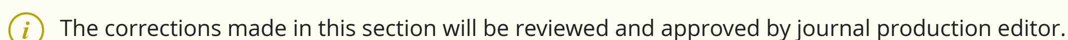
Unravelling the carbohydrate specificity of MelA from *Lactobacillus plantarum* WCFS1: An α-galactosidase displaying regioselective transgalactosylation



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Abstract

This comprehensive work addresses, for the first time, the heterologous production, purification, biochemical characterization and carbohydrate specificity of MelA, a cold-active α-galactosidase belonging to the Glycoside Hydrolase family 36, from the probiotic organism *Lactobacillus plantarum* WCFS1. The hydrolytic activity of MelA α-galactosidase on a wide range of *p*-nitrophenyl glycoside derivatives and carbohydrates of different molecular-weights showed its high selectivity and efficiency towards the α(1 → 6) glycosidic bonds involving the anomeric carbon of galactose and the C6-hydroxyl group of galactose or glucose units. MelA α-galactosidase also presented a high regioselectivity, efficiency and diversity in accommodating donor and acceptor substrates for the synthesis of α-GOS through transgalactosylation reactions. The catalytic mechanism of MelA for the production of α-GOS was elucidated, revealing its great preference for the transfer of galactosyl residues to the C6-hydroxyl group of galactose units to elongate the chain of α-GOS having either a terminal sucrose (raffinose family oligosaccharides, RFOS) or a terminal glucose (melibiose, manninotriose and verbascotetraose). Our findings indicate the feasibility of using MelA α-galactosidase from *Lactobacillus plantarum* WCFS1 in the hydrolysis of RFOS and

in the efficient and versatile synthesis of α-GOS with appealing functional properties in the context of food and nutraceutical applications.

Keywords: α-Galactosidase; Lactobacillus plantarum WCFS1; Prebiotics

1 Introduction

α-D-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) are glycoside hydrolases (GHs) that catalyze the hydrolysis of α-1,6 linked galactoside residues from non-reducing ends of different substrates. In view of the biotechnological and medical applications derived from their hydrolytic activity, α-D-galactosidases are suitable for increasing the yield of crystallized sugar [1], treating Fabry disease [2], or converting B-type blood antigens to O-type antigen [3]. In addition, transgalactosylation activity at high concentrations of substrate under adequate reaction conditions has been noticed for α-D-galactosidases [4,5]. These enzymes can catalyze the transfer of a galactosyl unit to suitable acceptors, forming α-galactooligosaccharides (α-GOS) which are a group of non-digestible oligosaccharides due to the absence of α-D-galactosidases expressed by somatic cells in the mammalian gastrointestinal tract [6]. The backbone of α-GOS is normally comprised by 2–10 monosaccharide units and its chemical structure is based on sequential galactose residues with a terminal sucrose (that is, raffinose family oligosaccharides (RFOS) which includes raffinose, stachyose or verbascose) or glucose, linked predominantly with α-(1-6) glycosidic bonds, although α-(1-3) or α-(1-4) linkages can also be present [7].

A variety of beneficial properties has been reported for α-GOS, such as immunostimulating ability [8] or a demonstrated prebiotic effect *in vitro* [9,10], in rodents [11,12] and in humans [13,14], warranting their potential use as functional food ingredients [15]. Nevertheless, some side-effects, such as abdominal discomfort, diarrhea and flatulence, have been described for RFOS due to their fermentation by anaerobic bacteria in the large intestine. In this sense, α-D-galactosidases derived from microorganisms [16–18], plants [19] or animals can be efficiently used to hydrolyze RFOS and, consequently, to avoid these side-effects [20,21]. Particularly, microorganisms offer high potential for the production of α-galactosidases due to their advantages such as regio- and estereoselectivity, efficiency, diversity and sustainability for the design of oligosaccharides with new properties [22]. Several probiotic bacteria like *Bifidobacterium* and lactic acid bacteria (LAB) as *Lactobacillus* are ideal candidate sources in terms of their antimicrobial activity, survival in stimulated gastric environment, viability during storage and higher growth profile. Concretely, an α-galactosidase from *Lactobacillus plantarum* MTCC 5422 was efficient for the removal of RFOS [23]. Moreover, α-galactosidases from several strains of *Lactobacillus reuteri* showed high transgalactosylation activity for producing α-GOS [7,24].

Lactobacillus plantarum WCFS1 is the first strain at species level whose genome was fully sequenced [25] and is encountered in a variety of environmental and appealing niches, including some fermented products and the human intestinal tract [26]. The complete genome of *L. plantarum* WCFS1 was determined from a single colony originally isolated from human saliva, and a high number of genes were identified that could encode enzymes involved in the hydrolysis, synthesis or modification of carbohydrates [27]. In *L. plantarum* the *melA* gene codes for a protein classified as an α-galactosidase belonging to the GH family 36 and involved in the

hydrolysis of melibiose into galactose and glucose [28,29], although this protein has not been biochemically nor functionally characterized so far following its expression in *Escherichia coli*. The *mel*A gene is a convenient target for single-locus mutagenesis because it is predicted to encode a nonredundant function in *L. plantarum* WCFS1, and its phenotype could be measurable both quantitatively (by hydrolysis of a chromogenic substrate) and qualitatively (by the absence of growth on melibiose as a carbon source) [29]. Apart from MelA, only a few GHs from *L. plantarum* having α-glucosidase [30], β-glucosidase [31,32], β-galactosidase [33,34], β-fructofuranosidase [35] or α-rhamnosidase [36] activities have been described up to date, according to the CAZy database (http://www.cazy.org/b125.html) [37].

In this study, we choose the model probiotic organism *L. plantarum* WCFS1 [38] as a suitable source to produce glycosidases, and particularly α-galactosidases, with appealing applications as described above. In order to confirm that the annotation of the *melA* gene (*lp_3485* locus) is correct, in the present study we address the heterologous production of MelA in *Escherichia coli*, and its subsequent purification and biochemical characterization. The carbohydrate specificity of MelA is, then, determined by assessing its hydrolytic activity on a wide range of *p*-nitrophenyl derivatives and carbohydrates, as well as its α-transgalactosylation activity for the synthesis of functional oligosaccharides like α-GOS, whose use is increasingly promoted because of their health benefits.

2 Materials and methods

2.1 Bacterial strains, plasmids and enzymes

L. plantarum WCFS1 was kindly provided by Prof. M. Kleerebezem (Wageningen University & Research, The Netherlands). *E. coli* DH10B and *E. coli* BL21 (DE3) were used as transformation and expression hosts in the pURI3-Cter vector, respectively [39]. The *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and shaking at 140 rpm. When required, ampicillin was added to the medium at a concentration of 100 mg L^{-1}.

