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Optimized of sonication-assisted extraction of α-L-fucosidase from *Bifidobacterium spp*. for the synthesis of fucosylated oligosaccharides

Optimización de la extracción de α-L-fucosidasa de *Bifidobacterium spp*. mediante sonicación para la síntesis de oligosacáridos fucosilados

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Abstract

Fucosylated oligosaccharides structurally related to human milk oligosaccharides (HMOs) are emerging as functional ingredients that can modulate gut microbiota and support immune development. Due to their limited availability from natural sources, microbial enzymatic synthesis offers a sustainable alternative. This study aimed to optimize the sonication-assisted extraction of intracellular α-L-fucosidase from *Bifidobacterium* spp. and evaluate its application in the synthesis of fucosylated oligosaccharides. Cultivation with inulin as the sole carbon source increased α-L-fucosidase activity by 28-60% in *B. bifidum*, *B. longum*, and *B. longum subsp. infantis*. A Box-Behnken design identified optimal extraction conditions consisting of a 10 mL cell suspension, 50% amplitude, and 8 minutes of sonication, recovering approximately 35% of the whole-cell α-L-fucosidase activity. The resulting enzymatic extract catalyzed the synthesis of fucosylated di- and trisaccharides using *p*NP-fucose as donor and several acceptors, including lactose, lactulose, galactose, and fructose. The highest product concentration (61 mM) was obtained with fructose, yielding fucosyl-fructose, confirmed by MALDI-TOF/TOF and acid hydrolysis. These results demonstrate that optimized sonication conditions improved enzyme recovery within the evaluated experimental range while maintaining catalytic activity. This study highlights the potential of bifidobacterial α-L-fucosidase as a biocatalyst for the sustainable production of HMO-like oligosaccharides with possible applications in infant nutrition and health-related formulations.

Keywords: Fucosidase, Bifidobacteria, Sonication, fucosylated oligosaccharides.

Resumen

Los oligosacáridos fucosilados estructuralmente relacionados con los oligosacáridos de la leche humana (OLH) están emergieron como ingredientes funcionales capaces de modular la microbiota intestinal y favorecer el desarrollo del sistema inmunológico. Debido a su limitada disponibilidad en fuentes naturales, la síntesis enzimática microbiana representa una alternativa sostenible. El objetivo de este estudio fue optimizar la extracción asistida por sonicación de la α-L-fucosidasa intracelular de *Bifidobacterium spp*. y evaluar su aplicación en la síntesis de oligosacáridos fucosilados. El cultivo con inulina como única fuente de carbono incrementó la actividad de α-L-fucosidasa entre 28 y 60% en *B. bifidum, B. longum* y *B. longum subsp. infantis.* Un diseño Box-Behnken permitió identificar condiciones óptimas de extracción, consistentes en una suspensión celular de 10 mL, una amplitud de 50% y 8 minutos de sonicación, recuperando aproximadamente el 35 % de la actividad total de α-L-fucosidasa en células completas. El extracto enzimático catalizó la síntesis de di- y trisacáridos fucosilados usando *p*NP-fucosa como donador y lactosa, lactulosa, galactosa y fructosa como aceptores. La mayor concentración de producto (61 mM) se obtuvo con fructosa, confirmándose la formación de fucosil-fructosa por MALDI-TOF/TOF e hidrólisis ácida. Estos resultados demuestran que las condiciones optimizadas de sonicación mejoraron la recuperación enzimática dentro del intervalo experimental evaluado, manteniendo la actividad catalítica. Este estudio destaca el potencial de la α-L-fucosidasa de bifidobacterias como biocatalizador para la producción sostenible de oligosacáridos tipo OLH, con posibles aplicaciones en nutrición infantil y formulaciones orientadas a la salud.

Palabras clave: Fucosidasa, Bifidobacteria, Sonicación, oligosacáridos fucosilados.

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1 Introduction

Human milk (HM) provides both short- and long-term nutritional benefits to infants, including protection against gastrointestinal diseases and a reduced risk of obesity, diabetes, atopic dermatitis, and childhood leukemia (Karbasi *et al*., 2023; Trujillo-Cárdenas, *et al*., 2019). HM contains approximately 8% carbohydrates, of which around 2% correspond to human milk oligosaccharides (HMO), making them the third most abundant solid component after lactose and lipids (Chen *et al*., 2025; Okburan & Kızıler, 2023).

HMOs act as metabolic substrates for specific bacterial species and are considered the first prebiotics in the human diet (Cai *et al*., 2025; Walsh *et al*., 2020). Breastfed infants typically develop a gut microbiota dominated by Bifidobacterium and Bacteroidetes within the first week of life. In the following days, bifidobacteria may represent up to 80–90% of the intestinal microbiota (Bottacini *et al*., 2014).

More than 10% of the *Bifidobacterium* genome is involved in carbohydrate metabolism and encodes glycosyl hydrolases (GHs), enzymes responsible for degrading complex carbohydrates into monosaccharides, that are subsequently metabolized through the bifid shunt pathway (Arboleya *et al*., 2018; Pokusaeva *et al*., 2011).

