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Article

Reuse of Immobilized *Komagataella phaffii* Cells for the Elimination of D-Glucose in Syrups of Bioactive Carbohydrates

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ABSTRACT: During the synthesis of prebiotic carbohydrates such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), or isomaltooligosaccharides (IMOS), D-glucose is released as a side-product of the transglycosylation process. It is desirable to remove glucose from these sugar mixtures due to its caloric contribution and its effect on caries and diabetes. In this work, we have investigated the use of immobilized *Komagataella phaffii* (formerly *Pichia pastoris)* for elimination of D-glucose and D-fructose in several sugar syrups. *K. phaffii* cells were immobilized in calcium alginate beads to facilitate the separation of the yeast cells from the reaction medium and reuse of the biocatalyst. The immobilized yeasts were successfully reutilized for at least 20 cycles (of 195 min) to remove D-glucose (62.3 g/L) and D-fructose (5.5 g/L) in a FOS syrup, without affecting the concentration of oligosaccharides. Excellent selectivity was also found for elimination of D-glucose (57.2 g/L) in IMOS syrups. The methodology is versatile and easy to scale-up, as demonstrated in the removal of D-glucose (97.5 g/L) and D-fructose (142 g/L) for the purification of heteroglucooligosaccharides synthesized by *Metschnikowia reukaufii* α-glucosidase. In addition, D-glucose (50 g/L) was selectively removed by *K. phaffii* beads in the presence of D-galactose (50 g/L) for at least 20 cycles of 150 min and applied to GOS purification.

KEYWORDS: *Immobilized yeasts, Glucose removal, Pichia pastoris, Bioactive carbohydrates, Alginate entrapment, Whole-cell biocatalysis*

INTRODUCTION

Prebiotics are food components (basically oligosaccharides) that are not digested in the small intestine and reach the colon, where they serve as preferential substrates for beneficial microorganisms of the microbiota, such as lactobacilli and bifidobacteria.[1,2] As a result, certain metabolic substrates (particularly short-chain fatty acids) and micronutrients are produced, which exert beneficial effects to the health of the host.[3] Accepted prebiotics, those whose properties in humans have been scientifically tested, include inulin-type fructans, fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose, and human milk oligosaccharides (HMOs).[4]

Most of the first- and second-generation prebiotic oligosaccharides are produced from disaccharides using either typical glycosyltransferases or hydrolytic enzymes reacting under conditions that favor the transglycosylation reaction in detriment of the substrate hydrolysis. FOS are synthesized from sucrose by fructosyltransferases[5] or β-fructofuranosidases,[6,7] GOS are produced from lactose by β-galactosidases,[8,9] and α-glucosidases are used to transform maltose into isomaltooligosaccharides (IMOS).[10,11]

Because of their intrinsic transglycosylation mechanism, these enzymes release D-glucose as a side-product of the process. D-Glucose causes an increase in sweetness (not always desired) and caloric contribution and promotes the formation of dental caries. In addition, the presence of D-glucose is also a serious threat to the health of people suffering diabetes. This is particularly critical because the consumption of prebiotic ingredients could be considered a tool to prevent and manage type 2 diabetes.[12] Therefore, it is convenient to remove glucose from carbohydrates mixtures of industrial application, in particular in the field of prebiotics.[13] This is extendable to the use of prebiotics in animal nutrition.[14]

Several strategies have been proposed for the elimination of glucose. Chromatographic separation of monosaccharides from complex sugars is expensive and difficult to scale-up.[15] Nanofiltration is a cost-effective and easy-maintenance methodology for the purification and concentration of oligosaccharides.[16] Nanofiltration harnesses their differences in size and utilizes membranes with molecular weight cutoff (MWCO) in the range 200−1000 Da,[17] which are particularly efficient to remove monosaccharides. However, the requirement of specific devices and the use of pressure limit the applications of nanofiltration.

Enzymatic biotransformations constitute an alternative to physicochemical methods to specifically remove D-glucose. D-Xylose isomerase (EC 5.3.1.5) catalyzes the reversible isomerization of D-xylose into D-xylulose but is also referred to as glucose isomerase due to its ability to convert D-glucose

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to D-fructose.[18] Glucose isomerase has been employed to diminish the amount of glucose in carbohydrate syrups and increase the sweetening power, but total elimination of glucose is not feasible.[19,20] Glucose oxidase is also able to remove residual glucose in foods and beverages, but it decreases the pH by formation of gluconic acid and the byproduct H_2O_2 needs to be eliminated using a second enzyme, catalase.[13]

