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Synthesis of Fucose-Containing Disaccharides by Glycosylhydrolases from Various Origins

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Abstract

Glycosylhydrolases of various origins were used to produce fucose-containing disaccharides with prebiotic potential using different donor substrates and L-fucose as the acceptor substrate. Eight different disaccharides were synthesized as follows: three β-D-galactosyl-L-fucosides with glycosidase CloneZyme Gly-001-02 using D-lactose as a donor substrate, two with a structure similar to prebiotics; one β-D-galactosyl-L-fucose with β-D-galactosidase from *Aspergillus oryzae* using D-lactose as a substrate donor; and four α-D-glucosyl-L-fucosides with α-D-glucosidase from *Saccharomyces cerevisiae* using D-maltose as a donor substrate. All disaccharides were purified and hydrolyzed. In all cases, an L-fucose moiety was present, and it was confirmed for β-D-galactosyl-L-fucose by mass spectrometry. High concentrations of L-fucose as the acceptor substrate enhanced the synthesis of the oligosaccharides in all cases. The three enzymes were able to synthesize fucose-containing disaccharides when L-fucose was used as the acceptor substrate, and the highest yield was 20% using β-D-galactosidase from *Aspergillus oryzae.*

Keywords Glycosylhydrolases · Fucose-containing disaccharides · Disaccharides synthesis · Transglycosylation

Introduction

The synthesis of oligosaccharides is currently receiving a great deal of attention because of the important roles of oligosaccharides in many biological processes. There are two

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groups of enzymes used in the synthesis of oligosaccharides: glycosyltransferases and glycosylhydrolases. Glycosyltransferases exhibit excellent regioselectivity, but they are limited by their instability and availability as well as the need for expensive substrates, such as nucleotide sugars. In contrast, glycosylhydrolases are generally more available and less expensive than glycosyltransferases and do not require expensive sugar nucleotide donors substrates [1, 2]. In particular, several researchers have reported the synthesis of oligosaccharides using galactosidase. Giacomini et al. [3] developed the synthesis of galactosyl-xylose using β-D-galactosidase from *Aspergillus oryzae*. Miyasato and Ajisaka [4] investigated the regioselective transglycosylation catalyzed by β-1-3-D-galactosidase using D-mannose as the acceptor substrate. Cruz-Guerrero et al. [5] reported the synthesis of galacto-oligosaccharides using a hyperthermophilic glycosidase in an organic solvent. The synthesis of galacto-oligosaccharides at very high L-lactose concentrations with immobilized β-D-galactosidase from *Aspergillus oryzae* has also been reported [6]. Lu et al. [7] reported the synthesis of galactosyl-sucralose by β-D-galactosidase from *Lactobacillus bulgaricus*. Sandoval et al. [1] used recombinant β-D-galactosidase from *Thermus thermophilus* HB27 for the synthesis of galactosyl-β-(1-4)-*N*-acetyl-D-glucosamine. Rodríguez-Díaz et al. [8] employed the recombinant fucosidases AlfB and AlfC from *Lactobacillus casei* BL23 to synthesize fucose-containing disaccharides. AlfB allowed the formation of α-1,3 links with a yield of 23%, while AlfC allowed the formation of α-1,6 links with a yield of 56%.

Currently, there is a growing interest in the availability of human milk oligosaccharides, especially fucosyl-oligosaccharides, to protect newborns from enteric pathogens during early development. Specifically, 2′-fucosyllactose is a major component of human milk oligosaccharides. Ruiz-Palacios et al. [9] have shown that fucosyl-oligosaccharides found in human milk inhibit the colonization of human intestinal mucosal cells by *Campylobacter jejuni*, which is a well-known enteropathogen. Despite their involvement in critical biological roles, fucosyl-oligosaccharides remain relatively inaccessible to scientific and pharmaceutical research due to the difficulties that are associated with their synthesis. Substantial advances in chemical oligosaccharide synthesis have been achieved [10]. Nonetheless, synthesis on a cost-effective scale remains a major obstacle. For instance, commercially available fucosyl-lactose is currently extracted from human milk; the use of this source is complicated by the possibility of contamination by human pathogens [11].

Therefore, the aim of this work was to study the synthesis of fucose-containing disaccharides with potential prebiotic and anti-infective properties using glycosylhydrolases from various origins and L-fucose as an acceptor substrate.

