Supporting Information

Characterization of a Novel α-L-fucosidase from *Truepera sp.* for efficient transfucosylation and 2'-fucosyllactose biosynthesis

Hamed I. Hamouda [a,b], Mohamed H. El-Sayed [c], Hussein N. Nassar [b,d,e], Nour Sh. El-Gendy [b,d], Samah Baleegh Meawad [f], Wenxia Wang [a], Tang Li [a,*], and Heng Yin [a,*]

[a] Dalian Engineering Research Center for Carbohydrate Agricultural Preparations, Dalian Technology Innovation Center for Green Agriculture, Liaoning Provincial Key Laboratory of Carbohydrates, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

[b] Processes Development Department, Egyptian Petroleum Research Institute (EPRI), Nasr City 11727, Cairo, Egypt

[c] Department of Biological Sciences, College of Science, Northern Border University, Arar 73213, Saudi Arabia

[d] Center of Excellence, October University for Modern Sciences and Arts (MSA), 6th of October City 12566, Giza, Egypt

[e] Faculty of Physical Therapy, October University for Modern Sciences and Arts (MSA), 6th of October City 12566, Giza, Egypt

[f] Forensic medicine and clinical toxicology department, Faculty of medicine for girls, Al-Azhar University, Nasr City 11754, Cairo, Egypt

[*] **Corresponding authors**

H. Yin, E-mail: yinheng@dicp.ac.cn Tel.: +86-532-84379060

T. Li, E-mail: tangli@dicp.ac.cn Tel.: +86-532-84379060

1. Material and Methods

1.1 Cloning, expression, and purification of the True-Fuc

The encoded gene of True-Fuc was synthesized by BGI (Beijing, China), and the code was optimized according to the *E. coli* expression system. The synthesized gene was inserted into pGEX6p-1 through double digestion using *Eco*RI/*Xho*I and transformed into *E. coli* BL21(DE3) competent cells. *E. coli* transformants carrying the recombinant plasmid pGEX6p-1-True-Fuc were grown overnight at 37°C in Luria–Bertani (LB) medium containing 50 μg/mL kanamycin. A 5-mL aliquot of this culture was transferred to 0.5 L of LB medium supplemented with kanamycin and incubated at 37°C with shaking at 180 rpm until the optical density at 600 nm (OD_{600}) reached 0.6–0.9. Protein expression was induced by adding 400 μL of 500 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG), followed by incubation at 20°C for 16 h. Cells were harvested by centrifugation at 10,000×g for 5 min at 4°C and resuspended in 25 mL of washing buffer (buffer A) (300 mM NaCl, 50 mM Tris-HCl, pH 8.0). Following sonication on ice to lyse the resuspended cells, the lysate was centrifuged at 12,000×g for 50 min at 4°C. The recombinant True-Fuc was purified using Glutathione Sepharose 4B beads (GE Healthcare). The clarified lysate was incubated with the beads at 4°C for 3 h, followed by thorough washing with buffer A at a flow rate of 1 mL/min to remove non-specifically bound components. The GST tag was cleaved on-column using PreScission Thrombin in buffer A at 4°C for 3 h. The de-tagged True-Fuc was collected, concentrated using ultrafiltration with a 30 kDa Millipore filter, and stored at -80°C. Purity and molecular mass were assessed by SDS-PAGE, and protein concentration was assayed using the Bradford assay [1].

1.2 Optimal pH and temperature for True-Fuc activity

The optimal pH for True-Fuc was determined by incubating the reaction mixtures containing 20 μL of the True-Fuc and 180 μL *p*NP-*α*-Fuc solutions using various 50 mM buffers: glycine–HCl (pH 3.0–5.0), acetic acid-sodium acetate (pH 4.0–6.0), NaH_2PO_4-Na_2HPO_4 (pH 6.0–8.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 9.0–11.0). The relative activity of the True-Fuc at the optimum reaction pH was defined as 100%. True-Fuc was incubated in these buffers at 4 °C for 12 h to assess pH stability, and their residual activities were evaluated.

Analogously, the optimal temperature for True-Fuc was evaluated by incubating the reaction mixtures containing 20 μL of the purified enzyme and 180 μL fucoidan solutions across a range of 20 to 60 °C at pH 8.0 for 5 min. After incubation at temperatures ranging from 20 to 60 °C, residual enzyme activity was measured to evaluate thermostability. Additionally, thermal inactivation was studied by incubating the enzymes at 20, 30, 40, 50, and 60 °C at pH 8.0 for up to 72 h. Enzyme samples were taken at various time points, and their residual activities were assessed by measuring the change in absorbance at 405 nm.

