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Galactooligosaccharide Production from *Pantoea anthophila* Strains Isolated from "Tejuino", a Mexican Traditional Fermented Beverage

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Abstract: Two *Pantoea anthophila* bacterial strains were isolated from "tejuino", a traditional Mexican beverage, and studied as β-galactosidase producers for galactooligosaccharides synthesis. Using 400 g/L of lactose, 50 °C, and 15 U/mL of β-galactosidase activity with ethanol-permeabilized cells, the maximum galactooligosaccharides (GOS) yield determined by High performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) was 136 g/L (34% *w*/*w* of total sugars) at 96% of lactose conversion for Bac 55.2 and 145 g/L (36% *w*/*w* of total sugars) at 94% of lactose conversion for Bac 69.1. The main synthesized products were the disaccharides allolactose [Gal-β(1 → 6)-Glc] and 6-galactobiose [Gal-β(1 → 6)-Gal], as well as the trisaccharides 3′-galactosyl-lactose [Gal-β(1 → 3)-Gal-β(1 → 4)-Glc], 6-galactotriose [Gal-β(1 → 6)-Gal-β(1 → 6)-Gal], 3′-galactosyl-allolactose [Gal-β(1 → 3)-Gal-β(1 → 6)-Glc], and 6′-galactosyl-lactose [Gal-β(1 → 6)-Gal-β(1 → 4)-Glc]. The β-galactosidases present in both strains showed a high transgalactosylation activity and formed principally β(1 → 3) and β(1 → 6) linkages. Considering the stability and bifidogenic properties of GOS containing such types of bonds, *P. anthophila* strains Bac 55.2 and Bac 69.1 possess a high potential as novel biocatalysts for prebiotic industrial production.

Keywords: *Pantoea anthophila*; permeabilized cells; β-galactosidase; galactooligosaccharides; transglycosylation; prebiotics

1. Introduction

Actually, a growing interest has been observed in β-galactosidases with transgalactosylation activity for the commercially available production of galactooligosaccharides (GOS), which are added-value lactose derivatives [1]. The enzyme β-galactosidase (EC 3.2.1.23) hydrolyze the terminal non-reducing β-D-galactose in β-D-galactosides. Moreover, this enzyme can perform the transgalactosylation reaction in which the donor (galactose) is transferred to a nucleophilic acceptor different than water; almost any sugar present in the reaction medium can act as an acceptor of galactose, resulting in its elongation to a higher degree of polymerization, thus forming different GOS [2–4].

GOS are complex mixtures of nondigestible carbohydrates, which have a positive effect on the intestinal microflora with a wide variety of health benefits, such as anticarcinogenic effects, serum cholesterol reduction, colon cancer risk reduction, and improvement of intestinal health. These are well established prebiotic ingredients for the functional food industry (dietary fiber) [5–7].

The major application of GOS is its use in the formulation of infant milk formula and infant foods, as they are similar to human milk oligosaccharides [8]. Experimental evidence and clinical data demonstrate that infant milk formula supplemented with GOS regulates the intestinal flora and stimulates the immune system as human milk does [9].

For GOS production, the transgalactosylation reaction is supposed to be kinetically controlled with a maximum synthesis yield depending on the rate of transglycosylation and hydrolysis [8]. Thus, the ratio of the hydrolytic and transferase activities depends on the type of enzyme, concentration of substrate, and reaction conditions (pH, temperature, and time) [10]. Normally, transferase activity is favored at high lactose concentration and elevated temperature [11] and the type of glycosidic bond formed between the galactose moieties depends on the type of the enzyme [12]. Most of the galactosyltransferase enzymes tend to predominantly form GOS with β (1 → 6), β (1 → 3), or β (1 → 4) linkages [13].