Materials and Methods

Materials

D-Lactose, D-maltose, D-galactose, D-glucose, and L-fucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). The enzymes used were β-D-galactosidase from *Aspergillus oryzae* and α-D-glucosidase from *Saccharomyces cerevisiae* (both from Sigma-Aldrich) and glycosidase CloneZyme Gly-001-02 were obtained from Diversa (San Diego, CA, USA). All other chemicals that were used were of analytical grade.

Enzymatic Synthesis of Fucose-Containing Disaccharides

The synthesis of D-galactosyl-L-fucose with β-D-galactosidase from *A. oryzae* was performed using different concentrations of L-fucose (10, 50, and 100 mg/mL) as an acceptor substrate, and D-lactose (10 mg/mL) as a donor substrate. Enzymatic transgalactosylation reaction was accomplished in a stirred vessel in a total reaction volume of 10 mL. L-Fucose and D-lactose were dissolved in 50 mM acetate buffer (pH 4.5), and 3.8 U/mL β-D-galactosidase was added. The mixture was incubated at 60 °C for 4 h, and aliquots were collected at regular intervals. The reaction was stopped by heating at 100 °C for 5 min. Carbohydrates from the mixture were analyzed as described below.

The synthesis of D-galactosyl-L-fucose by glycosidase CloneZyme Gly-001-02 was performed using different concentrations of L-fucose (1, 5, and 10 mg/mL) as an acceptor substrate and D-lactose (10 mg/mL) as a donor substrate. Enzymatic reactions were carried out in 50 mM acetate buffer (pH 5.5), and 0.4 U/mL glycosidase was added. The mixture was incubated at 90 °C for 3 h, and aliquots were collected at regular intervals. Carbohydrates from the mixture were analyzed as described below.

The synthesis of D-glucosyl-L-fucose was performed with α-D-glucosidase from *S. cerevisiae* (100 U/mL) and 10 mL of a 10 mg/mL D-maltose solution in 50 mM phosphate buffer (pH 6.8) containing different concentrations of L-fucose (10, 30, and 50 mg/mL) as an acceptor substrate. The reaction was incubated at 37 °C for 2 h and stopped by heating at 100 °C for 5 min. Aliquots were collected at regular intervals. Carbohydrates from the mixture were analyzed as described below.

Analysis of Carbohydrates

The concentrations of carbohydrates in the reaction mixture (D-lactose, D-maltose, D-galactose, D-glucose, L-fucose, and fucose-containing disaccharides) were determined by HPLC (Lab Alliance, PA, USA) using a Rezex RHM monosaccharides column 300 × 7.8 mm and 8 μm (Phenomenex, Torrance CA, USA) with a light-scattering detector (Polymer Laboratories, Amherst, MA, USA). The column was maintained at a constant temperature of 75 °C. The elution phase was distilled and degasified water (at 75 °C) at a flow rate of 0.3 mL/min. The concentrations of carbohydrates were calculated using standard curves for each carbohydrate.

The presence of isomers of the fucose-containing disaccharides was determined by HPLC using a Hypercarb® column 100 × 4.6 mm (Thermo Scientific, USA) and a light-scattering detector (Polymer Laboratories, Amherst, MA). The mobile phase used was acetonitrile-ammonia (96:4) at 60 °C with a flow rate of 1 mL/min.

Purification of Fucose-Containing Disaccharides

Every fucose-containing disaccharide was purified from reactions conducted under the previously described conditions, at the highest concentration of L-fucose and the maximum transglycosylation time. Reaction mixtures were lyophilized (Labconco Freezone 4.5, USA), and an appropriate dilution of each sample was injected into the HPLC using a Rezex RHM monosaccharide column (as described in the analysis of carbohydrates section). Relevant fractions were collected and lyophilized again and injected into the HPLC using a Hypercarb® column (as described in the analysis of carbohydrate section), thus obtaining purified disaccharides.

Composition of Synthesized Disaccharides

The purified fucose-containing disaccharides were subjected to acid hydrolysis, performed at 100 °C for 2 h using 2 M HCl solution. L-Fucose, D-galactose, and D-glucose released after hydrolysis were quantified by HPLC using a Rezex RHM monosaccharide column (as described in the analysis of carbohydrates section).