1.3 Effect of metal ions on True-Fuc

The impact of metal ions and various chemicals on the activities of True-Fuc was assessed, including Na^{+} ($NaCl$), K^{+} (KCl), Ca^{2+} ($CaCl_2$), Ba^{2+} ($BaCl_2$), Co^{2+} ($CoSO_4$), Cu^{2+} ($CuSO_4$), Mn^{2+} ($MnCl_2$), Mg^{2+} ($MgCl_2$), Ni^{2+} ($NiSO_4$), Zn^{2+} ($ZnCl_2$), Fe^{3+} ($FeCl_3$), sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA). True-Fuc was pre-incubated with these chemical reagents (at concentrations of 1 and 10 mM) at 4°C and pH 8 for 60 min. The remaining enzymatic activities were then evaluated using the previously mentioned protocol in the material and methods section, with a control assay performed without any metal ions or chemicals.

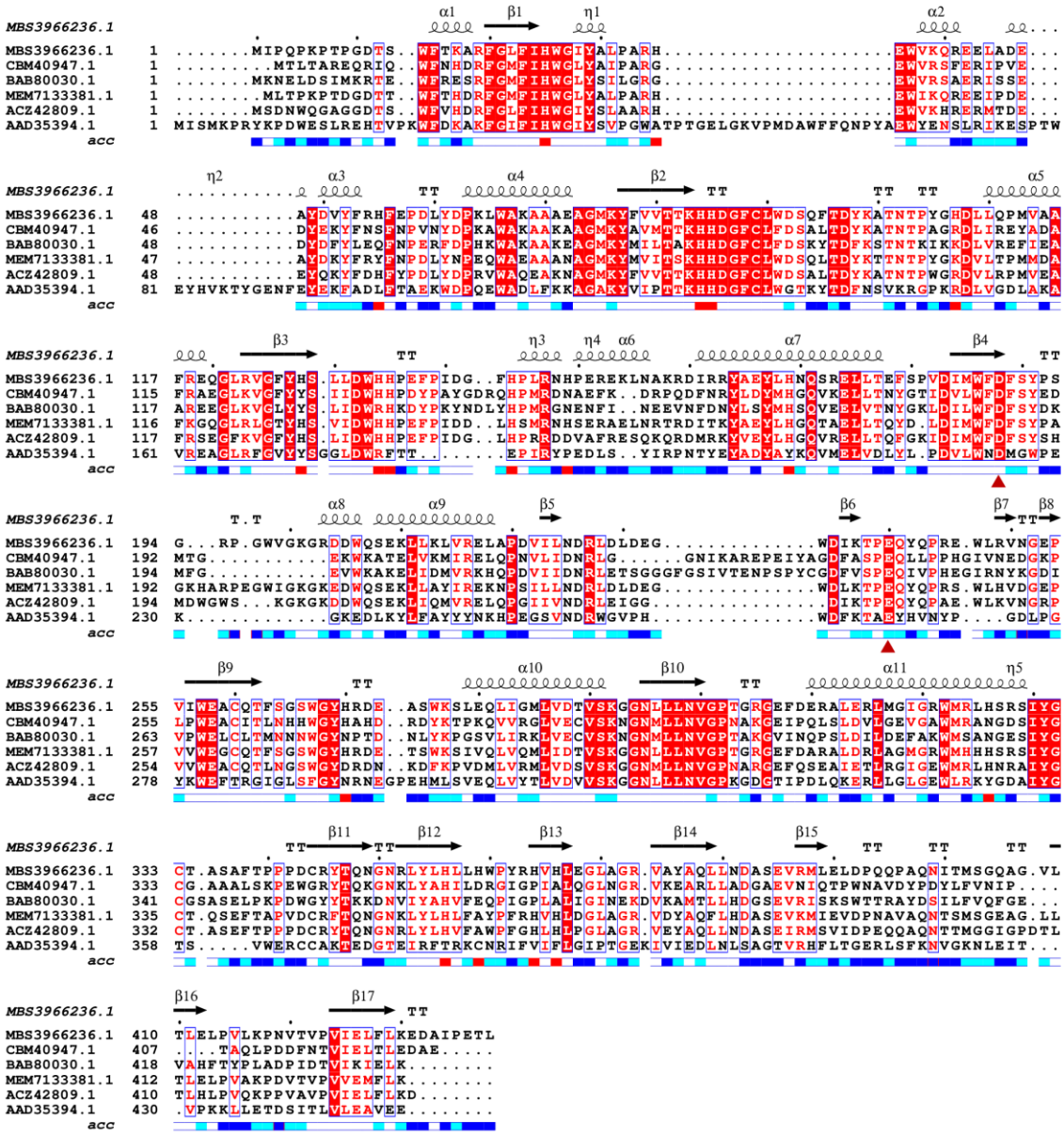

Figure S1. Amino acid sequence alignment of *α*-L-fucosidase True-Fuc from *Truepera* sp. and its homologous GH29 enzymes. The amino acid sequences compared are as follows: *α*-L-fucosidase aLfuk1 from *P. thiaminolyticus* CCM 3599 (CBM40947.1), *α*-L-fucosidase CPE0324 from *C. perfringens* str. 13 (BAB80030.1), *α*-L-fucosidase True-Fuc from *Truepera sp.* (MBS3966236.1), *α*-L-fucosidase from *Chloroflexota bacterium* (MEM7133381.1), *α*-L-fucosidase Tter_1903 from *T. terrenum* ATCC BAA-798 (ACZ42809.1), and *α*-L-fucosidase Tmari_0304 from *T. maritima* MSB8 (AAD35394.1). Red triangles indicate residues involved in catalysis.

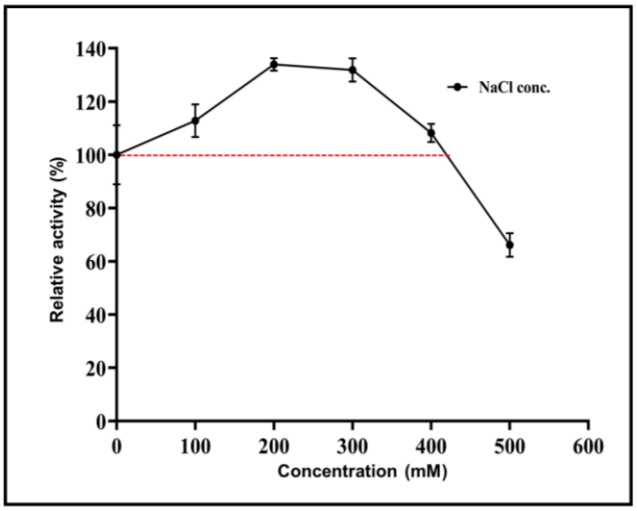

Figure S2. Influence of sodium chloride on enzyme stability and activity.