β-Galactosidases can be isolated from various sources as they are widely distributed in nature; however, microbial enzymes still being the choice for commercial purposes [14]. The most studied β-galactosidases for GOS synthesis from lactose are those from strains of *Kluyveromyces* [15,16], *Aspergillus* [17,18], *Bacillus* [19,20], and *Bifidobacterium* [5,21]. GOS production with microbial β-galactosidases could be performed using crude, purified, recombinant, or immobilized enzymes and also whole cells [2,8]. In comparison with soluble enzymes, the use of whole cells as a biocatalyst avoids the need for enzyme isolation that can be costly and requires additional processing steps after the microbial culture [13]. Recently, the permeabilized cells of *Kluyveromyces lactis* [22] and *Kluyveromyces marxianus* [23] have been used for GOS synthesis, showing higher stability than soluble β-galactosidase.

Natural substrates rich in lactose, such as milk and their fermented products (Dairy industry), constitute the principal microbial sources of β-galactosidases [10]. Nevertheless, it has been found that some other substrates with starch as the principal carbon source could induce β-galactosidase activity on microorganisms, such as the case of potato starch [24,25] and corn fermented grains [26,27]. Therefore, traditional corn fermentations could be a source of these enzymes, such as the case of Tejuino, which is a traditional Mexican beverage produced from the 2–3 days fermentation of natural nixtamal (maize dough obtained by milling heat treated maize with lime), mixed with water and brown sugar cane [28]. In this type of spontaneous fermentations, it has been isolated a wide variety of microorganisms such as lactic acid bacteria (LAB) and non-LAB fungi and yeasts [29]. Therefore, in order to find new biocatalysts for GOS production, the microbiota of Tejuino was screened. Thus, in the present work, bacterial strains isolated from this beverage were tested for GOS synthesis. The ability of permeabilized cells (ethanol) of two *Pantoea anthophila* strains as biocatalysts for GOS synthesis from lactose was studied. Since the reaction mixtures typically contain numerous oligosaccharides varying in DP and glycosidic linkages, a kinetic study of the transgalactosylation reaction was carried out and the structure of synthesized GOS was analyzed.

2. Results and Discussion

2.1. Specificity of GOS Synthesis with Permeabilized Cells of P. anthophila Strains

Several bacteria (200) from different genera (*Brochothrix*, *Exiguobacterium*, *Pseudomonas*, *Corynebacterium*, *Kurthia*, *Enterococcus*, *Acetobacter*, *Klebsiella*, *Aeromonas*, *Serratia*, *Enterobacter*, *Lactobacillus*, *Leuconostoc*, *Acinetobacter*, *Weissella*, *Trabulsiella*, *Pantoea*, *Staphylococcus*, *Bacillus*) have been isolated from Tejuino, from these strains, non-LAB bacteria strains, which had not been studied previously for the GOS synthesis, were tested as β-galactosidase producers. *P. anthophila* Bac 55.2 and Bac 69.1 strains were selected to study their ability for GOS production since they gave blue colonies on the agar plates containing 2% X-gal. The interest was to evaluate if a β-galactosidase from a different source than the commercial enzymes used in dairy industry may have distinctive characteristics in the nature of the synthesized GOS. Thus, to assess the potential of new β-galactosidases from *P. anthophila*

strains for GOS synthesis, the influence of temperature, pH, and initial β-galactosidase activity was explored using permeabilized cells at 400 g/L of lactose (50 mM sodium phosphate buffer). A factorial design 2^3 was accomplished to investigate the performance of the synthesis reaction by analyzing the GOS yield as a response. According to the literature [1,10], the levels of the variables investigated were temperature: 30 and 50 °C, pH: 5.5 and 7.5, and initial volumetric activity of 5 and 15 U/mL (Supplementary Materials, Tables S1–S4). For both biocatalysts, the highest GOS yield was achieved at 50 °C, pH 7.5, and 15 U/mL. These conditions were used to perform the kinetic study of GOS synthesis in detail by HPAEC-PAD.