Synthesized D-galactosyl-L-fucose was also subjected to mass analysis. For this purpose, the purified disaccharide was concentrated to 1 mg/mL and analyzed by an Autoflex speed MALDI-TOF/TOF system (Bruker, MA, USA) with a 1000 Hz Smart beam II laser. 2,5-Dihydroxybenzoic acid (DHB) was used as a matrix (5 mg/100 mL in 50% ACN:H_2O), and 0.01 M NaCl was added as a cation dopant to increase signal sensitivity. The sample was spotted on a stainless-steel target plate, followed by the NaCl dopant and the matrix. The spot was dried in a vacuum prior to mass spectrometry analysis. MALDI-TOF MS via collision-induced dissociation was then performed to confirm the mass of the disaccharide. The tandem mass (MS/MS) spectrum was obtained at a 1-keV collision energy with argon gas.

Results and Discussion

Synthesis of β-D-Galactosyl-L-Fucose

The synthesis of β-D-galactosyl-L-fucose by using different concentrations of L-fucose as the acceptor substrate was studied. Figure 1 shows the synthesis by β-D-galactosidase from *A. oryzae*, and in Fig. 2, the synthesis using the glycosidase CloneZyme Gly-001-02 is shown, which has been previously reported for their ability to produce oligosaccharides from D-lactose at high temperatures [5]. It was observed that the synthesis of disaccharides was favored when the L-fucose concentration in the reaction was increased. The highest production of β-D-galactosyl-L-fucose (0.95 mg/mL) was observed with β-D-galactosidase from *A. oryzae* after reacting for 3 h, while, with glycosidase CloneZyme Gly-001-02, the production was

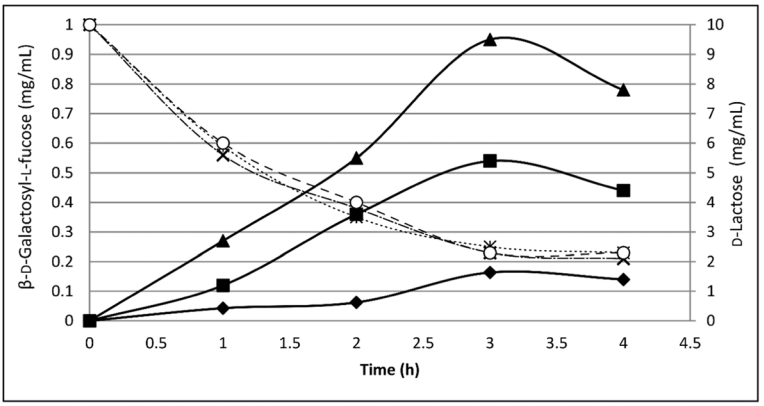

Fig. 1 Synthesis of β-D-galactosyl-L-fucose and consumption of D-lactose by β-D-galactosidase from *Aspergillus oryzae*. The reaction was performed using different concentrations of L-fucose as an acceptor substrate at 10 (◊), 50 (□), and 100 mg/mL (Δ) with 10 mg/mL D-lactose as a donor substrate and 3.8 U/mL β-D-galactosidase. Consumption of D-lactose at different concentrations of L-fucose was assayed as follows: 10 (×), 50 (*), and 100 mg/mL (O). The reaction conditions were pH 4.5 at 60 °C for 4 h

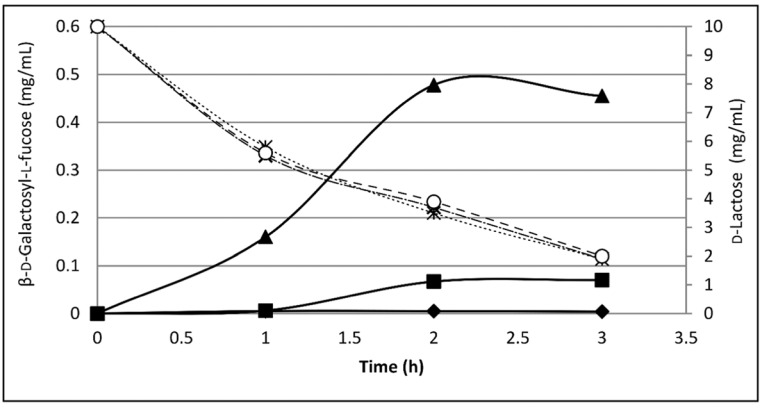

Fig. 2 Synthesis of β-D-galactosyl-L-fucose and consumption of D-lactose by glycosidase CloneZyme Gly-001-02. The reaction was performed using different concentrations of L-fucose as an acceptor substrate at 1 (◊), 5 (□), and 10 mg/mL (Δ) with 10 mg/mL D-lactose as a donor substrate and 0.4 U/mL glycosidase. Consumption of D-lactose at different concentrations of L-fucose was assayed as follows: 1 (×), 5 (*), and 10 mg/mL (O). The reaction conditions were pH 5.5 at 90 °C for 3 h