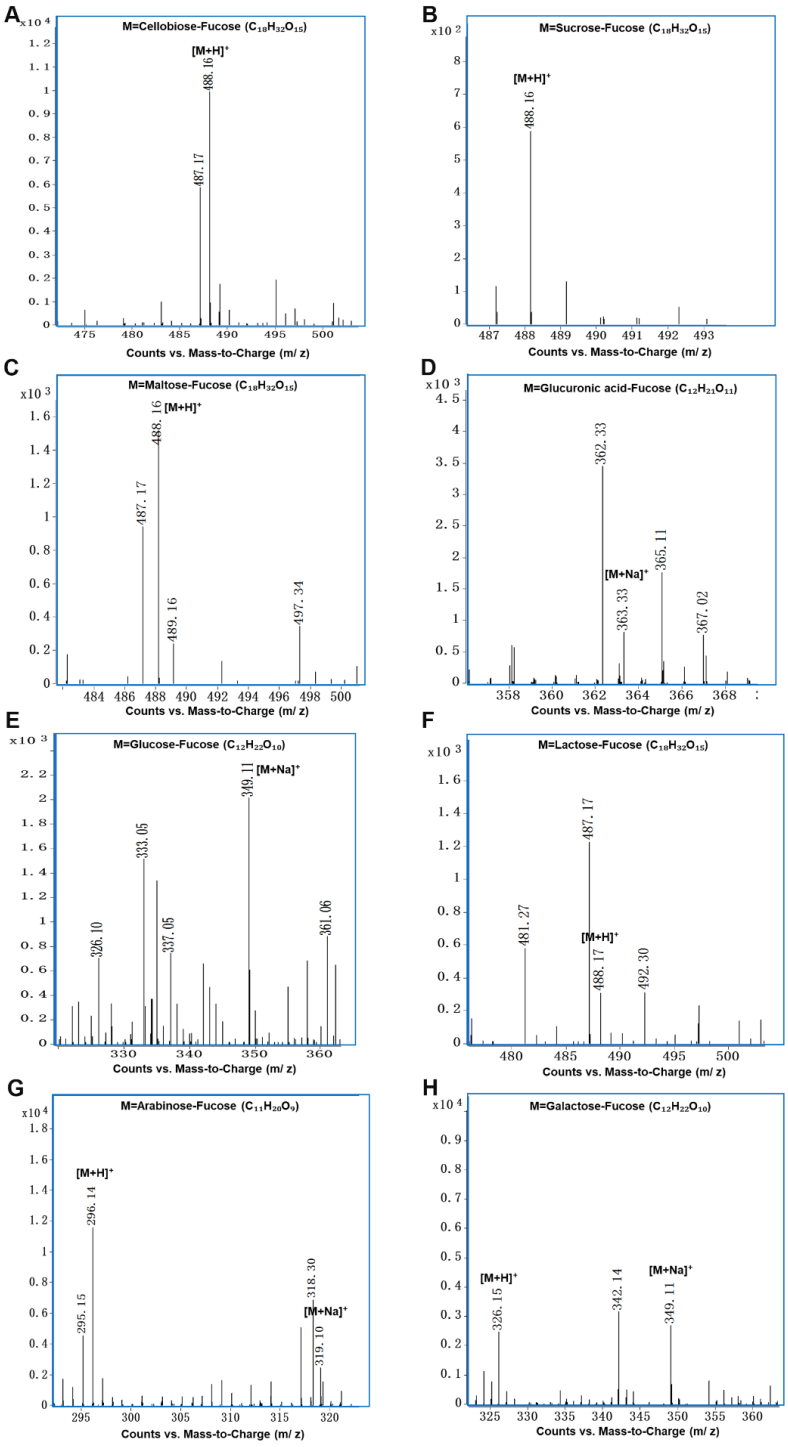

Figure S3. ESI-MS spectra of transfucosylated products generated by True-Fuc using different acceptor sugars: cellobiose (A), sucrose (B), maltose (C), D-glucuronic acid (D), glucose (E), lactose (F), arabinose (G), and galactose (H).

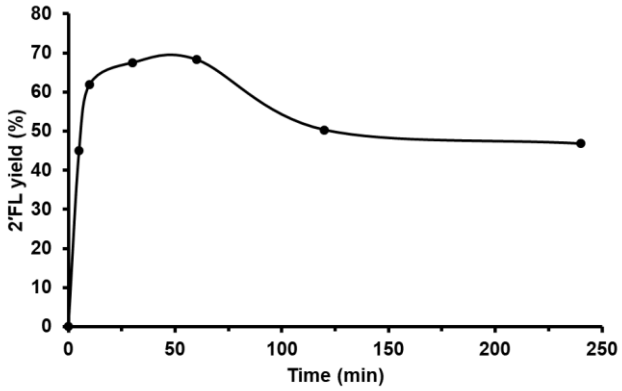

Figure S4. Time-course analysis of 2′FL production via transfucosylation catalyzed by True-Fuc. Product yields are presented as a percentage relative to the initial donor concentration (20 mM *p*NP-*α*-Fuc).

Table S1. Summary of the purification procedures of the recombinant True-Fuc.

	Volume (mL)	Total protein (mg)	Total activity (U)	Recovery yield (%)	Specific activity (U/mg)	Purification fold
Crude extracts	25	21.1	858.77	100.00	40.7±1.4	1.00
GST column	0.5	4.86	412.13	47.9	84.8±0.8	2.08

Table S2. Comparative analysis of True-Fuc and previously characterized GH29 α-L-fucosidases in 2′FL production.

Enzyme Name	GH Family	Yield (%)	Fucose Donor Substrate	Reference