2.2 Chemical and reagents

The substrates used in this study are listed in Table S1 (Supplementary Material). The complex mixture called AlphaGOS® is composed of 3.8% melibiose, 43.6% manninotriose and 49.5% verbascotetraose, in dry matter.

2.3 Production and purification of *L. plantarum* MelA

The *melA* (*lp_3485*) gene from *L. plantarum* WCFS1 was PCR-amplified by Advantage HD DNA polymerase (TaKaRa) by using primers 1699 (5 *TAACTTTAAGAAGGAGATATACATatg*gcagtaacgttgcagcaaactt) and 1700 (5 *GCTATTAATGATGATGATGATGATG*gtccttagccttgaagtaatgtac) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *melA* gene sequence are written in lowercase letters). Purified PCR product was inserted into the pURI3-Cter vector using a restriction enzyme- and ligation-free cloning strategy [39]. This vector produces recombinant protein having a six-histidine affinity tag in their C-termini. *E. coli* DH10B cells were transformed, recombinant plasmids were isolated, and those

containing the correct insert were identified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression.

E. coli BL21 (DE3) was transformed with pURI3-Cter-MelA recombinant plasmid. *E. coli* cells were grown in LB medium containing 100 mg L^{-1} ampicillin until an optical density at 600 nm of 0.4 was reached and then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.4 mM final concentration. Following induction, the cells were grown at 22 °C for 18 h and collected by centrifugation (8,000*g*, 15 min, 4 °C). The cells were resuspended in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl. Crude extracts were prepared by French press lysis of the cell suspension. The insoluble fraction of the lysate was removed by centrifugation at 47,000*g* for 40 min at 4 °C, and the supernatant was filtered through a 0.45 μm pore-size filter and then applied to a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 7) containing 300 mM NaCl and 10 mM imidazole. The bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His6-tagged MelA were pooled and dialyzed against 50 mM sodium phosphate buffer, 300 mM NaCl, pH 7 at 4 °C using dialysis membranes (OrDial D35-MWCO 3,500, Orange Scientific, Braine-l'Alleud, Belgium) of 3.5 kDa pore diameter. MelA was analyzed for glycosyl hydrolase activity. The amino acid sequence of MelA (UniProtKB code: F9UUF1) can be found at https://www.ncbi.nlm.nih.gov/protein/WP_011102184.1.

2.4 Determination of MelA glycosidase activities

2.4.1 Hydrolytic activity of MelA on p-nitrophenyl glycoside derivatives

The hydrolytic activity of MelA α-galactosidase from *L. plantarum* WCFS1 was determined by using a library of 24 *p*NP-glycoside derivatives (Table S1). A stock solution of each *p*NP-glycoside was prepared in water. The standard conditions chosen for the assay were 4 μg of MelA in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl and 1 mM DTT at 30 °C for 10 min. MelA protein (4 μg) was added to a pre-warmed reaction mix including the substrate (75 μl). The assay was performed in a 96-well Flat Bottom plate (Sarstedt) where each well contains a different substrate (10 mM). The reaction was stopped by the addition of a reaction volume (75 μl) of sodium carbonate 1 M at pH 9.0. Hydrolysis of each *p*NP-glycoside derivative was colorimetrically measured by liberation of *p*-nitrophenolate (pNP) at 420 nm using an extinction coefficient of $\varepsilon = 9310\ M^{-1}\ cm^{-1}$ in a microplate spectrophotometer PowerWave HT (Bio-Tek, USA) by using Gene 5 version 2.06 software. Blanks without enzyme for spontaneous hydrolysis of the tested substrates were carried out and data were collected in triplicate and the average activities were quantified. One unit (U) of MelA activity was defined as the amount of enzyme required to release 1 μmol of *p*NF per minute at saturated *p*-NF-galactopyranoside concentration, at 30 °C in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl.

2.4.2 Hydrolytic activity of MelA on carbohydrates

The hydrolytic activity of MelA was tested using 59.3 U in 1 mL of reaction on different carbohydrates (sixteen di-, three tri-, two tetrasaccharides and a commercial mixture of α-GOS (AlphaGOS®) comprised by one tri- and one tetrasaccharide) dissolved at 200 g L^{-1} in 50 mM of MOPS buffer supplemented with 20 mM of NaCl (pH = 7) at 22 °C (Table S1). In the case of galactotetraose, 2-fucosyl-lactose, isomaltose, nigerose

and maltulose the concentration was 0.5 g L^{-1} and, in the case of planteose, the concentration was 2 g L^{-1}. The reactions were checked by GC-FID at 24 h. For those substrates that were hydrolyzed by MelA, additional reactions were carried out by taking aliquots at suitable time intervals (i.e., 0, 2, 4, 8, 24 and 48 h). The enzymatic reactions were carried out by triplicate and stopped by heating at 100 °C for 5 min.

2.4.3 Transgalactosylation activity of MelA on selected carbohydrates.

The transgalactosylation activity of MelA was also carried out at 22 °C in 50 mM of MOPS buffer supplemented with 20 mM of NaCl (pH = 7) using melibiose, raffinose, stachyose and AlphaGOS® at a concentration of 400 g L^{-1} and, in the case of melibiose an additional concentration of 800 g L^{-1} was also tested. Reactions using galactose at 400 g L^{-1} were also assessed to observe the capability of MelA to directly transfer galactosyl units and produce α-GOS. The reactions were monitored by GC-FID after taking aliquots from the reaction mixture at time intervals of 0, 1, 2, 4, 6, 8 and 24 h. The enzymatic reactions were stopped by heating at 100 ^{0}C for 5 min.

2.5 Biochemical and kinetic properties of MelA

The properties of MelA were determined using *p*NP-α-D-galactopyranoside as substrate (1 mM final concentration) under the standard conditions previously described. The effects of pH and temperature on the hydrolytic activity of MelA on *p*NP-α-D-galactopyranoside were studied. The optimal pH was determined by using citrate (pH 3), acetic acid-sodium acetate (pH 4–6), MOPS (pH 6.5 and 7) and Tris-HCl (pH 8) buffers (50 mM). The optimal temperature was assayed by incubating purified MelA α-galactosidase in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl, 1 mM DTT at different temperatures (4, 10, 22, 30, 37, 45, and 65 °C). For temperature stability measurements, MelA α-galactosidase was incubated in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl, 1 mM DTT at 22, 30, 37, 45, and 65 °C for 30 min and 1, 2, 4, 6, and 20 h. Aliquots were withdrawn at these incubation times to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%). The analyses were performed in triplicate.