0.478 mg/mL after 2 h. However, compared with the synthesis of fucose-containing disaccharides using 10 mg/ml L-fucose, the glycosidase CloneZyme Gly-001-02 was greater, which suggests that this enzyme has a higher transglycosylation capacity. Moreover, the decrease in the concentration of synthesized β-D-galactosyl-L-fucose at the end of the reaction is the result of hydrolysis, which is the opposite of transglycosylation; this phenomenon has been previously reported for other glycosidases [2, 5]. In addition, the hydrolysis of the product could be slower compared to lactose due to the specificity of the β-galactosidases. Ajisaka and Yamamoto [12] reported transglycosylation using α-L-fucosidase from *Penicillium multicolor*, which regioselectively produced a 49% yield of fucosyl-α-(1-3)-*N*-acetyl-D-glucosamine using 4-nitrophenyl-α-L-fucopyranoside (*p*NP-Fuc) as a donor substrate and *N*-acetyl-D-glucosamine (GlcNac) as an acceptor substrate. In addition α-(1-3)-linked disaccharide was hydrolyzed more slowly than *p*NP-Fuc.

Moreover, both products were subjected to analysis by HPLC (Fig. 3a), and, to show that the synthesized compound was D-galactosyl-L-fucose, it was purified (Fig. 3b) and subjected to acid hydrolysis, obtaining fucose and galactose moieties (Fig. 3c) and thus demonstrating the composition of the synthesized disaccharide. Purification by HPLC allowed β-D-galactosyl-L-fucose to be recovered with a purity of 97.6%. Other investigations have used chromatographic techniques to purify carbohydrates. For example, Mikkelson et al. [13] used size-exclusion chromatography to isolate oligosaccharides, from an enzymatic reaction, with a purity of 86–95%. In addition, β-D-galactosyl-L-fucose purified was subjected to mass spectrometry analysis. As shown in Fig. 4a, an ion with a strong signal of $m/z = 360.9$ Da may correspond to the mass of $[\text{Fuc-Gal}+2\text{H}_2\text{O}-\text{H}]^+$. The presence of L-fucose in the molecule of the compound was confirmed in the MS/MS spectrum of 360.9 Da ion (Fig. 4b), which showed two signals at 197.274 and 360.121 Da. The difference between these signals is 162.847 Da and corresponds to the mass of a moiety of L-fucose without a hydrogen atom. This result confirms the presence of L-fucose in the disaccharide, as previously demonstrated by HLPC. Giacomini et al. [3] studied the synthesis of galactosyl-xylose in an aqueous medium using β-D-galactosidase from *A. oryzae*; they reported that increasing the acceptor substrate concentration favored the