Figure 1 shows the chromatogram of the reaction mixture with Bac 55.2 at 10 h, identifying the presence of a least 14 components. Peaks 1, 2, and 5 corresponded to the sugars galactose, glucose, and lactose, respectively. The products of transgalactosylation identified with pure standards were the disaccharides allolactose [Gal-β(1 → 6)-Glc], 6-galactobiose [Gal-β(1 → 6)-Gal], 3-galactosyl-glucose [Gal-β(1 → 3)-Glc], 3-galactobiose [Gal-β(1 → 3)-Gal], and the trisaccharides 6′-galactosyl-lactose [Gal-β(1 → 6)-Gal-β(1 → 4)-Glc], 3′-galactosyl-lactose [Gal-β(1 → 3)-Gal-β(1 → 6)-Glc], and Gal-β(1 → 3)-Gal-β(1 → 3)-Glc. Based on recent data (unpublished) obtained with other β-galactosidases, peaks 8 and 13 were identified as the trisaccharides 6-galactotriose [Gal-β(1 → 6)-Gal-β(1 → 6)-Gal], and 3′-galactosyl-allolactose [Gal-β(1 → 3)-Gal-β(1 → 6)-Glc], respectively. The structures of the main products are presented in Figure 2.

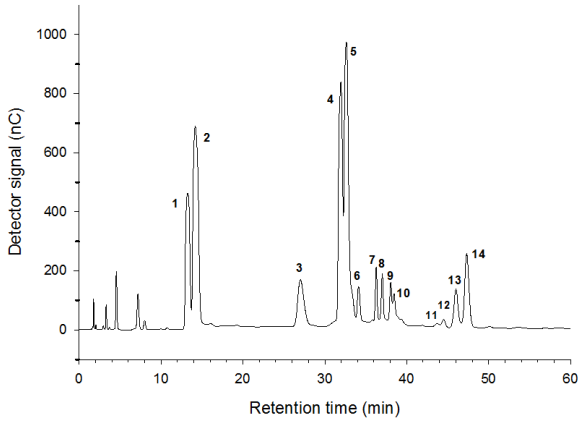

Figure 1. HPAEC-PAD chromatogram of the reaction mixture at 10 h using Bac 55.2 permeabilized cells as a biocatalyst for galactooligosaccharide (GOS) synthesis. Peaks: (1) Galactose; (2) Glucose; (3) Gal-β(1 → 6)-Gal (6-galactobiose); (4) Gal-β(1 → 6)-Glc (allolactose); (5) Lactose; (6) Gal-β(1 → 3)-Gal (3-galactobiose); (7) Gal-β(1 → 6)-Gal-β(1 → 4)-Glc (6′-galactosyl-lactose); (8) Gal-β(1 → 6)-Gal-β(1 → 6)-Gal (6′-galactosyl-6-galactobiose); (9) Gal-β(1 → 3)-Glc (3-galactosyl-glucose); (10) Unknown; (11) Gal-β(1 → 4)-Gal-β(1 → 4)-Glc (4′-galactosyl-lactose); (12) Gal-β(1 → 3)-Gal-β(1 → 3)-Glc; (13) Gal-β(1 → 3)-Gal-β(1 → 6)-Glc (3′-galactosyl-allolactose); (14) Gal-β(1 → 3)-Gal-β(1 → 4)-Glc (3′-galactosyl-lactose).