Several GHs involved in HMO metabolism, including fucosidase, sialidase, galactosidase, hexosaminidase, and lacto-N-biosidase are produced by certain bifidobacterial species, such as *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum* (Singh, 2019). In the case of *Bifidobacterium longum* subsp. *infantis*, up to 5 genes encoding α-L-fucosidase have been identified (Sela *et al*., 2012). These hydrolases are often localized intracellularly due to the association of their genes with transport systems involved in the uptake of fucosylated oligosaccharides (Garrido *et al*., 2016). For example, in *B. longum subsp. infantis* ATCC15697, Garrido *et al*. (2016) reported the expression of several genes encoding solute-binding proteins from the F1 family (F1SBP), which are part of the ABC transporters and are associated with the import of HMOs. In addition, α-L-fucosidases from *B. longum* lack the N-terminal signal peptide required for export through bacterial secretion pathways, unlike several α-L-fucosidases from *B. bifidum*, which possess signal peptides associated with cell-surface localization or extracellular activity (Ashida *et al*., 2009). In contrast, *B. bifidum* possesses several membrane-associated glycosyl hydrolases, including multiple α-L-fucosidase (Ashida *et al*., 2009). These enzymes hydrolyze fucosylated oligosaccharides extracellularly, generating smaller carbohydrate fragments that can be transported into the cell. This strategy explains why *B. bifidum* contains fewer genes encoding oligosaccharide transporters and contributes to microbial mutualism by releasing hydrolyzed carbohydrates that can be utilized by other microorganisms in the gut microbiota (Singh, 2019).

Due to their well-documented health benefits and limited industrial availability, the synthesis of HMOs has attracted increasing interest (Enam & Mansell, 2019). In this context, the isolation and extraction of α-L-fucosidases, such as those produced by bifidobacteria, represent a promising strategy for the synthesis of fucosylated oligosaccharides with structures similar to HMOs (Sela *et al*., 2012).

Intracellular enzymes can be extracted using several approaches, including chemical methods based on detergents or solvents, enzymatic treatments, and physical methods such as osmotic shock, high pressure, homogenization, and sonication (Loan *et al*., 2024; Wang *et al*., 2020). Among these techniques, sonication, based on the application of acoustic waves in the range of 20 kHz to 1 MHz, generates cavitation, shear forces, and micro-mixing effects that disrupt the cell membrane and facilitate enzyme release (Brennan, 2006; Sanchez-Madrigal *et al*., 2017; Zhang *et al*., 2019). Sonication has been widely applied as an efficient extraction technology for recovering intracellular and bioactive compounds from biological matrices. Recent studies published in the Revista Mexicana de Ingeniería Química have also demonstrated the effectiveness of ultrasound-assisted processes for the extraction and recovery of valuable biomolecules (Loan *et al*., 2024; Marín-Cortez *et al*., 2026).

Therefore, this study aimed to obtain intracellular α-L-fucosidase extracts from *B. bifidum* UAMX, *B. longum* UAMX, and *B. longum* subsp. *infantis* ATCC 17930. In addition, the sonication-assisted extraction of α-L-fucosidase from *B. longum* UAMX was optimized using response surface methodology. The resulting enzymatic extract was subsequently applied to the synthesis of fucosylated oligosaccharides structurally related to those present in human milk. To the best of our knowledge, this is the first study reporting the optimization of sonication-assisted extraction of α-L-fucosidase from *Bifidobacterium spp*. and the subsequent use of the obtained intracellular extract for the synthesis of fucosylated oligosaccharides. This work provides a biotechnological approach for improving enzyme recovery and expands the potential use of bifidobacterial α-L-fucosidases as biocatalysts for the sustainable production of HMO-like oligosaccharides with potential applications in infant nutrition.

2 Materials and methods

2.1 Materials

Meat extract, proteose peptone, and yeast extract were purchased from Bioxon^{TM} (Mexico City, Mexico). Ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, monopotassium phosphate, dipotassium phosphate, sodium hydroxide, lactose, lactulose, galactose, and fructose were obtained from JT Baker (Estado de México, Mexico). Inulin was supplied by Imperial Suiker Unie (Dinteloord, Netherlands), and MRS broth was obtained from Difco^{TM} (Mexico City, Mexico). p-nitrophenyl-α-L-fucopyranoside (pNP-Fuc) and p-nitrophenol (pNP) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Bacterial strain and preservation

The *Bifidobacterium* strains used in this study were obtained from the strain repository of the Metropolitan Autonomous University, Xochimilco campus. The strains included *Bifidobacterium longum* UAMX, *Bifidobacterium longum subsp. infantis* ATCC 17930, and *Bifidobacterium bifidum* UAMX. Cultures were preserved in freezing medium consisting of MRS broth and glycerol (1:1 v/v) and stored at -20 °C until use.

2.2.1 Culture conditions

Bifidobacteria were reactivated by inoculating 500. μL of the thawed cell suspension into sealed serological bottles containing 10 mL of MRS broth. The carbon source in the medium was replaced with 2% (w/v) inulin. Cultures were flushed with CO_2 to maintain anaerobic conditions, and the pH was adjusted to 6.8 using 1 M NaOH. Fermentations were carried out at 37 °C for 24 h with rotary shaking at 140 rpm. Control cultures were grown in standard MRS broth under the same conditions. All experiments were performed in triplicate.

2.3 Preparation of cell suspension

After incubation, cultures were centrifuged at 5500 × *g* for 12 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 10 mL of phosphate buffer (0.1 M, pH 7), followed by a second centrifugation under the same conditions. The washed cell pellet was then resuspended in the same buffer to obtain a final concentration of 150 mg cells/mL. This preparation was designated as the cell suspension.

Cell concentration was determined spectrophotometrically at 600 nm and expressed in mg/mL using a calibration curve ranging from 1 to 12 mg/mL.