Fermentation with different microorganisms is also an alternative for elimination of D-glucose and other sugars.[21] In this context, Beerens et al. employed baker's yeast to metabolize the contaminating carbohydrates (sucrose, glucose, fructose, and maltose) during the synthesis of kojibiose (not consumed by the yeast).[22] *Saccharomyces cerevisiae* was also used to remove glucose and galactose in mixtures of GOS.[23] *Kluyveromyces marxianus* was also able to remove lactose in GOS mixtures, apart from glucose and galactose.[24] The bacterium *Zymomonas mobilis* ferments specifically glucose, fructose, and sucrose to ethanol and carbon dioxide with minimal byproduct formation.[25]

Interestingly, *Komagataella phaffii* (formerly *Pichia pastoris*) can ferment monosaccharides but not disaccharides and higher oligosaccharides, so it is an excellent choice for glucose elimination without diminishing the amount of carbohydrates of higher size. It was demonstrated that *K. phaffii* can selectively remove glucose and fructose from FOS mixtures.[26,27] Our group reported that *K. phaffii* was very efficient in eliminating D-glucose from a milk whey hydrolysate for the production of D-tagatose.[28] The key point was that the concentration of D-galactose, which is required for the isomerization reaction, remained constant in the presence of *K. phaffii*.

The immobilization of enzymes and whole cells allows the reutilization of biocatalysts and makes product recovery easier because the enzymes/cells can be separated from the reaction mixture.[29] Immobilization allows one to enhance the resistance of biocatalysts against inactivation by high temperatures, extreme pHs, organic cosolvents, inhibitors, or shearing forces.[30] In the case of cells, entrapment in calcium alginate is one of the most successful strategies.[31,32]

In this work, we have analyzed the reutilization of *K. phaffii* cells immobilized in alginate beads for the elimination of D-glucose present in different sugars mixtures, mostly related with prebiotics.

MATERIALS AND METHODS

Enzymes and Reagents. Pectinex Ultra SP-L and the β-galactosidase from *Bifidobacterium bifidum* (Saphera, Novozym 46091) were gently donated by Novozymes A/S (Denmark). *Komagataella phaffii* GS115 (his4) was acquired by Invitrogen (Carlsbad, CA, U.S.A.). D-Galactose and D-glucose were purchased from Sigma-Aldrich, D-fructose from Merck (Darmstadt, Germany), and lactose from Fisher Chemical. 1-Kestose was from TCI Europe (Zwijndrecht, Belgium). Sucrose was from Panreac (Spain). Sodium alginate SG3000 was from Degussa Texturant Systems (Spain). All other reagents and solvents were of the highest purity grade available.

Cultures of *Komagataella phaffii*. *K. phaffii* was initially cultured for 24 h at 30 °C and 1200 rpm in 5 mL of yeast extract peptone dextrose (YEPD) medium containing 0.5% (w/v) yeast extract, 1% (w/v) peptone and, 2% (w/v) D-glucose in distilled water. Chloramphenicol (25 μg/mL) was added to avoid bacterial contamination. The 5 mL of culture was used to inoculate a 500 mL flask containing 50 mL of fresh YEPD medium. After incubation for 16 h at 30 °C and 1200 rpm, the cells were centrifuged (5000×*g* for 10 min) and washed three times with distilled water to remove the remaining medium.

Elimination of D-Glucose and D-Fructose by *Komagataella phaffii*. Several amounts of *K. phaffii* cells (between 0.5 and 2 g of wet weight) were added to 2 mL of a solution containing 170 g/L D-glucose and 10 g/L D-fructose under orbital stirring at 30 °C. Aliquots were withdrawn at 1, 2, and 3 h. The samples were centrifuged at 5000×*g* for 3 min and the concentration of both sugars in the supernatant was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Immobilization of *Komagataella phaffii* Cells. A 4% (w/v) sodium alginate solution was prepared in distilled water, stirred until a homogeneous clear solution was obtained, and allowed to settle for 2 h to eliminate air bubbles. The alginate solution was gently mixed in a ratio 1:1 (w/w) with a suspension of *K. phaffii* cells in distilled water (1:1, w/v). Using a peristaltic pump (P-1, GE Healthcare), the mixture was dropped onto a 0.2 M $CaCl_2$ solution. The drops instantly formed gel beads by ionotropic gelation,[33] which were maintained under magnetic stirring at 100 rpm. After 10 min, the beads were transferred to a fresh 0.2 M $CaCl_2$ solution and kept for another 10 min with mild agitation. The gel beads were then separated from the solution and washed twice with 250 mL of water.