MelA kinetics were studied by using 0.2 µg MelA and *p*NP-α-D-galactopyranoside as substrate at 30 °C in 50 mM MOPS buffer pH 7.0 containing 20 mM NaCl. K_m and V_{max} values were determined by nonlinear regression analysis fitting to Michaelis-Menten curves of formation rates of *p*NP as a function of the concentration of substrates from 0 to 20 mM by using the software SigmaPlot version 13.0. The analyses were performed in triplicate.

To study the effect of metal ions, detergents, reductants, and inhibitors on α-galactosidase activity, MelA was incubated in the presence of different additives at a final concentration of 1 mM for 5 min at room temperature. Then, *p*NP-α-D-galactopyranoside was added, and the reaction mixture was incubated under standard conditions. The residual hydrolytic activity was measured after the incubation of purified enzymes with each additive. The analysed additives were KCl, $CaCl_2$, $HgCl_2$, $ZnCl_2$, $CuCl_2$, $NiCl_2$, $FeCl_2$, $MnCl_2$, Triton-X-100, Tween 20, Tween 80, SDS, urea, DMSO, cysteine, β-mercaptoethanol, PMSF, DEPC and EDTA. Hydrolytic activity measured in the absence of any additive was taken as control (100%). Experiments were done in triplicate.

2.6 Gas chromatography with a flame ionization detector (GC-FID)

The carbohydrate composition of the reaction mixtures was determined by GC-FID in an Agilent Technologies 7820A Gas Chromatograph (Agilent Technologies, Wilmington, DE, EEUU) equipped with a flame ionization detector (FID). Carbohydrates were analyzed as trimethyl silylated oximes (TMSO) following the method of Sanz, Villamiel and Martínez-Castro [40] using a fused silica capillary column DB-5HT, bonded, crosslinked phase (5% phenyl-methylpolysiloxane; 15 m × 0.32 mm i.d., 0.10 µm film thickness) (J&W Scientific, Folson, California, USA). The oven temperature was initially 150 °C increasing at a rate of 3 °C/min to 380 °C and held this temperature during 76 min. The injector and detector temperatures were at 280 °C and 385 °C, respectively. Injections were carried out in split mode (1:20) using nitrogen as carrier gas at a flow rate of 1 mL/min. Data acquisition and integration were performed using Agilent ChemStation software (Wilmington, DE, USA). Quantification of each sugar was performed by internal standard calibration using phenyl-β-glucoside (0.5 mg/mL). Mixture of standard solutions of glucose, galactose, fructose, lactose, raffinose and nystose were prepared over the expected concentration range to calculate the response factors of each sugar. Identification of α-GOS disaccharides was performed by comparing the retention times ~~(Rt)~~ with standard α-1,6-galactobiose (α-D-Gal-(1 → 6)-α-D-Gal).

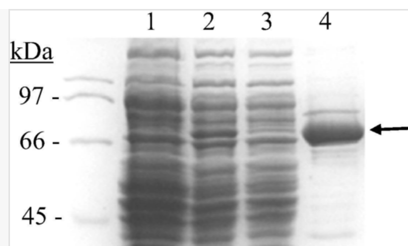
3 Results and discussion

3.1 Biochemical characterization of MelA from *L. plantarum* WCFS1

LAB have long been used as starters in food fermentation where they exert technologically important functions on raw agricultural products. The knowledge acquired on the metabolism of these bacteria opens the way to new applications, as the production of health-beneficial compounds such as prebiotic oligosaccharides. Genes encoding α-galactosidases are present in the genome of most lactobacilli [41], and proteins having α-galactosidase activity have been characterized in some of them, including *Lactobacillus plantarum* [42–44].

The *melA* gene from *L. plantarum* ATCC 8014 was previously expressed in *E. coli* and the α-galactosidase activity of the encoded protein was demonstrated [28]. However, the MelA α-galactosidase was neither purified nor biochemically characterized. *L. plantarum* WCFS1 possesses a MelA protein whose amino acid sequence is 99.86% identical to MelA from *L. plantarum* ATCC 8014. Both 738-amino acid residues proteins are identical except in the residue 672 where a conservative change Leu to Phe appeared (data not shown). The *melA* (*lp_3485*) gene from *L. plantarum* WCFS1 was cloned into the pURI3-Cter expression vector, and the recombinant plasmid obtained was transformed into *E. coli* BL21 (DE3). Cell extracts were used to detect the presence of overproduced proteins. SDS-PAGE analysis showed that there was one major band protein, approximately of 80 kDa, consistent with the theoretical molecular weight expected for MelA. The recombinant protein was observed as a single band on SDS-PAGE (Fig. 1, lane 4). Routinely, about 13.5 mg of purified protein from 1 L culture was obtained.

Fig. 1

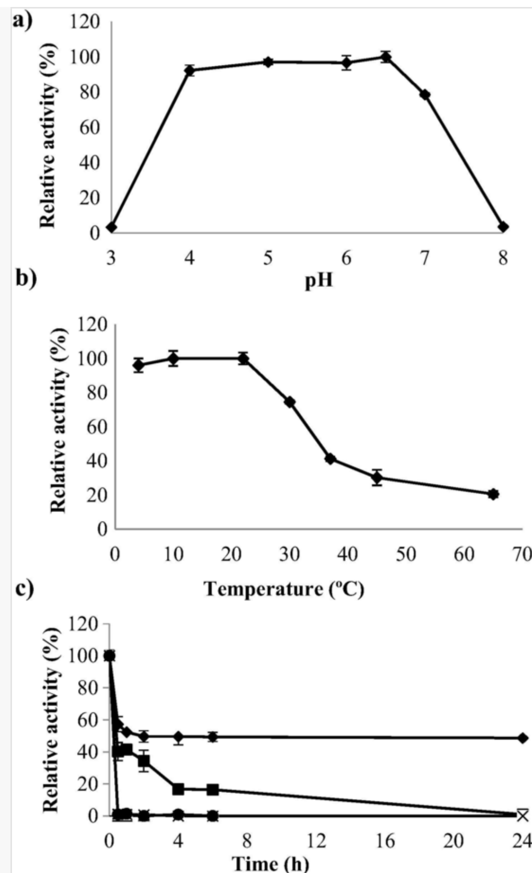


Purification of MelA α-galactosidase from *L. plantarum* WCFS1. SDS-PAGE analysis of soluble cell extracts of IPTG-induced *E. coli* BL21 (DE3) (pURI3-Cter) (lane 1), *E. coli* BL21 (DE3) (pURI3-Cter-MelA) (lane 2), flowthrough (lane 3) and MelA protein eluted after affinity resin (lane 4). The gel was stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE Standards, Bio-Rad).