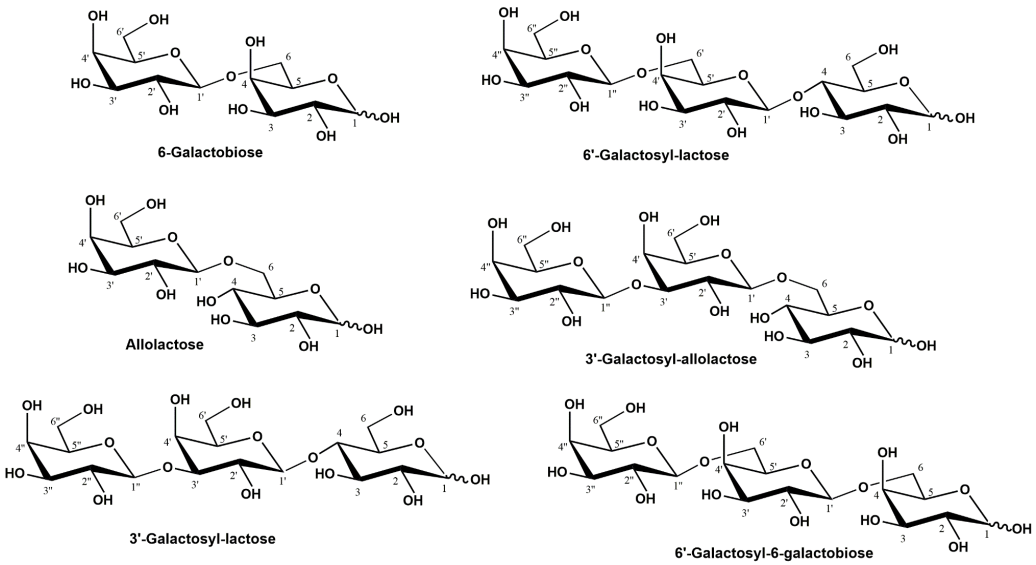

Figure 2. Structures of main six GOS obtained in the reaction with *P. anthophila* permeabilized cells.

2.2. Kinetics and GOS Production Profile with Permeabilized Cells of P. anthophila

Figure 3 illustrates the GOS kinetics with Bac 55.2 and Bac 69.1 *P. anthophila* strains (Figure 3A,B), and Figure 4 shows the GOS formation during lactose conversion by *P. anthophila* permeabilized cells. After 70% of lactose conversion, the rate of GOS synthesis significantly decreased. The maximum GOS concentration was 135.6 and 145 g/L for Bac 55.2 and Bac 69.1, respectively, corresponding to 34% and 36% of the total carbohydrate mixture (Figure 3). These yields were obtained at a lactose conversion between 94% and 96% (Figure 4), in contrast with the behaviour exhibited by most β-galactosidases (e.g., *Bacillus circulans*, *Aspergillus oryzae*), which showed a maximum GOS yield when approximately 50% lactose was consumed [4]. After this point, hydrolysis prevailed over trangalactosylation, and the total GOS concentration was progressively decreased until lactose conversion was around 100% [8]. This GOS yield is comparable with other reported bacterial β-galactosidases, 38% for β-galactosidase from *Lactobacillus reuteri* [30], 31% for β-galactosidase from *Lactobacillus pentosus* [31], 41% for β-galactosidase from *Lactobacillus sakei* [32], and 41% for β-galactosidase from *Bacillus circulans* [33]. In the literature, the typical yields are between 30% and 40% (*w*/*w*).

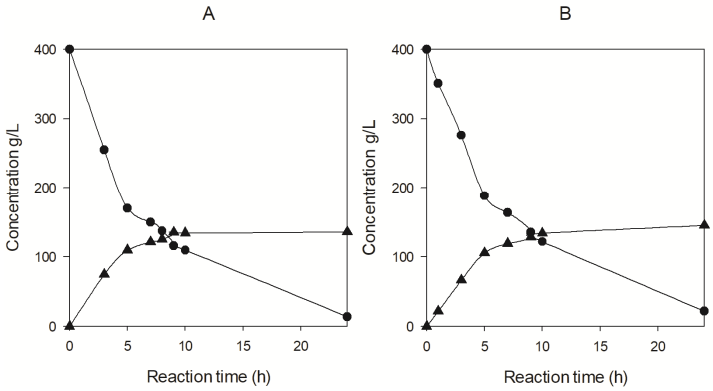

Figure 3. Kinetics of GOS formation at 15 U/mL and pH 7.5, using 400 g/L lactose catalyzed by *P. anthophila* permeabilized cells from: (**A**) Bac 55.2; (**B**) Bac 69.1: (●) Lactose; (▲) Total GOS.