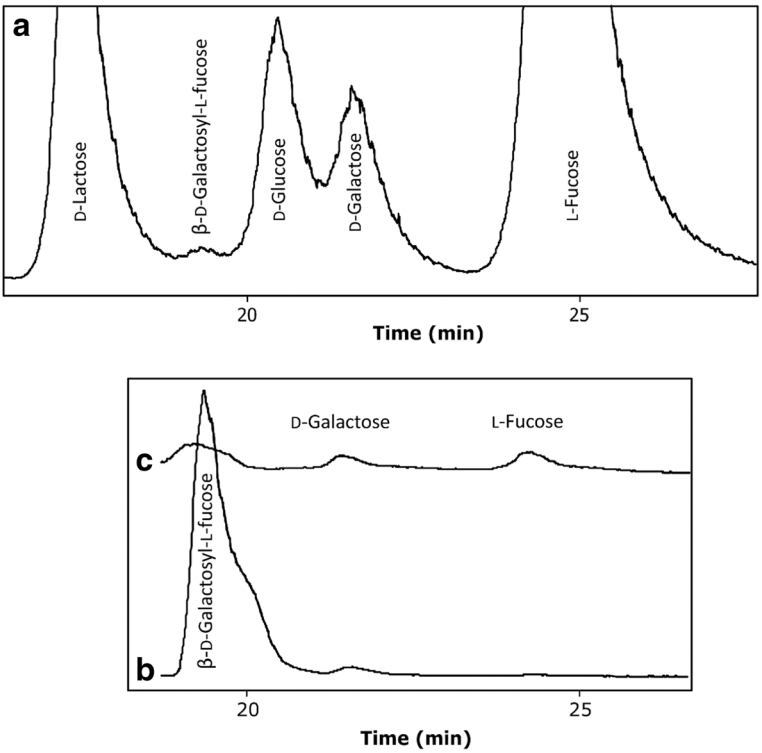

Fig. 3 Typical chromatograms from the synthesis reaction of β-D-galactosyl-L-fucose by β-D-galactosidase from *A. oryzae* or glycosidase CloneZyme Gly-001-02 (a), purified disaccharide (b) and monosaccharides obtained after acid hydrolysis of purified disaccharide (c). Acid hydrolysis was conducted with 2 M HCl, for 3 h at 90 °C. Chromatography was performed in an HC-75 (H+) column at 75 °C, using Milli-Q® water as the mobile phase with a flow rate of 0.3 mL/min

synthesis. Miyasato and Ajisaka [4] reported transgalactosylation using β-D-galactosidase from *Bacillus circulans* employing D-mannose as the acceptor substrate and *p*NP-galactose as the donor substrate. This enzyme synthesized galactosyl-mannose isomers with β 1-3, β 1-4, and β 1-6 links. Usvalampi et al. [14] used commercial β-D-galactosidase from *Bacillus circulans* to synthesize fucose-containing oligosaccharides from D-lactose and L-fucose. The main product obtained was galactosyl-β-1-3-fucose followed by minor quantities of galactosyl-β-1-4-fucose and galactosyl-β-1-2-fucose. Rodríguez-Díaz et al. [8] used two recombinant α-fucosidases (AlfB and AlfC) from *Lactobacillus casei* BL23 to synthesize fucosyl-GlcNac from *p*NP-Fuc as a donor substrate for L-fucose and GlcNac as an acceptor substrate. AlfB formed an α-1-3 link (yield of 23%) while AlfC synthesized α-1-6 link (yield of 56%). Guzmán-Rodríguez et al. [15] used α-L-fucosidase from *Thermotoga maritima* to synthesize L-fucosyl-D-lactose from *p*NP-Fuc as a donor substrate and D-lactose as an acceptor substrate. The highest yield reported was 32.5%.

Synthesis of α-D-Glucosyl-L-Fucose

Figure 5 shows the influence of L-fucose concentration on the synthesis of α-D-glucosyl-L-fucose where it was observed that the synthesis of the disaccharide is