The MelA protein purified by the affinity resin was biochemically characterized. Substrate specificity was determined using 24 *p*-nitrophenyl-linked carbohydrates of various types of α- or β- linkages (Table S1). The enzyme was only active for *p*NP-α-D-galactopyranoside, indicating clearly its α-galactosidase activity (624 U mg^{-1}). As compared to α-galactosidases from other LAB, the specific activity showed by MelA is remarkably higher than the activity exhibited by α-galactosidases ~~fs2w3zom~~ from *Carnobacterium piscicola* (2.3 U mg^{-1}) [45], and *Lactobacillus curvatus* (5.71 U mg^{-1}) [46].

The influence of pH on MelA α-galactosidase activity was studied at 30 °C (Fig. 2a). The enzyme showed its maximal activity at the pH range from 4.0 to 6.5. This result is in agreement with the optimal pH exhibited by α-galactosidases from LAB, which varies from a pH close to neutral to an acidic pH. Similar to MelA, *Lactobacillus curvatus* α-galactosidase exhibited its optimal pH at 6.5 [46], however, the optimal pH of *L. reuteri* and *L. fermentum* α-galactosidases were more acidic, being around 4.8 [7,47].

Fig. 2



Biochemical properties of MelA α-galactosidase. (a) MelA pH activity profile. (b) MelA temperature activity profile. (c) MelA thermal stability after incubation at 22 °C (♦), 30 °C (■), 37 °C (▲), 45 °C (×) and 65 °C (●) in MOPS buffer (50 mM, 20 mM NaCl, 1 mM DTT, pH 7) at indicated times. The mean value and standard error are shown (n = 3). The percentage of residual activity was calculated by comparing to the non-incubated enzyme.

In relation to temperature, MelA is a cold-active α-galactosidase because, from the temperatures assayed, its maximal activity was at 4, 10 and 22 °C (Fig. 2b). When incubation temperature increased, MelA activity decreased. At 30 and 37 °C, MelA exhibited only 75 and 40% of its maximal activity, respectively. As far as we known, this is the first cold-active α-galactosidase described in lactic acid bacteria. α-Galactosidases from *L. reuteri* [7] and *L. curvatus* [46] exhibited optimal temperatures ranging from 30 to 37 °C. In contrast to MelA α-galactosidase from *L. plantarum*, *L. fermentum* possesses a thermostable MelA enzyme which showed optimal temperature at 50 °C [47]. The protein sequence of *L. plantarum* MelA is only 36.3% identical to MelA from *L. fermentum*; this amino acid sequence variability could explain the different thermal behaviour of both MelA proteins. Moreover, [Instruction: In pdf version there is an incomplete line (last line of third paragraph) from
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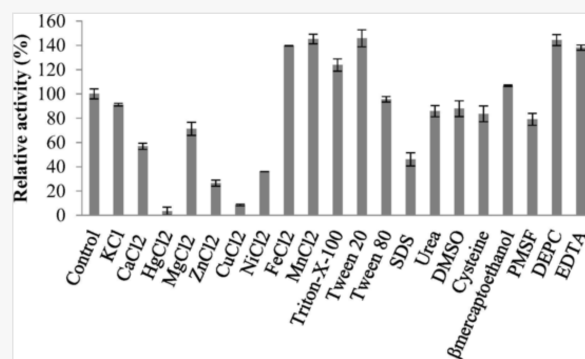
]*L. plantarum* MelA is a thermolabile enzyme, when it was preincubated at different temperatures only at 22 °C its relative activity remained around 50% throughout the 24 h of incubation (Fig. 2c). Among the α-galactosidases from LAB, *L. plantarum* MelA is the most thermolabile enzyme, since *L. curvatus* α-galactosidase maintained a relative activity above 65% after its preincubation at 40 °C during 3 h [46].

The effect of several metal ions, surfactants, reductants, and inhibitors on *L. plantarum* MelA activity is shown in Fig. 3. Enzymatic activity was increased 145% and 139% by Mn^{2+} and Fe^{2+} ions, respectively. In good agreement with our results, the presence of these two metal ions also increased the activity of *L. reuteri* α-galactosidase [48]. In addition to these metal ions, protease inhibitors DEPC and EDTA, and the detergents Tween 20, Triton-X-100 also increased MelA activity around 40%. These additives could activate MelA due to protein conformational changes, to a depletion of inhibitory cations, or to their surfactant effect [49]. In contrast, Hg^{2+} and Cu^{2+} greatly inhibited MelA activity to 3 and 9%, respectively.

Fig. 3



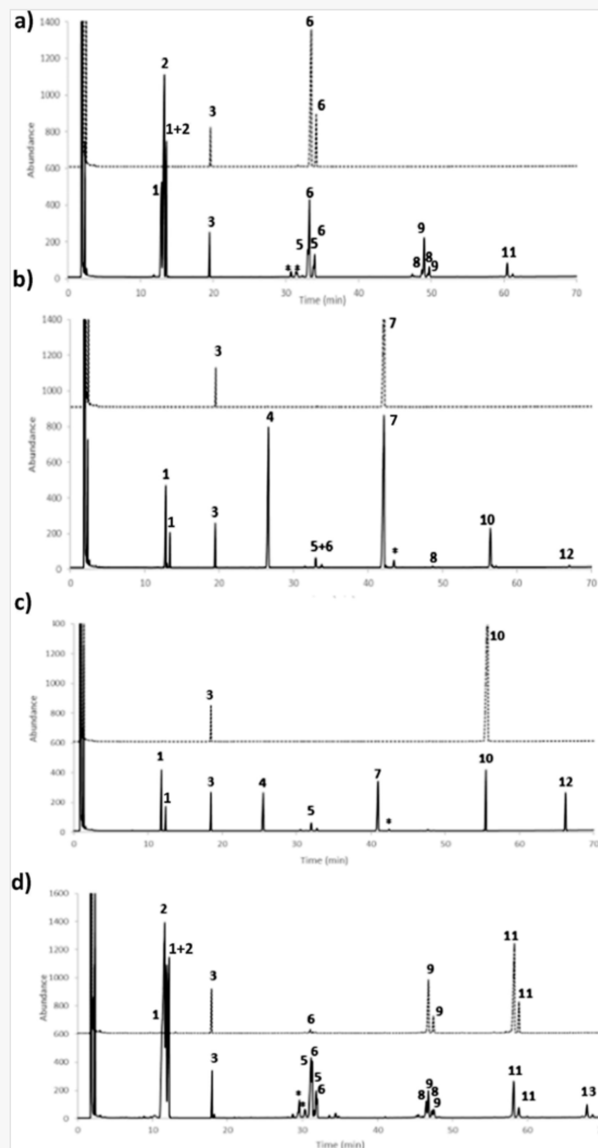
Effect of additives at 1 mM on *L. plantarum* WCFS1 MelA α-galactosidase relative activity. The activity of MelA in the absence of additives was defined as 100%. The mean value and standard error are shown (n = 3).