2.4 Enzyme activity assay

α-L-Fucosidase activity was determined according to the method described by Escamilla-Lozano *et al.* (2019), with minor modifications. A 3.4 mM solution of *p*NP-Fuc was prepared in phosphate buffer (0.1 M, pH 7). Whole-cell α-L-fucosidase activity was determined using intact bacterial cells suspended in phosphate buffer prior to sonication or enzyme extraction. Throughout this manuscript, the term "whole-cell α-L-fucosidase activity" refers to the activity measured in intact cells and corresponds to the enzyme activity associated with the bacterial biomass. Whole-cell α-L-fucosidase activity was measured in 1.5 mL microcentrifuge tubes (Axygen^{TM}) containing 450 μL of the *p*NP-Fuc solution and 50 μL of cell suspension. The reaction mixtures were incubated at 37 °C. Independent reaction tubes were prepared for each incubation time and processed separately. At the corresponding incubation time, the enzymatic reaction was stopped by adding 50 μL of 0.5 M NaOH to the entire reaction mixture. Samples were then centrifuged at 3900 × *g* for 10 min, and the supernatant was collected for spectrophotometric quantification of released *p*-nitrophenol at 410 nm using a UV-1800 spectrophotometer (Shimadzu). Intracellular α-L-fucosidase activity was determined under the same conditions using 400 μL of *p*NP-Fuc solution and 100 μL of intracellular enzymatic extract.

Enzyme activity was calculated using the molar extinction coefficient of *p*-nitrophenol (7.8 $mM^{-1}\,cm^{-1}$) reported by Escamilla-Lozano *et al.* (2019). One unit (U) of α-L-fucosidase activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under the assay conditions (pH 7.0, 37 °C). Specific activity was expressed as U/mg of cells. All assays were performed in triplicate.

2.5 Extraction of α-L-fucosidase by sonication

Preliminary experiments were conducted to determine appropriate sonication amplitude and time intervals prior to the response surface experimental design (data not shown). A Box-Behnken design was implemented using Statgraphics software (Version 16.1.11; StatPoint Technologies, Inc.) to optimize the extraction process. The design included three variables at three levels: sonication time (5, 8, and 11 min), amplitude (38%, 50%, and 62%), and cell suspension volume (10, 15, and 20 mL) (Table 1). The sonication procedure was adapted from the method described by Momin *et al.* (2018), with minor modifications. Cell suspensions were placed in a 25 mL beaker immersed in an ice bath and subjected to sonication using a Vibra-Cell VCX130 sonicator (Sonics & Materials, USA) equipped with a 6 mm probe. The probe tip was

positioned approximately 3 mm above the bottom of the vessel. Sonication was applied in pulsed mode with cycles of 18 s "on" and 42 s "off" per minute. After sonication, the samples were centrifuged at 5500 × *g* for 22 minutes at 4 °C. The resulting supernatant was collected and designated as the intracellular enzymatic extract. The pellet was resuspended in phosphate buffer (0.1 M, pH 7.0) to the same volume used prior to centrifugation. For comparison, whole-cell activity was also measured in non-sonicated samples. Residual activity (RA) of the intracellular enzymatic extracts was calculated as:

$$RA(\%) = [IA/CA] * 100 \quad (1)$$

where:

IA: specific activity of the intracellular enzymatic extract (U/mg)

CA: specific activity of the non-sonicated whole-cell enzyme (U/mg)

2.6 *Synthesis of* fucosylated oligosaccharides

Fucosylated oligosaccharides were synthesized using α-L-fucosidase from the intracellular enzymatic extract of *B. longum* UAMX. Transfucosylation reactions were conducted in 1.5 mL microtubes with a total volume of 300 μL. The reaction mixture consisted of the donor substrate *p*NP-Fuc (3.4 mM), the acceptor substrate (lactose, lactulose, galactose, or fructose; 14 mM), and 30 μL of intracellular enzymatic extract. Substrates were prepared in 5 mM phosphate buffer (pH 7.0), and the enzymatic extract was adjusted to a final activity of 15 U/mL. Independent reaction tubes were prepared for each incubation time and incubated at 37 °C. At the corresponding time point, the entire reaction mixture was stopped by adding 30 μL of 0.5 M NaOH.

2.7 *Quantification of fucosylated oligosaccharides*

Reaction products were analyzed by HPLC using a LabAlliance system (State College, PA, USA) equipped with an HC-75+ column (305 × 7.75 mm, Hamilton) and an evaporative light-scattering detector (300S ELSD, SofTA Corporation, Chrom Tech, Minnesota, USA). The ELSD was operated with a nitrogen flow of 62.5 psi, a drift tube temperature of 45 °C, and a spray chamber temperature of 10 °C. Elution was performed using deionized water at a flow rate of 0.3 mL/min. The column was maintained at 75 °C, and the detector at 110 °C. Fucosylated trisaccharides were quantified using 2'-fucosyllactose as standard, whereas lactose was used as standard for fucosylated disaccharides. Monosaccharides (fructose and fucose) were quantified using individual standard curves.

2.8 *Composition of Synthesized Fucosyl-fructose*

To confirm the composition of the synthesized fucosyl-fructose, the purified disaccharide was submitted to acid hydrolysis using 2 M HCl for 2 h at 100 °C. Released L-Fucose and D-fructose were quantified by HPLC employing a Rezex RHM monosaccharide column, following the conditions described in the quantification section.