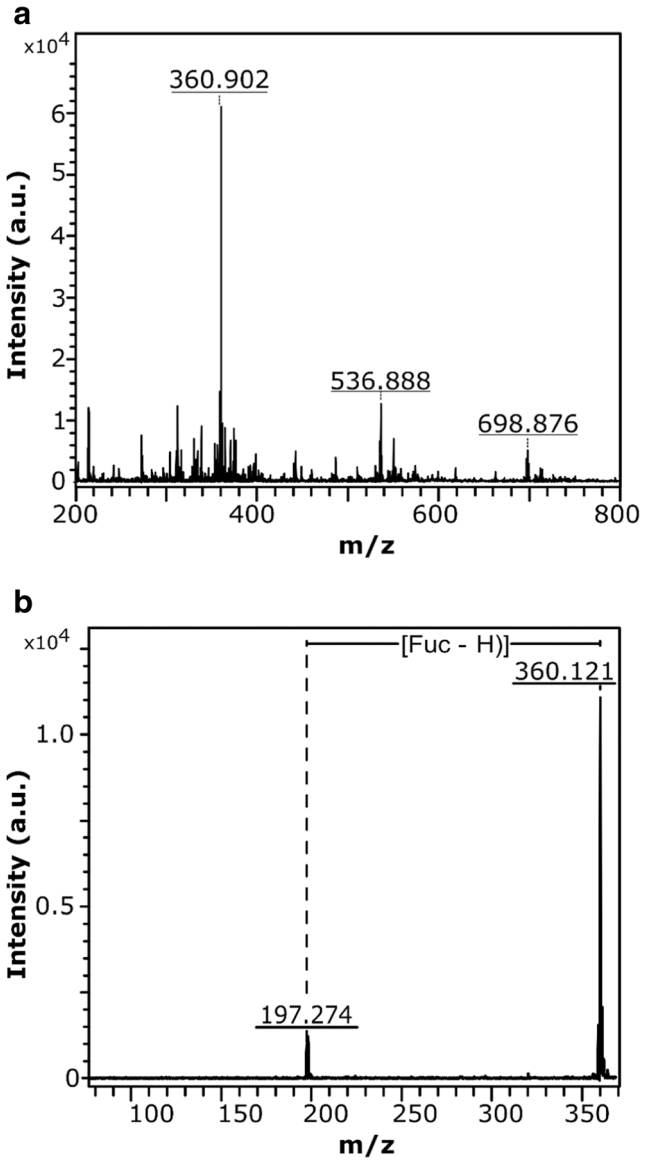

Fig. 4 MALDI-TOF MS analysis of purified β-D-galactosyl-L-fucose (**a**) MS spectrum of purified disaccharide, (**b**) MS/MS spectrum of the precursor ion (*m/z* 360.902). DHB was used as a matrix (5 mg/100 mL in 50% ACN:H_2O), and 0.01 M NaCl was added as dopant. The MS/MS spectrum was obtained at a 1 keV collision energy with argon gas

favored when the L-fucose concentration was 30 and 50 mg/mL. The highest α-D-glucosyl-L-fucose production (0.3 mg/mL) was observed after 30 min of reaction. In this case, α-D-glucosidase from *S. cerevisiae* was used to transfer one molecule of D-glucose obtained from D-maltose to an available -OH group of L-fucose. To show that the synthesized compound was α-D-glucosyl-L-fucose, it was purified and subjected to acid hydrolysis, obtaining L-fucose and D-glucose moieties, which indicate the

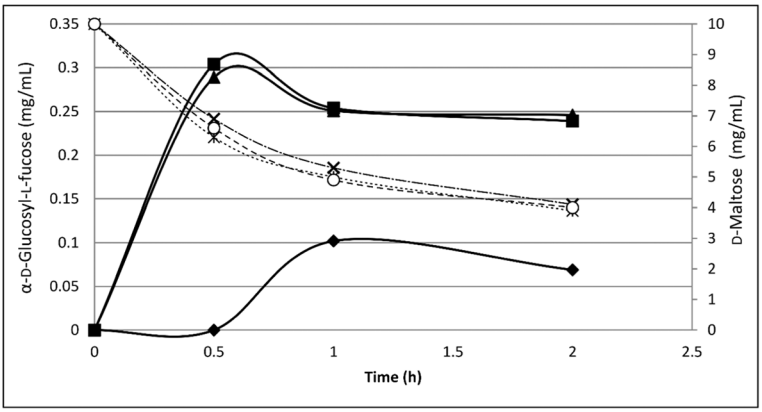

Fig. 5 Synthesis of α-D-glucosyl-L-fucose and consumption of D-maltose by α-D-glucosidase from *Saccharomyces cerevisiae*. The reaction was performed using different concentrations of L-fucose as an acceptor substrate at 10 (◊), 30 (□), and 50 mg/mL (Δ) with 10 mg/mL D-maltose as a donor substrate, and 100 U/mL α-glucosidase. Consumption of D-maltose at different concentrations of L-fucose was assayed as follows: 10 (×), 30 (*), and 50 mg/mL (O). The reaction conditions were pH 6.8 at 37 °C for 2 h

formation of the disaccharide. Fernández-Arrojo et al. [16] studied the transglycosylation activity of α-D-glucosidase from *Xanthophyllomyces dendrorhous* using D-maltose as the glucosyl donor. The enzyme synthesized oligosaccharides with α 1-2, α 1-4, and α 1-6 links, and when increasing the D-maltose concentration, maximum production of tri- and tetrasaccharides was achieved. Nimpiboon et al. [17] investigated the α-D-glucosidase activity of *Bacillus licheniformis* TH4-2 and explored its potential for use in transglycosylation reactions for the synthesis of novel prebiotic. This enzyme was able to synthesize oligosaccharides by transglycosylation from sucrose donor substrate using various saccharides as acceptor substrates.