Kinetic studies performed in the conditions described previously, indicated that *L. plantarum* MelA for *p*NP-α-D-galactopyranoside showed a K_m 0.65 ± 0.07 mM, and V_{max} 0.1247 ± 0.0018 μmol min^{-1} (624 U mg^{-1}) values. From these data, the K_{cat} and catalytic efficacy (K_{cat}/K_m) were calculated, being the K_{cat} 52125 ± 0.0018 min^{-1} and the catalytic efficacy (K_{cat}/K_m) 80328 ± 8405 mM^{-1} min^{-1}. This catalytic efficacy is low as compared to a *Bacillus stearothermophilus* α-galactosidase which had a K_{cat}/K_m of 2.6×10^5 mM^{-1} min^{-1} [50].

3.2 Hydrolytic activity of MelA on carbohydrates

The hydrolytic activity of MelA was tested on sixteen disaccharides, including lactose, sucrose and maltose and some of their isomers among others, being melibiose (Gal-α(1 → 6)-Glc) the only hydrolyzed disaccharide (peak 6 in Fig. 4a). These results pointed out the high selectivity of *L. plantarum* MelA and also confirmed the *melA* gene's annotation as a gene encoding a α-galactosidase (EC 3.2.1.22) involved in the hydrolysis of melibiose into galactose and glucose [29].

Fig. 4



GC-FID profiles resulting from the hydrolysis of melibiose (a), raffinose (b), stachyose (c) and AlphaGOS® (d) at 200 g L^{-1} at 0 h (dashed line) and 8 h (solid line) catalyzed by *Lactobacillus plantarum* WCFS1 MelA. Peaks identification: 1, galactose; 2, glucose; 3, internal standard; 4, sucrose; 5, α-1,6-galactobiose; 6, melibiose; 7, raffinose; 8, α-1,6-galactotriose; 9, manninotriose; 10, stachyose; 11, verbascotetraose; 12, verbascose; 13, verbascopentaose; *unknown α-galactooligosaccharides.

The only trisaccharide hydrolyzed by *L. plantarum* MelA was raffinose (Gal-α(1 → 6)-Glc-α(1 ↔ 2)β-Fru, peak 7 in Fig. 4b) which was broken at the melibiose moiety, thus, releasing sucrose (Glc-α(1 ↔ 2)β-Fru, peak 4 in Fig. 4b) and galactose (peak 1 in Fig. 4b), whereas planteose (Gal-α(1 → 6)-Fru-β(2 ↔ 1)α-Glc) and 2-fucosyl-lactose (Fuc-α(1 → 2)-Gal-β(1 → 4)Glc) remained unaltered upon the enzymatic reaction (data not shown). Therefore, MelA α-galactosidase has the ability to efficiently hydrolyze the α(1 → 6) bond linking a galactose unit located at the non-reducing end with glucose but not with fructose.

In order to gain more knowledge on the carbohydrate specificity of *L. plantarum* MelA, two tetrasaccharides as stachyose (Gal-α(1 → 6)-Gal-α(1 → 6)-Glc-α(1 ↔ 2)β-Fru, peak 10 in Fig. 4c) and a galactotetraose[Instruction: Section 3.2. third paragraph last line:

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] (Gal-α(1 → 3)-Gal-β(1 → 4)-Gal-α(1 → 3)-Gal), as well as a commercial mixture of α-GOS having a terminal glucose instead of sucrose (i.e., AlphaGOS® which are mainly comprised by manninotriose (Gal-α(1 → 6)-Gal-α(1 → 6)-Glc, peak 9 in Fig. 4d) and verbascotetraose (Gal-α(1 → 6)-Gal-α(1 → 6)-Gal-α(1 → 6)-Glc, peak 11 in Fig. 4d) were subjected to hydrolytic incubation with MelA α-galactosidase. The efficient hydrolysis of either stachyose, by releasing mainly raffinose, sucrose and galactose (Fig. 4c), or AlphaGOS®, by releasing mainly galactose and glucose (peak 2 in Fig. 4d), revealed that *L. plantarum* MelA also readily hydrolyzed α(1 → 6) bonds linking consecutive galactose units from non-reducing ends.

In contrast, the assayed galactotetraose, which lacks α(1 → 6) linkages, was hardly hydrolyzed (data not shown), reinforcing the high selectivity of MelA α-galactosidase towards the α(1 → 6) glycosidic bonds involving the anomeric carbon of galactose and the C6-hydroxyl group of galactose or glucose units.

Therefore, only four out of twenty two tested carbohydrates were hydrolyzed by MelA α-galactosidase at a starting substrate concentration of 200 g L^{-1}, being α-GOS having either a terminal glucose (melibiose and AlphaGOS®) or a terminal sucrose (raffinose and stachyose) the positive substrates. Remarkably, these four hydrolysis reactions were highly efficient and yielded a percentage of degradation ≥ 93% in weight respect to the initial amount of substrate after 48 h of enzymatic reaction.

Lastly, in addition to the carbohydrates derived from the hydrolytic activity, new peak(s) eluting at longer retention times than the starting substrates and, consequently, corresponding to chain-elongated carbohydrates, could be detected during the hydrolysis of melibiose (see peaks 8, 9 and 11 in Fig. 4a), raffinose (peaks 8, 10 and 12 and an unknown minor trisaccharide marked with an asterisk in Fig. 4b), stachyose (peak 12 in Fig. 4c) and AlphaGOS® (peak 13 in Fig. 4d). This fact could be attributed to the transgalactosylation activity of MelA. Therefore, further experiments using higher starting concentrations of these carbohydrates were warranted and are reported in the next section.

3.3 Transgalactosylation activity of *L. plantarum* MelA on selected carbohydrates.

The substrate concentration is one of the critical factors coming into play when optimizing the oligosaccharide production during transglycosylation reactions catalyzed by GHs. Normally, the reduction of water concentration through the increase in total dissolved carbohydrates in the reaction favors the transglycosylation activity over the hydrolytic capacity of GHs [51,52]. Therefore, two additional initial concentrations of melibiose, that is 400 and 800 g L^{-1}, and one (400 g L^{-1}) of raffinose, stachyose, AlphaGOS® or galactose were tested for exploring the transgalactosylation activity of MelA α-galactosidase.