In Addition, mass spectrometric analysis was conducted to verify the molecular structure. The disaccharide was concentrated to 1 mg/mL and analyzed using an Autoflex speed MALDI-TOF/TOF system (Bruker, MA, USA) with a 1000 Hz Smart beam II laser. A 2,5-dihydroxybenzoic acid (DHB) matrix (5 mg/100 mL in 50% ACN:H_2O) was employed, and 0.01 M NaCl was added as a cation dopant to enhance ionization efficiency. For sample preparation, the analyte was spotted on a stainless-steel target plate, followed sequentially by the NaCl dopant and the DHB matrix. The sample was dried under vacuum before analysis. MALDI-TOF mass spectrometry was performed using collision-induced dissociation to confirm the disaccharide´s molecular mass. Tandem mass spectrum (MS/MS) were acquired at a collision energy of 1 keV with argon as the collision gas.

2.9 *Statistical analysis*

All experiments were performed in triplicate, and results are reported as means ± standard deviation. Statistical analyses were conducted using Statgraphics software. Analysis of variance (ANOVA) followed by Duncan's multiple range test was used to assess differences among treatments at 95% confidence level ($p < 0.05$). Response surface models were fitted using NCSS 12 software (2018, NCSS, LLC, Kaysville, UT, USA).

3 Results and discussion

3.1 *Effect of carbon source on α-L-fucosidase production*

As can be seen in Fig. 1, all three *Bifidobacterium* species exhibited whole-cell α-L-fucosidase activity. Enzyme activity was higher in cells grown in inulin-supplemented media than in those cultivated with glucose. The highest specific activity was observed in *B. longum* UAMX, which was significantly different from the activities measured for *B. bifidum* UAMX and *B. longum* subsp. *infantis* ATCC 17930. No statistically significant differences were observed between the latter two strains ($p < 0.05$).

Table 1. Residual activity (RA) of the intracellular enzymatic extract obtained in Box-Behnken design.

Treatment	Time (min)	Volume (mL)	Amplitude (%)	Intracellular extract RA (%)
1	5	10	38	$6.7^{g} \pm 0.8$
2	5	10	62	$8.2^{f,g} \pm 1.4$
3	5	15	50	$9.2^{e,f,g} \pm 0.0$
4	5	20	38	$14.0^{d} \pm 0.7$
5	5	20	62	$8.8^{f,g} \pm 0.6$
6	8	10	50	$35.5^{a} \pm 0.4$
7	8	15	38	$7.6^{f,g} \pm 1.0$
8	8	15	62	$13.4^{d,e} \pm 0.1$
9	8	15	50	$10.8^{d,e,f,g} \pm 2.4$
10	8	20	50	$23.9^{b} \pm 1.4$
11	11	10	38	$13.8^{d} \pm 1.7$
12	11	10	62	$24.3^{b} \pm 0.6$
13	11	15	50	$18.9^{c} \pm 2.4$
14	11	20	38	$9.2^{e,f,g} \pm 1.3$
15	11	20	62	$11.3^{d,e,f} \pm 1.8$

Different letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$).

Previous studies have reported α- L-fucosidase activity in *B. bifidum*, *B. longum*, and *B. longum subsp. infantis* (Arboleya *et al*., 2018; Ashida *et al*., 2009; Perna *et al*., 2023; Sela *et al*., 2012). In some cases, *B. longum* exhibits higher GH activity, which may confer a competitive advantage during carbohydrate utilization (Jedrzejczak-Krzepkowska *et al*., 2011). Furthermore, α-L-fucosidase has been identified as a membrane-bound enzyme in *B. bifidum* (Curiel *et al*., 2021), whereas in *B. longum* and *B. longum subsp. infantis*, this enzyme has been reported as intracellular (Thomson *et al*., 2017).

Selective production of GH in *Bifidobacterium* species can be influenced by the carbon source used during cultivation. For example, fructose has been reported to enhance β-fructofuranosidase activity (Jedrzejczak-Krzepkowska *et al*., 2011), an enzyme necessary for hydrolyzing polysaccharides containing fructose as a monomeric unit, such as inulin. The results presented in Fig. 1 suggest that the production of other GH, including α-L-fucosidase, may also be modulated by the presence of inulin. Although inulin does not contain fucose residues, an increase in α-L-fucosidase activity was observed in all strains tested. The specific activity of α-L-fucosidase increased by 60.3% in *B. bifidum* UAMX, 40.4% in *B. longum* UAMX, and 28.6% in *B. longum subsp. infantis* ATCC 17930 in the presence of inulin. This effect may be explained by the ability of bifidobacteria to express α-L-fucosidase genes in response to variations in carbon sources. Sela *et al.* (2012) reported the enhanced expression of five α-L-fucosidase genes in *B. longum subsp. infantis* ATCC 15697 when grown with different carbon sources, including inulin. Similarly, Garrido *et al*. (2011) demonstrated that inulin could induce the biosynthesis of enzymes related to carbohydrate transport, including those involved in the uptake of fucosylated oligosaccharides.

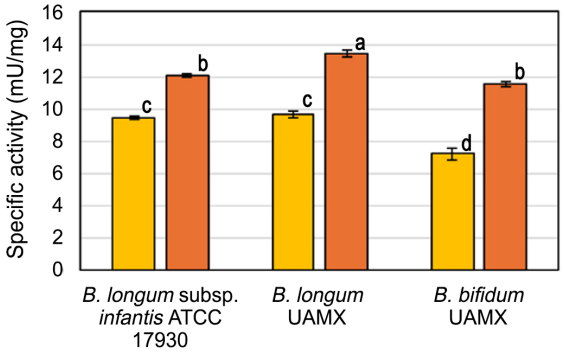

Figure 1. Whole-cell α-L-fucosidase specific activity of Bifidobacteria grown in media enriched with glucose (yellow) or inulin (orange). Different letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$).