Regioisomers of the Fucose-Containing Disaccharides

The presence of regioisomers of fucose-containing disaccharides was determined by HPLC using a Hypercarb® column to separate compounds based on their spatial configuration (Fig. 6). Regioisomers were only evaluated at the maximum production time points for each reaction. Three regioisomers of β-D-galactosyl-L-fucose were determined from the reaction with glycosidase CloneZyme Gly-001-02 (Fig. 6a). These regioisomers were subjected to the hydrolytic action of the same enzyme that is reported to hydrolyze the β 1-4 link. Regioisomer 1 was hydrolyzed, indicating that it contains a β 1-4 link. Regioisomers 2 and 3 were not hydrolyzed, which may indicate that these isomers have either a β 1-2 or β 1-3 link, making these disaccharides good candidates to be studied as potential prebiotic sugars. The human gut lumen does not express enzymes to cleave β 1-2 or β 1-3 links, while probiotic bacteria present in the intestine produce glycosidases to metabolize oligosaccharides with β 1-2 or β 1-3 links. The presence of β 1-2 or β 1-3 links on produced isomers is important, since they are the same type of links present in neutral fucosyl-oligosaccharides, suggesting that the synthesized products could exert the same biological effects [18].

Regarding the reaction with β-galactosidase from *A. oryzae*, only one regioisomer was detected after the chromatographic analysis with the Hypercarb® column

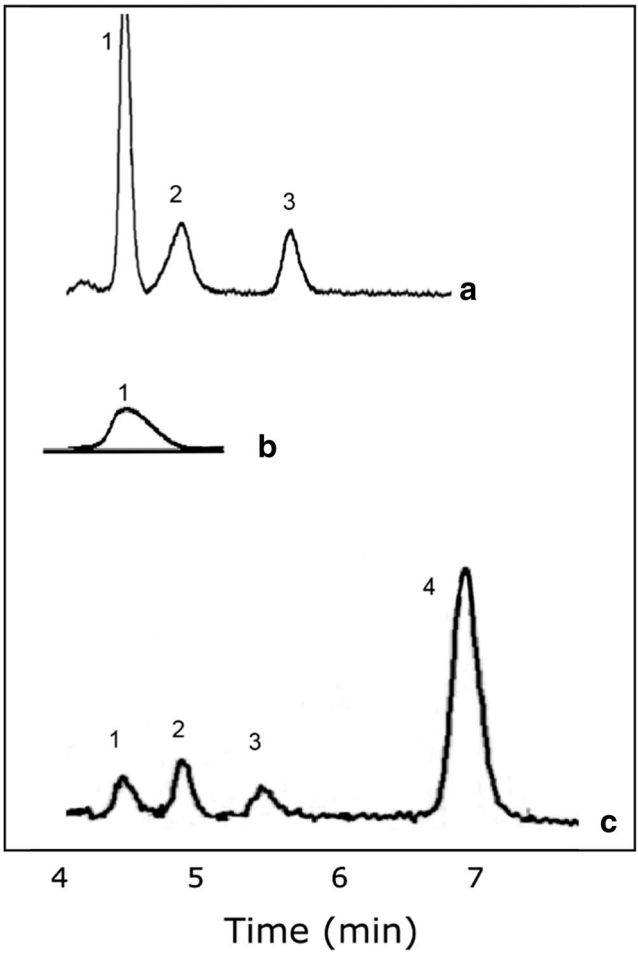

Fig. 6 Typical chromatograms of regioisomers synthesized by glycosidase CloneZyme Gly-001-02 (a), β-D-galactosidase from *A. oryzae* (b), and α-D-glucosidase from *S. cerevisiae* (c). Chromatography was performed using a Hypercarb® column

(Fig. 6b), and it was hydrolyzed by the same enzyme. This finding indicated that this regioisomer has a β 1-4 link.

The α-D-glucosyl-L-fucose disaccharide from the reaction of α-D-glucosidase from *S. cerevisiae* was separated into four fractions (Fig. 6c), which suggests the presence of at least four regioisomers. Moreover, these regioisomers were purified and subjected to α-D-glucosidase from *S. cerevisiae* because it has been previously shown that this enzyme is able to hydrolyze the α 1-4 link. It was observed that α-D-glucosidase did not hydrolyze regioisomers 1, 2, and 3, suggesting that they do not have an α 1-4 link. However, for regioisomer 4, the total hydrolysis of the disaccharide by α-D-glucosidase was observed to liberate D-glucose and L-fucose, suggesting that it has an α 1-4 link.