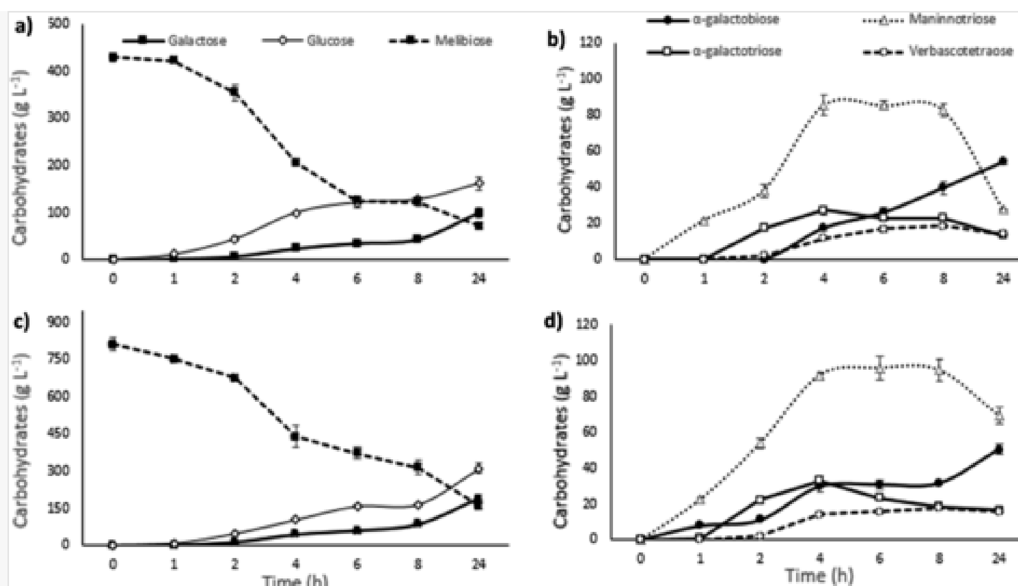
3.3.1 Synthesis of α-GOS from galactose

In reactions using galactose at 400 g L^{-1}, no synthetic products were observed, in agreement with Tzortis, Jay, Baillon, Gibson and Rastall [24], who used solutions of 60–80% of galactose in the presence of an α-galactosidase from *Lactobacillus reuteri* NCIMB 41152. In contrast, Yamashita, Hashimoto, Fujita, Okada, Mori and Kitahata [53] reported high yields of α-GOS by an α-galactosidase from fungal origin (i.e., *Aspergillus niger*) using supersaturated solutions of galactose (90%) at 60 °C. In our case, the use of supersaturated solutions of galactose was unworkable because the optimum temperature of *L. plantarum* MelA (22 °C) affected considerably the solubility of the monosaccharide in the reaction buffer (data not shown).

3.3.2 Synthesis of α-GOS from melibiose

Melibiose was also efficiently hydrolyzed after 24 h by MelA α-galactosidase at higher concentrations, since 83.6% and 80.5% of starting melibiose at 400 and 800 g L^{-1} respectively, was hydrolyzed (Fig. 5a and c), reinforcing, thus, the fact that melibiose is an excellent donor substrate for MelA α-galactosidase. Moreover, regardless of the starting concentration of melibiose, the main transgalactosylated product was manninotriose (Gal-α(1 → 6)-Gal-α(1 → 6)-Glc) (Fig. 5b and d) as a result of the transfer of the galactosyl residue released from the hydrolysis of melibiose to the C6-hydroxyl group of the galactose unit of melibiose, indicating that melibiose has the capacity to act both as donor and acceptor of galactosyl moieties. The transgalactosylation activity of *L. plantarum* MelA was also supported by the fact that glucose content was also higher than galactose levels throughout the enzymatic reaction (Fig. 5a and c). Likewise, three additional α-GOS compounds were observed although at a much lesser extent (around 4-fold lower) than that of manninotriose, and they were identified as i) α-1,6-galactobiose (Gal-α(1 → 6)-Gal); ii) α-1,6-galactotriose (Gal-α(1 → 6)-Gal-α(1 → 6)-Gal); and iii) verbascotetraose (Gal-α(1 → 6)-Gal-α(1 → 6)-Gal-α(1 → 6)-Glc) whose synthesis was due to the ability of manninotriose to also act as acceptor of galactosyl residues (Fig. 5b and d). In consequence, MelA α-galactosidase displayed a great regioselectivity as the only formed glycosidic linkage was the α(1 → 6), and manninotriose alone accounted for around two thirds of the total α-GOS products formed upon the enzymatic reaction.

Fig. 5



Concentration of monosaccharides, melibiose and formed α-galactooligosaccharides upon transgalactosylation reaction catalyzed by *Lactobacillus plantarum* WCFS1 MelA α-galactosidase using melibiose at 400 g L^{-1} (a and b) and 800 g L^{-1} (c and d), as starting substrate. Vertical errors represented standard deviations (n = 3).

Overall, the synthesis of α-GOS from melibiose was steadily increased during the first 4 h of reaction and then achieved a plateau until 8 h, followed by a decrease until the end of the reaction (24 h). The only exception to this behavior was the disaccharide α-1,6-galactobiose which exhibited a constant increase that was sharper between 8 and 24 h of reaction, suggesting that the formation of α-1,6-galactobiose can be also driven, especially from the eighth hour of the reaction, by the hydrolysis of the rest of transfer products. This trend was observed either by using 400 (Fig. 5b) or 800 g L^{-1} (Fig. 5d) of starting melibiose.

Table 1 summarizes the quantitative data of the transfer products formed upon transgalactosylation reaction catalyzed by *L. plantarum* MelA using melibiose at different concentrations (200, 400, or 800 g L^{-1}). The increase in starting melibiose concentration from 200 to 400 g L^{-1} substantially promoted the synthesis of α-GOS, whereas the maximum concentration of total α-GOS remained fairly constant when the melibiose concentration was increased up to 800 g L^{-1}. In consequence, the highest yield in α-GOS, i.e. 38.0%, was obtained by using 40% of starting melibiose. Tzortzis, Jay, Baillon, Gibson and Rastall [24] reported a lower maximum yield (26%) of α-GOS from melibiose and did not find substantial differences in the corresponding yields when melibiose was used at 200 g L^{-1} or at 700 g L^{-1} using an α-galactosidase from *Lactobacillus reuteri* NCIMB 41152. Furthermore, Wang, Black, Curtis and Gänzle [7] also demonstrated the transferase activity of an α-galactosidase from *Lactobacillus reuteri* 100–23 and *Lactobacillus reuteri* 100–16 when melibiose was present at 600 g L^{-1}, although no data on yields were reported.