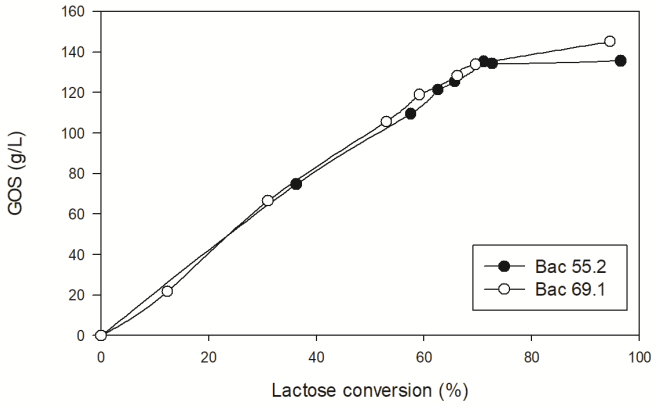

Figure 4. Galactooligosaccharide formation vs. lactose conversion by *P. anthophila* permeabilized cells.

Tables 1 and 2 summarize the carbohydrate composition (in weight) for Bac 55.2 and Bac 69.1, respectively, as a function of reaction time. At the point of maximum GOS concentration (24 h) for both strains (Bac 55.2 and Bac 69.1), the mixture of the total sugars was formed by the monosaccharides glucose and galactose (63% and 58%), lactose (3% and 5%), 6-galactobiose (9% and 8%), allolactose (13% and 15%), and other GOS (12% and 13%), respectively.

Analyzing the GOS profiles produced by the two *P. anthophila* permeabilized cells throughout the reaction, it can be seen that they were quite similar with slight differences in the concentration of individual GOS components. The main disaccharides produced were allolactose (63–65.9 g/L) and 6-galactobiose (33.8–34 g/L) at 70 and 95% of lactose conversion (approx. 10–24 h), while the predominant trisaccharide was 3′-galactosyl-lactose, reaching a maximum (29 g/L) when the lactose conversion was near to 50% (approx. 3–5 h). Thus, the concentration of the different type of GOS produced depended on the reaction time.

The disaccharides 6-galactobiose and allolactose and trisaccharides 3-galactosyl-lactose and 6-galactosyl-lactose have been also described as the principal transgalactosylation products using β-galactosidases from *K. lactis* [22], *Aspergillus aculeatus* [34], and *L. sakei* [32], among others, while in the case of the trisaccharide 6-galactotriose (6′-galactosyl-6-galactobiose), it has not been reported before as a product of enzymatic synthesis by β-galactosidases [35], and its presence in commercial GOS is not common [36]. As it can be seen, this trisaccharide was accumulated at the end of reaction, and the concentration of 6-galactobiose was increased (Tables 1 and 2). It has been found that β-galactosidases from different microbial sources can use other acceptors other than lactose, such as monosaccharides (glucose and galactose) [35]. Thus, it could be possible that the synthesis of 6-galactotriose by permeabilized cells of *P. anthophila* was carried out with 6-galactobiose as acceptor, which is usually produced as a product of the enzymatic transgalactosylation of lactose [35].

The fact that the maximum GOS yield was obtained at nearly complete lactose conversion for the *P. anthophila* strains could be related to the specificity of the involved enzymes. Thus, the major transgalactosylation products (6-galactobiose, allolactose, 6′-galactosyl-lactose, 3′-galactosyl-lactose, 6′-galactosyl-6-galactobiose, and 3′-galactosyl-allolactose) contain β (1 → 6) and β (1 → 3) linkages, which are more resistant to hydrolysis than β(1 → 4) bonds present in lactose or certain GOS like 4′-galactosyl-lactose (minor product in Figure 1) [37]. However, the trisaccharide 3′-galactosyl-lactose was prone to hydrolysis since it reached a notable concentration (approx. 29 g/L) at 3–5 h and it further decreased to nearly 2 g/L at 24 h.