The increase in α-L-fucosidase activity observed in cultures supplemented with inulin may reflect a broader adaptive response of bifidobacteria to complex carbohydrate utilization rather than a direct induction by fucosylated substrates. Since inulin requires the coordinated expression of transport systems and glycosyl hydrolases for its metabolism, the presence of this carbohydrate may stimulate regulatory networks associated with carbohydrate scavenging and utilization. Consequently, enzymes not directly involved in inulin hydrolysis, such as α-L-fucosidase, may also be upregulated as part of a generalized strategy to increase metabolic flexibility under conditions where complex carbohydrates constitute the primary carbon source.

3.2 *Extraction optimization by sonication*

Sonication is widely used for the extraction of biologically active molecules produced by *Bifidobacterium* species, such as GH, polysaccharides, and other compounds (Cuevas-Juárez *et al*., 2017;

Han *et al*., 2014; Hsu *et al*., 2006; Jedrzejczak-Krzepkowska *et al*., 2011). This method is particularly suitable for accessing cytoplasmic proteins due to the mechanical disruption generated by acoustic cavitation (Malherbe *et al*., 2019; Marín-Cortez *et al*., 2026). The results obtained from the experimental design are presented in Table 1. The highest residual activity (RA) of the intracellular enzymatic extract was observed in treatment 6 (35.5%), followed by treatments 10 (24.3%) and 12 (23.9%), with no statistically significant difference between the latter two ($p < 0.05$).

Treatments 6 and 10 differed only in the volume of the cell suspension used (10 mL vs. 20 mL, respectively). The higher RA of the intracellular enzymatic extract was obtained from the smaller volume, which aligns with previous observations indicating that the propagation of acoustic energy is more efficient in smaller liquid volumes. The outcome of sonication may be affected by both the volume and viscosity of the cell suspension, which depend on cell concentration (Brennan, 2006). Depending on the conditions, sonication waves can generate bubbles and eddies of different sizes. Conversely, a larger number of cells subjected to sonication is expected to release a greater quantity of enzyme (Yuan *et al*., 2012).

In treatment 12, both sonication time and amplitude were higher than those used in treatment 6, while the suspension volume remained constant. The application of higher power and longer sonication duration resulted in a lower RA compared to treatment 6. Under these conditions, the RA decreased, suggesting that excessive energy input may lead to partial enzyme inactivation due to structural destabilization during prolonged sonication (Costa-Silva *et al*., 2018; Huang *et al*., 2017).

The observed decrease in residual activity under conditions of higher sonication amplitude and longer treatment time can be explained by the dual effect of ultrasound on microbial cells and proteins. Sonication promotes cell disruption through acoustic cavitation, a phenomenon involving the formation, growth, and collapse of microbubbles within the liquid medium. The implosion of these bubbles generates localized regions of high temperature, pressure gradients, intense shear forces, and microjets that facilitate membrane disruption and intracellular enzyme release. However, excessive cavitation energy may also negatively affect enzyme stability.

Under prolonged sonication or high-amplitude conditions, the mechanical and physicochemical stresses generated during cavitation can induce conformational changes in proteins, promote aggregation, or disrupt non-covalent interactions required for catalytic activity. In addition, the formation of reactive radicals and localized heating events may contribute to partial enzyme inactivation.

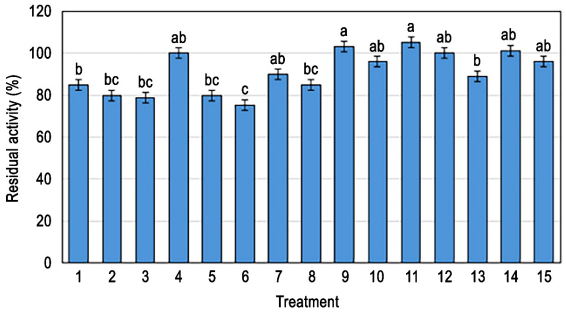

Figure 2. Residual whole-cell α-L-fucosidase activity from *B. longum* UAMX after sonication treatment. Different letters indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$).

Therefore, although increasing sonication intensity generally enhances cell disruption, an optimum operating window exists in which enzyme release is maximized while preserving catalytic functionality. The lower residual activity observed at the highest energy inputs evaluated in this study suggests that partial inactivation of α-L-fucosidase occurred simultaneously with cell disruption, resulting in reduced recovery of active enzyme.

Residual whole-cell activity after sonication is shown in Fig. 2. The lowest value was observed in treatment 6, which also exhibited the highest intracellular activity (RA) in the previous experiment (Table 1). In contrast, the mean residual whole-cell activity in treatments 9, 11, and 14 was slightly higher than in non-sonicated cells (100%). These treatments had previously shown moderate to low intracellular RA.

In treatments where higher residual whole-cell activity was detected, the applied sonication conditions were likely insufficient to achieve complete cell disruption, although they may have been strong enough to induce structural alterations in the cell membrane. Such alterations could increase membrane permeability, facilitating substrate transport as well as metabolite diffusion and accumulation (Huang *et al*., 2017). Similarly, Ku *et al.* (2015) applied sonication to *Bifidobacterium* cells without achieving complete disruption and reported an increase in enzymatic activity in whole cells. The authors concluded that sonication could represent a more practical method than traditional cell lysis followed by multiple purification steps.

Furthermore, previous studies have demonstrated that sonication-assisted extraction and purification of enzymes from *B. longum* can enhance enzymatic activity. For example, Jedrzejczak-Krzepkowska *et al*. (2011) used this method to extract fructofuranosidase, reporting a specific activity of 6.47 U/mg. Similarly, Hsu *et al*. (2005) extracted galactosidase with a specific activity of 10.8 U/mg, while Kim *et al*. (2004) reported a specific activity of 261 U/mg for the

intracellular bile salt hydrolases.