Table 1

i The presentation of Tables and the formatting of text in the online proof do not match the final output, though the data is the same. To preview the actual presentation, view the Proof.

Production and yield of α-GOS upon transgalactosylation reaction catalyzed by *Lactobacillus plantarum* WCFS1 MelA α-galactosidase using melibiose, raffinose, stachyose and AlphaGOS® at different initial concentrations (200, 400, or 800 g L^{-1}).

		α-GOS	
Starting substrates	**Concentration (g L^{-1})**	**Maximum concentration (g L^{-1})**	**Yield (%)**[b]
Melibiose	200	59.6 (4 h)[a]	28.0
	400	162.9 (8 h)	38.0
	800	168.3 (4 h)	20.8
Raffinose	200	42.4 (8 h)	22.6
	400	103.3 (8 h)	25.3
Stachyose	200	30.4 (24 h)	15.4
	400	88.6 (8 h)	20.7
AlphaGOS®	200	39.0 (4 h)	20.9
	400	114.7 (24 h)	30.5

Table Footnotes

[a]Reaction time at the maximum concentration of transfer products.

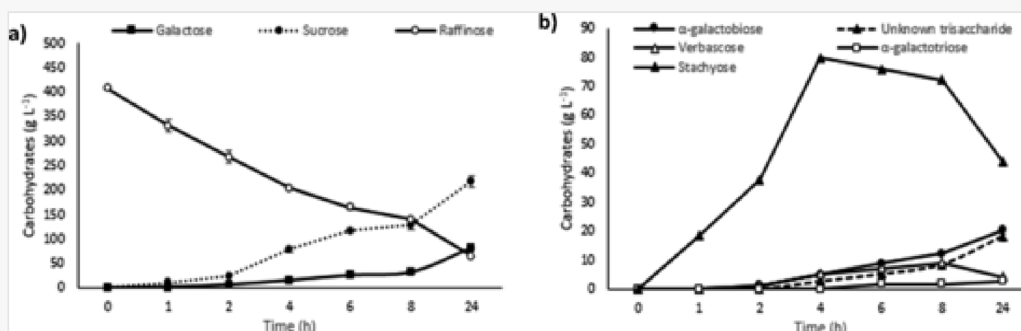
[b]Yield (g transfer products/100 g starting substrate) represents the maximum mass of transfer products obtained during the synthesis per unit mass of initial substrate.

3.3.3 Synthesis of α-GOS from raffinose

Raffinose at 400 g L^{-1} was also efficiently hydrolyzed (that is, only 15.6% of starting raffinose remained intact after 24 h of enzymatic reaction) by *L. plantarum* MelA to galactose and sucrose (Fig. 6a), revealing to be an efficient donor of galactosyl residues for MelA α-galactosidase. In good agreement with the mechanism described above for the formation of manninotriose from melibiose, *L. plantarum* MelA mainly transferred galactose moieties at the C-6 hydroxyl group of the galactosyl residue of raffinose, producing high amounts of stachyose and whose maximum formation (79.7 g L^{-1}) was obtained at 4 h of enzymatic reaction (Fig. 6b). Moreover, the formation of stachyose was even more predominant than in the case of manninotriose from melibiose, since 86.4% of the transfer products obtained from 400 g L^{-1} of raffinose at 4 h of reaction corresponded exclusively to stachyose. The rest of α-GOS compounds was comprised by the minor presence of: (i) the pentasaccharide verbascose (due to the transgalactosylation of stachyose) and whose maximum formation was 9.0 g L^{-1} at 8 h; (ii) α-1,6-galactotriose (whose maximum formation of 2.5 g. L^{-1} was at

24 h); and (iii) α-1,6-galactobiose (maximum formation of 22.6 g L^{-1} at 24 h); (iv) as well as an unknown trisaccharide that might correspond to an isomer of raffinose. Stachyose concentration started to decrease from the fourth hour of reaction whereas the decrease in verbascose was observed from the eight hour. In contrast, the remaining three minor α-GOS compounds increased during the whole enzymatic reaction (Fig. 6b). Wang, Black, Curtis and Gänzle [7] reported the synthesis of four α-GOS compounds when raffinose was present at 300 g L^{-1} using an α-galactosidase from a strain of *Lactobacillus reuteri.* Those products included not only stachyose but also another tetrasaccharide and two trisaccharides following the transfer of galactosyl residues through α(1 → 3) and α(1 → 4) linkages.

Fig. 6



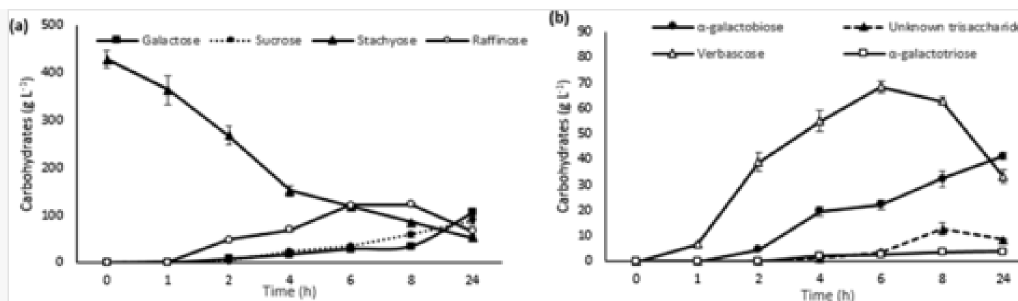
Concentration of galactose, sucrose, raffinose (a) and formed α-galactooligosaccharides (b) upon transgalactosylation reaction catalyzed by *Lactobacillus plantarum* WCFS1 MelA α-galactosidase using raffinose at 400 g L^{-1} as starting substrate. Vertical errors represented standard deviations (n = 3).

Overall, the increase of the starting raffinose from 200 to 400 g L^{-1} led to an increase of 2.4-fold both in the maximum formation of stachyose and in the formation of total α-GOS whose maximum yield was 25.3% (Table 1). Likewise, this increase substantially shortened the reaction time needed to reach the maximum levels in stachyose (from 8 to 4 h). Tzortis, Jay, Baillon, Gibson and Rastall [24], using an α-galactosidase from *L. reuteri*, reported a maximum yield of 18% for the synthesis of α-GOS from raffinose.