There are previous reports in which human milk oligosaccharides with an L-fucose moiety located in their reductive end exert biological properties. For example, 3-fucosyllactose, with an L-fucose attached to the unit of D-glucose (reductive end), inhibits the adherence of some

pathogens (enteropathogenic *Escherichia coli* and *Pseudomonas aeruginosa*) to human intestinal and respiratory cell lines [19].

The anomeric selectivity of glycosidases is absolute because it is determined by the reaction mechanism. In contrast, glycosidases often show broad selectivity for the donor substrate and particularly for the acceptor molecules. Regioselectivity in product formation varies by enzyme and is dependent on donor and acceptor substrate structures. For transglycosylation, the glycosyl donor must carry a good anomeric leaving group. Disaccharides, such as lactose, can be sufficiently activated [2]. β-Galactosidases catalyze the hydrolysis of lactose by glycosyl transfer to water. Under certain conditions, they exhibit a transglycosylation activity forming galacto-oligosaccharides (GOS). The regioselectivity of the enzymes varies leading to different linkages of GOS. The linkage between the sugar units can be β 1-2, β 1-3, β 1-4, or β 1-6 according to the enzymes used [14, 20].

Miyasato and Ajisaka [4] studied the regioselectivity of different β-galactosidases, which changed due to the structural change in the acceptor molecule. For example, in the reaction by β-1,3-galactosidase from *B. circulans* using galactosyl-β-*p*NP as the donor substrate and D-mannose as the acceptor substrate, two disaccharides, galactosyl-β-(1-3)-mannose and galactosyl-β-(1-6)-mannose, with a ratio of approximately 2:1, were obtained, while in the presence of GlcNac as the acceptor substrate only galactosyl-β-(1-3)-GlcNac was synthetized. Consequently, for transgalactosylation, there are alterations or even a complete loss of established regioselectivities.

Table 1 shows a summary of the results obtained using different enzymes. It was demonstrated that the portion of donor substrate, which participates in transglycosylation, was less than the hydrolyzed portion. This indicates that the hydrolysis reaction was favored due to the use of an aqueous medium. However, for β-D-galactosidase from *A. oryzae*, the yield was 20%. Further studies should be performed to increase the yield of the transglycosylation reaction; solvent engineering could be a good approach to improve the synthesis of fucose-containing disaccharides, as previously reported by Cruz-Guerrero et al. [5] for the synthesis of galacto-oligosaccharides.

Table 1 Synthesis of fucose-containing disaccharides using different glycosylhydrolases and employing L-fucose as acceptor substrate

Enzyme	Glycosidase (CloneZyme GLY 001-02)	β-D-Galactosidase (*Aspergillus oryzae*)	α-D-Glucosidase (*Saccharomyces cerevisiae*)
Donor substrate	D-Lactose	D-Lactose	D-Maltose
Temperature reaction (°C)	90	60	37
Time max: transglycosylation (h)	2	3	0.5
Disaccharide max: concentration (mg/mL)	0.478 ± 0.02	0.95 ± 0.02	0.3 ± 0.02
Consumption donor substrate (%)	81.6 ± 1.8	77.3 ± 1.1	61.3 ± 1.7
Donor hydrolysis (%)[a]	94.2 ± 1.3	79.1 ± 1.9	96.3 ± 1.5
Transglycosylation yield (%)[b]	5.9 ± 0.8	20.1 ± 0.3	4.9 ± 0.2
Regioisomers	1 (β 1-4) 2 (β 1-2 or β 1-3) 3 (β 1-2 or β 1-3)	1 (β 1-4)	1 (link unknown) 2 (link unknown) 3 (link unknown) 4 (α 1-4)

[a] Donor hydrolysis was calculated in base of donor substrate consumed

[b] Transglycosylation yield was calculated in base of donor substrate consumed

Conclusions

Our results proved that the synthesis of fucose-containing disaccharides using glycosylhydrolases is an alternative for the production of these compounds. The highest yield was obtained with β-D-galactosidase from *A. oryzae*. Additionally, the presence of L-fucose in the synthesized disaccharides is important because it has a composition similar to that of the oligosaccharides present in breast milk. Therefore, oligosaccharides could provide the same benefits to human health as prebiotics and anti-infective compounds in human milk.

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Compliance with Ethical Standards

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

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