The maximum GOS yield was reached when the reaction was stopped at 24 h. This performance was similar to β-galactosidase from *K. lactis* [22], having a characteristic profile of reaction, with a maximum when a hydrolysis versus transglycosylation competition exists.

Table 1. Carbohydrate composition (g/L) in function of the reaction time using *P. anthophila* Bac 55.2 permeabilized cells as a biocatalyst [a].

Reaction Time (h)	Galactose	Glucose	Lactose	Allolactose	6-Galactobiose	6′-Galactosyl-lactose	3′-Galactosyl-lactose	6-Galactotriose	3′-Galactosyl-allolactose	Other GOS	Total GOS
0	0	0	400	0	0	0	0	0	0	0	0
3	20.0	50.3	255	31.3	4.0	3.3	29.1	0.8	2.5	3.7	74.7
5	35.9	84.6	170	51.6	9.9	4.7	25.3	2.8	6.1	9.1	109.5
7	38.9	90.0	150	53.8	12.6	5.4	24.7	3.7	7.4	13.7	121.3
8	42.5	95.0	137	54.8	15.0	5.8	23.9	4.4	8.2	13.2	125.3
9	45.9	103	116	65.9	16.3	5.8	20.8	4.8	8.3	13.3	135.2
10	49.3	107	109	60.4	18.0	6.2	19.0	5.6	9.0	16.0	134.2
24	95.6	155	13.9	53.3	34.0	6.4	1.5	10.4	5.1	24.9	135.6

[a] Experimental conditions: 400 g/L lactose in 50 mM sodium phosphate buffer (pH 7.5), 15 U/mL (4.7 mg cell mass/mL), 50 °C.

Table 2. Carbohydrate composition (g/L) of the reaction mixture using *P. anthophila* Bac 69.1 permeabilized cells as a biocatalyst [a].

Reaction Time (h)	Galactose	Glucose	Lactose	Allolactose	6-Galactobiose	6′-Galactosyl-lactose	3′-Galactosyl-lactose	6-Galactotriose	3′-Galactosyl-allolactose	Other GOS	Total GOS
0	0	0	400	0	0	0	0	0	0	0	0
1	7.5	19.9	351	0	0	1.3	19.6	0	0	0.8	21.7
3	15.9	41.4	276	26.5	3.0	2.8	28.6	0.6	2.2	2.8	66.5
5	31.4	74.9	188	45.4	9.0	4.8	29.1	2.6	5.7	9.0	105.6
7	34.9	82.7	163	54.8	11.1	5.1	26.9	3.3	6.6	11.1	118.9
9	41.7	94.7	135	59.3	14.3	5.5	23.7	4.2	7.9	13.4	128.3
10	44.2	100	122	63.0	15.9	5.7	21.6	4.8	8.3	14.5	133.8
24	85.7	147	21.9	58.1	33.8	6.4	2.9	10.4	6.5	27.0	145.1

[a] Experimental conditions: 400 g/L lactose in 50 mM sodium phosphate buffer (pH 7.5), 15 U/mL (4.7 mg cell mass/mL), 50 °C.

The microenvironment in the inside of permeabilized cells can exert an influence on the kinetics of the GOS synthesis reaction. Mass transfer limitations make the substrate and product concentrations in contact with the enzyme different from that in the bulk reaction medium, in which the process is being monitored [38]. As a result, a distinctive reaction profile—compared with the observed with soluble enzymes—can be obtained using whole cells. Considering that more than one β-galactosidase could be present in the permeabilized cells, the isolation, purification, and cloning of the enzymes for a deep characterization would be of great interest. It is well established that regiochemistry of the GOS synthesis is primarily controlled by the type of enzyme used [13]. Based on the detailed analysis of the carbohydrate mixture profile, the permeabilized cells of *P. anthophila* strains display a high preference for the formation of galactosyl β(1 → 6) and β(1 → 3) linkages. This result was similar to that obtained with β-galactosidases from LAB [30–32,39].