A limitation of the present study was the incomplete extraction of fucosidase. However, similar observations have been reported previously. Momin *et al*. (2018), for example, partially extracted intracellular arginase from *Bacillus licheniformis* using sonication. Similarly, Üstün-Aytekin *et al*. (2016) evaluated different cell disruption methods for the extraction of dipeptidyl aminopeptidase from *Lactococcus lactis*, reporting a specific activity of 102 mU/mL in the sonicated extract. Chanalia *et al*. (2018) also reported the extraction of a membrane-bound proline iminopeptidase, which was initially pretreated with lysozyme and subsequently subjected to sonication. In related studies, Bansal *et al*. (2008) and Ganesh and Lin (2011) used sonication to partially extract β-galactosidase from *Kluyveromyces marxianus* and proteins from *Paenibacillus*, respectively.

Although the response surface analysis identified conditions that maximized enzyme recovery within the evaluated experimental domain, the highest residual activity recovered in the intracellular extract corresponded to approximately 35% of the initial whole-cell activity. Therefore, the optimized conditions should be interpreted as the best sonication parameters among those tested rather than evidence of complete enzyme extraction. The remaining activity may be attributed to incomplete cell disruption, retention of enzyme associated with cellular structures, or partial loss of activity caused by mechanical and physicochemical stresses generated during sonication. Similar limitations have been reported for the extraction of intracellular and membrane-associated enzymes, where complete recovery is often difficult to achieve using a single disruption method. Consequently, future studies should evaluate complementary extraction strategies, such as enzymatic pretreatment, high-pressure homogenization, or combined disruption approaches, to further improve α-L-fucosidase recovery.

After the experimental phase, statistical analysis was performed using response surface methodology. A full quadratic model was selected to describe the response surface (R^2 = 0.87) using NCSS 12 software (2018, NCSS, LLC, Kaysville, Utah, USA). The most influential variables were volume and amplitude (quadratic terms), followed by their linear effects ($p < 0.05$). The interaction between volume and time was not statistically significant. The resulting model was as follows:

*RA = -102.6623 -10.06351*Volume + 5.978516*Amplitude + 10.04115*Time + 0.4299507*Volume^2 - 0.05839397*Amplitude^2 - 0.541248* Time^2 - 0.0311875*Volume*Amplitude - 0.2126667*Volume*Time + 0.05649306*Amplitude*Time*

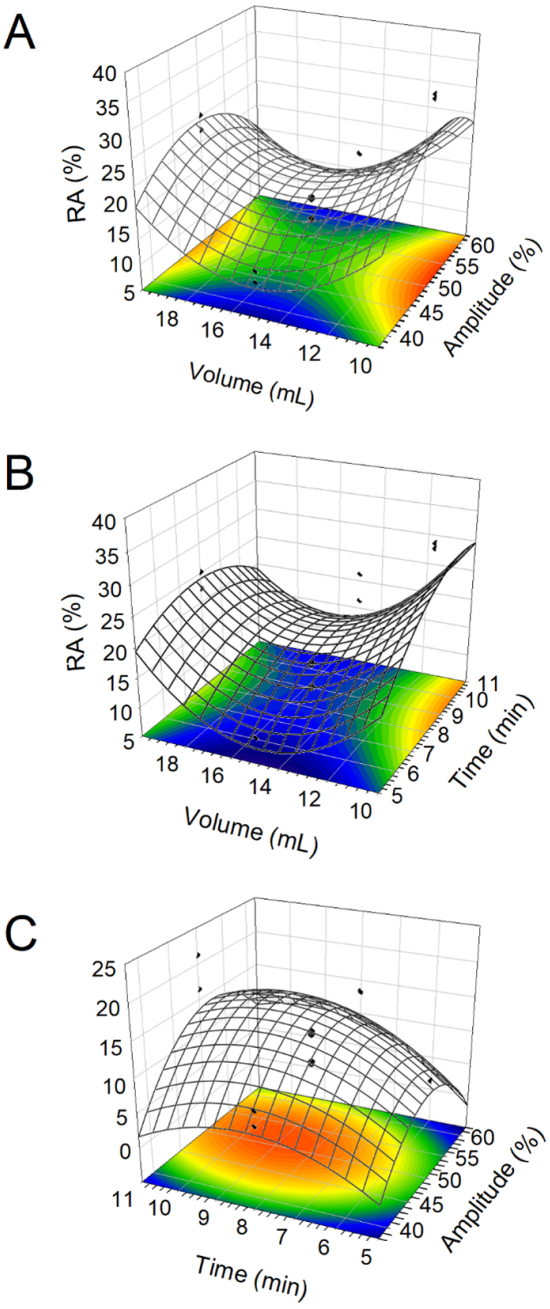

Figure 3. Effect of different sonication parameters on the extraction of intracellular α-L-fucosidase from *B. longum* UAMX: (a) sonication amplitude fixed at 50%, (b) sonication time 8 min, (c) sample volume 10 mL.

The response surfaces predicted by the model are shown in Fig. 3, where one variable was kept constant at its central level. When volume and amplitude were varied (Fig. 3A), the highest RA of α-L-fucosidase in the intracellular enzymatic extract was obtained at 10 mL and an amplitude between 46% and 55%. When time and volume were evaluated (Fig. 3B), the highest response was again observed at 10 mL and a sonication time between 8.4 and 11 minutes.

Table 2. Comparison of the extraction parameters obtained in the best experimental and theoretical conditions.

