg²⁺ enhanced Ftase activity. Moreover, the enzyme displayed kinetic properties presenting a K_m value of 79.51 mmol, v_{max} of 45.04 $\mu\text{mol min}^{-1}$ and k_{cat} value of 31.49 min⁻¹. The enzyme showed a high catalytic efficiency of 396 $\mu\text{mol}^{-1} \text{min}^{-1}$, with a relatively high carbohydrate content of 27.27%. Consequently, from the present study, the enzyme demonstration of high affinity to the substrate, high pH and thermal stability could be advantageous to industrial processes. Additionally, this study offers a different source (coprophilous

fungus) of Ftase producing fungus which showed the FOS production via sucrose biotransformation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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