Recently, the genome of *P. anthophila* [40] was obtained and the in silico analysis showed the presence of two genes that codified β-galactosidases from the GH2 (1045 AA) and GH42 families, respectively, the BLAST analysis revealed that both protein sequences have 89.6–89.8% and 94.2–93.7% identity, respectively, with β-galactosidases from *Pantoea* sp., *P. agglomerans,* and *P. vagans* (Data not shown). Thus, the cloning and expression of theses gene sequences could be interesting in order to know the catalytic properties of these two new enzymes as GOS producers.

2.3. Prebiotic Potential of GOS Synthesized with Permeabilized Cells of P. anthophila

Recent investigations highlighted a correlation between GOS structural characteristics and their prebiotic capability. The effect of the GOS structure on the fermentative properties of the prebiotic trisaccharides was studied and a probiotic bacteria preference towards β-galactosyl residues β(1 → 6) and β(1 → 1) was observed, whereas Bifibobacteria and some lactobacilli were not capable of growing on β(1 → 4) GOS as a carbon source [41]. In research performed with rats, trisaccharides with β(1 → 2) and β(1 → 6) linkages showed higher resistance to gastrointestinal digestion in vivo than those containing β(1 → 4) bonds, suggesting a correlation between linkage type and the prebiotic effect [42]. Moreover, it has been demonstrated that GOS administration to human volunteers with a mixture containing mainly β(1 → 3), β(1 → 4), and β(1 → 6) linkages produced by enzymes from *B. bifidum* exerts a high bifidogenic effect versus a commercial GOS mixture with only β(1 → 4) and β(1 → 6) linkages [5].

The predominant linkages between galactosyl residues in trisaccharides produced by *P. anthophila* permeabilized cells were β(1 → 3) and β(1 → 6), indicating that these oligosaccharides may have a significant prebiotic effect. In addition, previous studies carried out by some of the authors of this work [12] suggested that a disaccharide mixture (allolactose and 6-galactobiose) containing β(1 → 6) linkages displayed similarly in vitro bifidogenic properties than the GOS containing β(1 → 4) and β(1 → 6). In this study, the main disaccharides formed were allolactose and 6-galactobiose, suggesting that they could also contribute to the global prebiotic effect of the mixture. Recent findings in this field indicate that GOS with β(1 → 6) linkages are more recalcitrant than other GOS to degradation and hydrolysis during their passage through the upper gastrointestinal tract, and also might have less steric hindrance, which favors attack by the β-galactosidases produced by probiotic bacteria in the gut [43].

3. Materials and Methods

3.1. Materials

Glucose, galactose, lactose monohydrate, o-nitrophenyl-β-D-galactopyranoside (ONPG), and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) were from Sigma-Aldrich (St. Louis, MO, USA). 3-*O*-β-galactosyl-galactose (3-galactobiose), 4-*O*-β-galactosyl-galactose (4-galactobiose), 6-*O*-β-galactosyl-galactose (6-galactobiose), 6-*O*-β-galactosyl-glucose (allolactose), and 4′-*O*-β-galactosyl-lactose were acquired from Carbosynth (Berkshire, UK). Other galactooligosaccharide standards were purified

according to previous publications [4,22,44]. Other chemicals used for culture medium, buffers, and mobile phases were of analytical grade.

3.2. Bacterial Strains

Pantoea anthophila strains Bac 55.2 and Bac 69.1 were isolated from the tejuino fermentation in Guadalajara City (CIATEJ-collection).

3.3. Screening of β-Galactosidase in Solid Medium

The screening was carried out on agar plates containing 2% lactose, 1% bactopeptone, 1% yeast extract, and 40 µL of 2% X-gal and incubated for 24 h at 30 °C (all substrates were purchased in Sigma-Aldrich). The blue colonies appeared if the bacteria had β-galactosidase activity [45]. Experiments were performed in triplicate.