Elimination of D-Glucose/D-Fructose by Alginate-Entrapped *Komagataella phaffii*. Reutilization Studies. A solution (2 mL) containing 170 g/L D-glucose and 10 g/L D-fructose was mixed in an Erlenmeyer flask with 3.8 g (wet weight) of immobilized *K. phaffii* cells. The mixture was incubated at 30 °C and 240 rpm for 2 h 15 min in an orbital shaker (Orbitron, Infors HT, Surrey, UK). After this time, immobilized cells were separated from reaction medium by filtration and washed three times with water to remove any residual substrate and products. Subsequently, alginate gel beads were reused for the next batch. The liquid phase was inactivated at 90 °C for 10 min and analyzed by HPAEC-PAD using a method previously developed in our laboratory.[34] The same protocol was repeated during 20 cycles. The results were presented as percentage of consumed sugars.

Elimination of D-Glucose/D-Fructose in FOS Mixture by Alginate-Entrapped *K. phaffii*. FOS were synthesized from a concentrated sucrose solution by β-fructofuranosidase from *Aspergillus aculeatus* (Pectinex Ultra SP-L) as previously described.[6,35] The mixture (5 mL) was then treated with 3.8 g of *K. phaffii* cells immobilized in calcium alginate and incubated at 30 °C. Aliquots were harvested at different times and analyzed by HPAEC-PAD as described previously until glucose and fructose disappeared.[34] The operational stability of the biocatalyst was analyzed during 20 cycles using 5 mL of a FOS syrup containing 62.3 g/L of D-glucose and 5.5 g/L of D-fructose. For each cycle, the syrup was mixed with the *K. phaffii* gel beads in an Erlenmeyer flask and incubated at 30 °C for 3 h 15 min in an orbital shaker (Orbitron, Infors) at 240 rpm. After each cycle, the concentration of the different sugars was measured as described above. NeoFOS for HPAEC-PAD quantification were obtained in the laboratory as previously described.[33]

Elimination of D-Glucose in IMOS Mixture by Alginate-Entrapped *K. phaffii*. Isomaltooligosaccharides (IMOs) syrup was prepared from 200 g/L of maltose in 50 mM sodium acetate (pH 4.5) and α-glucosidase GAM1 from *Schwanniomyces occidentalis* (25 U/mL). Reaction time was 3 h. The syrup (5 mL) was mixed with 3.8 g of *K. phaffii* cells immobilized in calcium alginate and incubated at 30 °C and 240 rpm. Samples were taken every 30 min, inactivated at 95 °C for 10 min, and analyzed by HPAEC-PAD. To separate the reaction products, a gradient method employing 200 mM NaOH and a solution containing 100 mM NaOH and 320 mM sodium acetate as eluents was employed.

Elimination of D-Glucose/D-Fructose in Hetero-GlcOS Mixture by Alginate-Entrapped *K. phaffii*. Heteroglucooligosaccharides (hetero-GlcOS) were produced from 500 g/L sucrose by the recombinant α-glucosidase from *Metschnikowia reukaufii* expressed in *Escherichia coli* as described elsewhere.[36] The reaction mixture (25 mL) was then incubated at 30 °C with 10 g of *K. phaffii* cells immobilized in calcium alginate. Aliquots were harvested at different

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times and analyzed by HPAEC-PAD as described until glucose and fructose disappeared.[11]

Elimination of D-Glucose from D-Galactose/D-Glucose Mixture by Alginate-Entrapped *Komagataella phaffii*. Reutilization Studies. A solution (5 mL) containing 50 g/L of D-glucose and 50 g/L of D-galactose was treated with 3.8 g of immobilized *Komagataella phaffii* cells. The mixture was incubated at 30 °C for 2 h 30 min in an orbital shaker (Orbitron, Infors HT) at 240 rpm. Each batch was treated under the conditions specified above (Section 2.5). Samples were analyzed by HPAEC-PAD using a method previously reported.[28] The same protocol was repeated during 20 cycles.

Elimination of D-Glucose in GOS Mixture by Alginate-Entrapped *K. phaffii*. GOS were synthesized from concentrated lactose by commercial β-galactosidase Saphera from *Bifidobacterium bifidum* as previously described.[37] The mixture was then treated with *K. phaffii* cells immobilized in calcium alginate and incubated at 30 °C. Aliquots were harvested at different times and analyzed by HPAEC-PAD as described until complete disappearance of D-glucose was observed.[37]

General Conditions for HPAEC-PAD Analyses. Analysis of carbohydrates was carried out by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a ICS3000 Dionex system consisting of a SP gradient pump, an electrochemical detector with a gold working electrode, Ag/AgCl as a reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. Pellicular anion-exchange Carbo-Pack columns (4 × 250 mm; PA-1 for FOS, GOS, and hetero-GlcOS; PA-100 for IMOS) connected to the corresponding 4 × 50 mm CarboPac guard columns were used at 30 °C. Eluent preparation was performed with Milli-Q water, 50% (w/v) NaOH, and sodium acetate trihydrate. The elution methods for each family of compounds are described in previous publications.[11,34,37] The peaks were analyzed using Chromeleon software. Identification of the different carbohydrates was carried out employing synthesized and commercially available standards.