Variable	Best experimental parameters	Theoretically optimized parameters
Volume (mL)	10	10
Amplitude (%)	50	53
Time (min)	8	10.09
Residual activity (%)	35	30.97

Finally, the interaction between amplitude and time (Fig. 3C) showed that the highest RA of α-L-fucosidase was obtained with amplitudes between 48 and 56% and sonication times between 7.8 and 10.2 minutes. Overall, the response surface analysis suggests that a sample volume of 10 mL, a sonication amplitude of 46–56%, and a sonication time between 7.8 and 11 minutes maximize the extraction efficiency of α-L-fucosidase from *B. longum* UAMX.

Subsequently, the model was further optimized using the Microsoft Excel Solver add-in (2021, Microsoft Excel for Microsoft 365, version 2012), restricting the values within the experimental ranges evaluated for the three variables. The optimized parameters are summarized in Table 2. Good agreement was observed between the experimental and theoretical optimal conditions. The optimal volume was identical in both cases (10 mL), whereas the predicted amplitude and time were slightly higher than those obtained experimentally (53% vs. 50% and 11 min vs. 8 min, respectively). The experimentally determined residual enzymatic activity was also slightly higher than the value predicted by the model (35% vs. 31%).

A limitation of the present study is that the intracellular enzymatic extract was evaluated primarily in terms of α-L-fucosidase activity and its application in transfucosylation reactions. Although the extract demonstrated catalytic functionality, additional biochemical characterization would be required to fully understand the properties of the enzyme. Future studies should address protein purification, molecular weight determination, kinetic characterization, substrate specificity, and enzyme stability under different operational conditions. Such information would contribute to a more comprehensive assessment of the biocatalytic potential of bifidobacterial α-L-fucosidases for oligosaccharide synthesis.

3.3 Synthesis of fucosylated oligosaccharides

Fig. 4 shows the synthesis of fucosylated oligosaccharides using different acceptor substrates. As observed, when disaccharides such as lactose and lactulose were used as acceptor substrates, the formation of trisaccharide products was limited, particularly in the case of lactose. The low

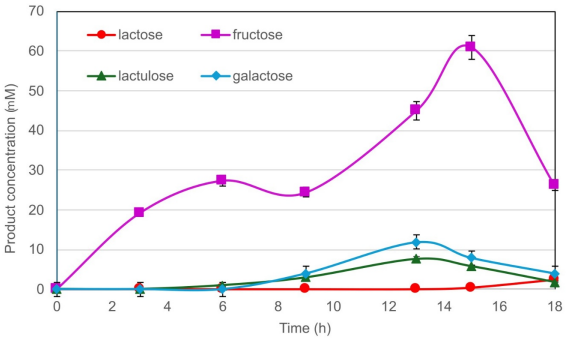

Figure 4. Synthesis kinetics of fucosylated oligosaccharides using pNP-Fuc as a donor substrate and different acceptor substrates: lactose, lactulose, galactose and fructose.

accumulation of fucosylated trisaccharides when lactose was used as acceptor substrate may also be explained by kinetic competition between transfucosylation and secondary hydrolysis reactions. In α-L-fucosidase-catalyzed reactions, the enzyme may transfer the fucosyl residue from *p*NP-Fuc to lactose, generating fucosyllactose; however, the newly formed trisaccharide can also behave as an enzyme substrate and be hydrolyzed during prolonged incubation. Therefore, the final concentration of fucosyllactose depends not only on the initial transfucosylation event but also on the balance between donor hydrolysis, acceptor-mediated fucosyl transfer, and secondary hydrolysis of the product. Under the conditions evaluated in this study, this balance may have favored hydrolysis over product accumulation, particularly for lactose-derived trisaccharides. In contrast, when monosaccharides were employed as acceptor substrates, a higher yield of disaccharide products was achieved. Furthermore, in the case of lactose, the trisaccharide product may correspond to fucosyllactose (either 2'-FL or 3-FL), as previously reported by Guzmán-Rodríguez *et al.* (2018), who identified similar products using mass spectrometry after conducting the same reaction by a *Thermotoga maritima* fucosidase.

n contrast, the highest product yield was achieved when fructose was used as the acceptor substrate. Similarly, Zhou *et al.* (2022) reported the synthesis of fucose–galactose and fucose–fructose disaccharides using a fucosidase from *Flavobacterium algicola* and 10 mM pNP-Fuc. Likewise, Benešová *et al.* (2015) demonstrated the capacity of a fucosidase from *Paenibacillus thiaminolyticus* to transfer fucose to different acceptor molecules.

The major product synthesized when fructose is used as the acceptor is consistent with the functional mapping study of α-L-fucosidases conducted by Perna *et al.* (2023), who demonstrated that certain *Bifidobacterium* α-L-fucosidases exhibit activity toward substrates containing branched galactose moieties, such as 3-FL and the Lewis antigen.