3.4. Permeabilized Cells Production

P. anthophila strains Bac 55.2, and Bac 69.1 were cultured in a shaker with different Erlenmeyer flasks (2 L) with 500 mL of medium (2% lactose, 1% bactopeptone, 1% yeast extract, and 0.5% NaCl) at 30 °C and 250 rpm. Cells were harvested from 3000 mL culture in exponential growth by centrifugation and washed with sterile distilled water. For the permeabilization procedure, the precipitated cells were resuspended in ethanol (50% *v*/*v*), stirred for 15 min at 4 °C [22], then washed with sterile distilled water and suspended in sodium phosphate buffer (50 mM, pH 7.5). This suspension was used as biocatalyst preparation.

3.5. β-Galactosidase Hydrolytic Activity Assay

The determination of β-galactosidase activity was performed with o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrates at 40 °C, 20 µL of biocatalyst preparation (appropriated dilution to have 1 mg of cell mass/mL) was added to 480 µL of 22 mM ONPG in 50 mM sodium phosphate buffer (pH 7.5). The reaction was stopped after 10 min by the addition of 750 µL of Na_2CO_3 (0.4 M). The release of o-nitrophenol (ONP) was measured by spectrophotometry at 420 nm. The molar extinction coefficient of o-nitrophenol in this solution was determined (757 M^{-1} cm^{-1}). One unit of activity was defined as 1 µmol of ONP hydrolyzed per minute under the assay conditions. Experiments were carried out in triplicate.

3.6. Production of Galactooligosaccharides from Lactose

The reaction was carried out with 400 g/L lactose and permeabilized cells as a biocatalyst (15 U/mL of β-galactosidase activity) in 50 mM sodium phosphate buffer (pH 7.5) at a final volume of 50 mL. These reactions were performed in Erlenmeyer flasks using an orbital shaker (200 rpm and 50 °C). At different times, aliquots of 1 mL were taken, and the reaction was stopped by heating samples at 100 °C during 10 min in a Thermomixer (Eppendorf, Hamburg, Germany). Samples were diluted with grade water (1:400) and filtered using 0.45 µm syringe filters, afterward it was analyzed by HPAEC-PAD. Experiments were carried out in triplicate and results were expressed as mean values.

3.7. HPAEC-PAD Analysis

The determination of the synthetized GOS was performed using an ICS3000 Dionex system, which consists of an SP gradient pump with an electrochemical detector (gold working electrode and Ag/AgCl as a reference electrode) with an autosampler (model AS-HV). All eluents were degassed with helium. A pellicular anion-exchange 4 × 250 mm Carbo-Pack PA-1 column (Dionex, Sunnyvale, CA, USA), which was connected to a Carbo-Pack PA-1 guard column at 30 °C. For eluent preparation, Milli-Q water was used with NaOH (50% *w*/*v*). The initial mobile phase was NaOH (15 Mm) at 1 mL/min for 28 min; then, a gradient of NaOH from 15 mM to 200 mM was carried out for 7 min

and, finally, NaOH at 200 mM was maintained during 25 min. Data acquisition and processing were performed using Chromeleon software. The identification and quantification of different carbohydrates were based on commercially available standards or products purified as described in previous papers [22,35,44].

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/7/8/242/s1. Statistical results of the experimental design for galactooligosaccharide synthesis with permeabilized cells of *Pantoea anthophila* Bac. 55.2 and 69.1 (Tables S1, S2, S3 and S4).

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Author Contributions: C.Y.-Ñ. designed and performed the experiments, analyzed data and wrote the manuscript. B.R.-C. performed and analyzed data from HPAEAC-PAD measurements. J.A., L.A.-D., and F.J.P. participated in the design of the study, contributed with reagents, analyzed data and drafted the manuscript. A.O.B. and A.G. participated as assessors and contributed to the discussion of results.

Conflicts of Interest: The authors declare no conflict of interest.

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