3.3.4 Synthesis of α-GOS from stachyose

Stachyose (Gal-α(1 → 6)-Gal-α(1 → 6)-Glc-α(1 ↔ 2)β-Fru) at 400 g L^{-1} was also an efficient donor of galactosyl residues following its hydrolysis by releasing galactose and raffinose which was, in turn, further hydrolyzed to give rise to sucrose and galactose as shown in Fig. 7a. Thus, 87.6% of stachyose was hydrolyzed after 24 h of enzymatic reaction. In addition, the higher formation of raffinose than that of sucrose observed during the first 8 h of reaction confirmed that *L. plantarum* MelA is an *exo*-alpha-galactosidase by catalyzing the hydrolysis of galactosyl residues from non-reducing ends.

Fig. 7



Concentration of galactose, sucrose, raffinose and stachyose (a) and formed α-galactooligosaccharides (b) upon transgalactosylation reaction catalyzed by *Lactobacillus plantarum* WCFS1 MelA α-galactosidase using stachyose at 400 g L^{-1} as starting substrate. Vertical errors represented standard deviations (n = 3).

Concerning the transfer products, the pentasaccharide verbascose (Gal-α(1 → 6)-Gal-α(1 → 6)-Gal-α(1 → 6)-Glc-α(1 ↔ 2)β-Fru) was the main oligosaccharide synthesized under the mechanism described in previous sections. The maximum formation (68.5 g L^{-1}) was observed at 6 h of enzymatic reaction and comprised 84% of the total synthesized α-GOS (Fig. 7b). As in the case of raffinose, three additional minor products, i.e. α-1,6-galactotriose, α-1,6-galactobiose and the same unknown trisaccharide detected during the synthesis of raffinose, were also found and quantified.

Finally, by increasing the concentration of starting stachyose from 200 to 400 g L^{1} a ~ 3-fold increase of either verbascose or total α-GOS was found, shortening the reaction time needed to reach the maximum levels, as well as yielding up to 20.7% of total α-GOS (Table 1).

3.3.5 Synthesis of α-GOS from AlphaGOS®

The use of AlphaGOS® at 400 g L^{-1} also demonstrated the ability of manninotriose and verbascotetraose to act as galactosyl donors by considering their respective hydrolysis degree of 83.7% and 77.9% after 24 h of enzymatic reaction. Thus, the monosaccharides glucose and galactose were the most abundant carbohydrates at the end of the reaction (Fig. 8a). Remarkably, the notable presence of the uncommon verbascopentaose[Instruction: In pdf version in 3.3.5. section line 7:
common verbascopentaose
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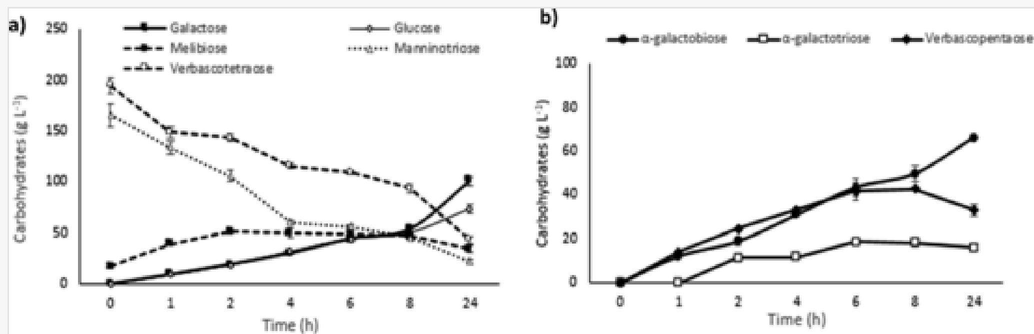
Is it possible to fill in this line with more words??] (Gal-α(1 → 6)-Gal-α(1 → 6)-Gal-α(1 → 6)-Gal-α(1 → 6)-Glc), whose maximum formation (42.6 g L^{-1}) was observed at 8 h of enzymatic reaction (Fig. 8b), revealed the ability of verbascotetraose, which comprised ~ 43 49.5% of the composition of the commercial AlphaGOS®, to act as a galactosyl-acceptor with good efficiency. In addition, the presence of verbascopentaose was already detected by GC-FID when AlphaGOS® was used as starting substrate at 200 g L^{-1} (peak 13 in Fig. 4d). As in previous sections, the content of α-1,6-galactobiose was steadily increased until the end of the reaction, likely due to the hydrolysis of α-GOS of higher degree of polymerization (Fig. 8b). Finally, both the maximum concentration and the yield in total α-GOS were greatly enhanced by increasing the initial concentration of AlphaGOS® from 200 to 400 g L^{-1} (Table 1).

Fig. 8



Concentration of monosaccharides and AlphaGOS® (a) and formed α-galactooligosaccharides (b) upon transgalactosylation reaction catalyzed by *Lactobacillus plantarum* WCFS1 MelA α-galactosidase using AlphaGOS® at 400 g L^{-1} as starting substrate. Vertical errors represented standard deviations (n = 3).

4 Conclusions

To the best of our knowledge, this work describes, for the first time, the biochemical characterization of MelA α-D-galactosidase from *Lactobacillus plantarum* WCFS1. An efficient hydrolysis of RFOS, such as raffinose and stachyose, was achieved, with a degradation degree greater than 84%, revealing that *L. plantarum* MelA has a high capacity to hydrolyze non-digestible RFOS. Likewise, MelA α-galactosidase showed a high regioselectivity, efficiency and great diversity in accommodating donor and acceptor substrates for the synthesis of α-GOS through transgalactosylation reactions. Thus, the enzyme exhibited a great preference for the transfer of galactosyl residues to the C6-hydroxyl group of galactose units to elongate the chain of α-GOS having either a terminal sucrose (i.e., RFOS) or a terminal glucose (melibiose, manninotriose and verbascotetraose). Under optimum pH, temperature and enzyme and starting substrate concentrations, the maximum yield in total α-GOS ranged from 20.7% (using stachyose as donor and acceptor substrate) to 38.0% (using melibiose as starting substrate). To conclude, our findings indicate the feasibility of using MelA from *Lactobacillus plantarum* WCFS1 in the hydrolysis of RFOS and in the efficient and versatile synthesis of α-GOS with appealing functional properties in the context of food and nutraceutical applications. Likewise, our results open up interesting possibilities for the further development of novel hetero-α-galactooligosaccharides using specific α-GOS compounds as donors and different acceptors to design new oligosaccharides exerting promising bioactive properties.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.10.237.

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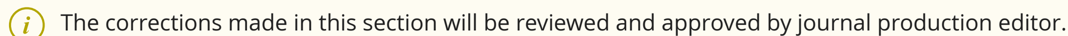

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Appendix A Supplementary data

The following are the Supplementary data to this article:

Multimedia Component 1

Supplementary Data 1

Queries and Answers

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