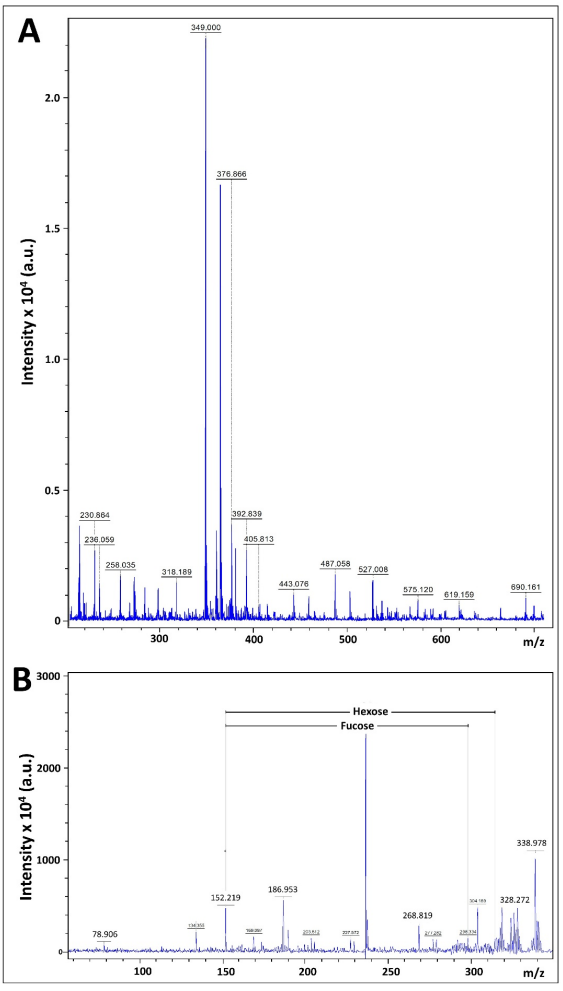

Figure 5. MALDI-TOF MS analysis of purified fucosyl-fructose. A) Mass spectrum (MS) of the purified disaccharide; B) Tandem mass spectrum (MS/MS) of the precursor ion at m/z 349.00, showing fragment ions consistent with fucose and fructose residues.

The superior performance of fructose as an acceptor substrate may be related to the accessibility and orientation of its hydroxyl groups within the active site of the enzyme. Successful transfucosylation requires not only acceptor binding but also effective competition with water for the glycosyl-enzyme intermediate. Under the reaction conditions employed, fructose may provide a more favorable configuration for nucleophilic attack than lactose, lactulose, or galactose, thereby increasing the probability of fucose transfer relative to hydrolysis. This observation suggests that acceptor structure plays a critical role in determining transfucosylation efficiency and may be exploited for the targeted synthesis of novel fucosylated carbohydrates.

Further optimization of transglycosylation parameters could improve fucosyllactose accumulation. Variables such as donor substrate concentration, acceptor substrate concentration, donor/acceptor molar ratio, enzyme load, reaction time, temperature, and water activity may significantly influence the competition between hydrolysis and transfucosylation. In particular, shorter reaction times or higher acceptor concentrations could reduce secondary hydrolysis and favor the accumulation of fucosylated trisaccharides.

To confirm the identity of the fucosylated disaccharide synthesized via the transfucosylation reaction using *p*NP-Fuc and fructose, the reaction product was collected, lyophilized, and subjected to acid hydrolysis. After hydrolysis, 31 mM of fructose and 36 mM of fucose were detected, corresponding to a near-equimolar ratio of 0.9:1. These results confirm that the enzymatic extract from *B. longum* was able to transfer fucose from *p*NP-Fuc to fructose, resulting in the formation of the disaccharide fucosyl-fructose.

As shown in Fig. 5A, a prominent ion was detected at m/z = 349.0 Da, which is consistent with the $[Fuc\text{-}Fruc+2H_2O]^+$ adduct. To further confirm the structural identity of the disaccharide, tandem mass spectrometry (MS/MS) was performed on the m/z = 349.0 Da precursor ion (Fig. 5B). The resulting fragmentation spectrum displayed signals corresponding to fucose and hexose. This structural confirmation aligns with the results previously obtained via HPLC analysis.

Although MALDI-TOF/TOF analysis and acid hydrolysis provided strong evidence supporting the formation of fucosyl-fructose, these techniques do not allow unambiguous determination of the glycosidic linkage position. Therefore, additional structural studies using NMR spectroscopy would be required for complete structural elucidation of the synthesized disaccharide. Nevertheless, the combined chromatographic, hydrolytic, and mass spectrometric evidence confirms the successful transfer of fucose from *p*NP-Fuc to fructose by the bifidobacterial α-L-fucosidase extract.

Conclusions

This study demonstrated that the expression and activity of α-L-fucosidase vary among *Bifidobacterium* species and increase when inulin is used as the sole carbon source, with *B. longum* showing the highest enzymatic activity. Sonication proved to be a useful method for recovering intracellular α-L-fucosidase from *B. longum* UAMX, although only partial enzyme recovery was achieved under the conditions evaluated.

The obtained enzymatic extract enabled the synthesis of fucosylated oligosaccharides using *p*NP-fucose as the donor substrate and several acceptor sugars, including lactose, lactulose, galactose, and fructose. The highest product yield was obtained when fructose was used as the acceptor substrate, resulting

in the formation of a fucosylated disaccharide whose identity was confirmed by HPLC and MALDI-TOF/TOF-MS. Acid hydrolysis of the product yielded fucose and fructose in near-equimolar proportions, confirming the formation of fucosyl-fructose. Overall, these results highlight the potential of bifidobacterial α-L-fucosidase as a biocatalyst for the synthesis of fucosylated oligosaccharides. The optimized extraction and reaction conditions described in this work provide a basis for future development of enzymatic platforms aimed at producing functional oligosaccharides with potential applications in infant nutrition and health-related formulations.

From an engineering perspective, the ability to recover catalytically active α-L-fucosidase through a relatively simple extraction procedure and subsequently employ the crude extract for oligosaccharide synthesis may reduce the need for extensive purification steps. This approach could contribute to the development of more cost-effective biocatalytic platforms for the production of HMO-like oligosaccharides and other value-added glycoconjugates.

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