True-Fuc	*Truepera sp.*	68.3	*p*NP-*α*-Fuc	This study
PbFuc	*Pedobacter* sp.	50	*p*NP-*α*-Fuc	[2]
PbFuc	*Pedobacter* sp.	31	XyG-oligos	[2]
Te2FT	*Thermosynechococcus elongatus*	95.5	*p*NP-*α*-Fuc	[3]
HpFucT	*Helicobacter pylori*	91.3	*p*NP-*α*-Fuc	[4]
OUC-Jdch16	*Flavobacterium algicola*	92.2	*p*NP-*α*-Fuc	[5]
FgFCO1	*Fusarium graminearum*	39	*3FL*	[6]
FgFCO1	*F. graminearum*	16	XyG-oligos (2mMFuc)	[6]
Fuc2358	*Streptococcus parasanguinis*	35	*p*NP-*α*-Fuc	[7]
α-L-fucosidase	*Paenibacillus* sp.	13	*p*NP-*α*-Fuc	[8]
Mfuc2	Soil metagenome	0.4	*p*NP-*α*-Fuc	[9]
Mfuc5	Soil metagenome	18	*p*NP-*α*-Fuc	[9]
Mfuc5	Soil metagenome	0.6	XyG(2mMFuc)	[6]
TfFuc1	*Tannerella forsythia*	0.7	XyG(2mMFuc)	[6]
TmαFuc	*Thermotoga maritima*	40	*p*NP-*α*-Fuc	[10]
TmαFuc	*T. maritima*	60	*p*NP-*α*-Fuc	[11]
EntFuc	*Enterococcus gallinarum* ZS1	35	*p*NP-*α*-Fuc	[12]
N423H	*Bifidobacterium bifidum*	88	*p*NP-*α*-Fuc	[13]

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