RESULTS

Elimination of D-Glucose/D-Fructose by *Komagataella phaffii* Cells Entrapped in Alginate. *K. phaffii* cells were cultivated in YEPD medium for 16 h to an exponential phase of growth and its effectiveness to utilize glucose and fructose was evaluated.[38] Different amounts of the yeast (between 0.5 and 2 g of wet weight) were added to a solution (2 mL) containing D-glucose (170 g/L) and D-fructose (10 g/L), which simulated a typical reaction mixture obtained in FOS synthesis.[39] The concentration of sugars was analyzed after 1, 2, and 3 h by HPAEC-PAD. We observed that approximately 2 g of cells per milliliter assured an efficient elimination of D-glucose and D-fructose in 2−3 h.

A 4% (w/v) sodium alginate solution was mixed in a ratio 1:1 (w/w) with an aqueous suspension containing 2 g of *K. phaffii* cells per milliliter. After pumping onto a concentrated $CaCl_2$ solution, gel beads with a high density of yeast cells were obtained (Figure 1). The resulting biocatalysts were tested as glucose (and eventually fructose) removers in different carbohydrate syrups.

The solution containing D-glucose (170 g/L) and D-fructose (10 g/L) was mixed with approximately 1.9 g of *K. phaffii* beads (per mL of syrup) for 2 h 15 min at 30 °C. After each reaction cycle, the beads were separated and the remaining concentration of the two monosaccharides was analyzed by HPAEC-PAD. The process was repeated 20 times. Figure 2 represents the percentage of removal of each sugar. As shown, the removal of D-glucose and D-fructose was complete during the first 11 cycles. During the next nine cycles, the disappearance of glucose and fructose was also very effective (99.8% and 95.0%, respectively).

Figure 1. Cells of *Komagataella phaffii* entrapped in calcium alginate. Small spheres of about 3 mm diameter were obtained.

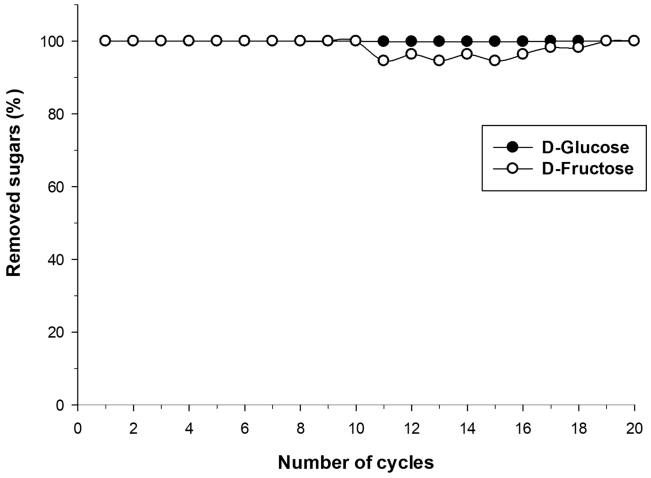

Figure 2. Reuse of *K. phaffii* beads for monosaccharide removal in a mixture of D-glucose (170 g/L) and D-fructose (10 g/L).

Interestingly, we did not find other metabolites in the chromatograms during the process of D-glucose and D-fructose removal, which suggested a complete degradation of the sugars to CO_2 and H_2O. This was confirmed by the air bubbles of carbon dioxide in the fixed-bed bioreactors packed with the alginate-entrapped *K. phaffii* (data not shown).

Elimination of D-Glucose/D-Fructose in FOS Syrups and Operational Stability. FOS of the inulin-type constitute one of the most established groups of prebiotics. They are currently produced at multiton scale from concentrated sucrose solutions using fungal transfructosylating enzymes such as those from *Aspergillus niger*, *Aspergillus oryzae*, and *Aureobasidium pullulans*, which yield a mixture of inulin-type FOS containing β(2 → 1) linkages. The synthesized syrups contain different amounts of glucose, fructose, and sucrose, which must be removed to increase the purity of the final product.

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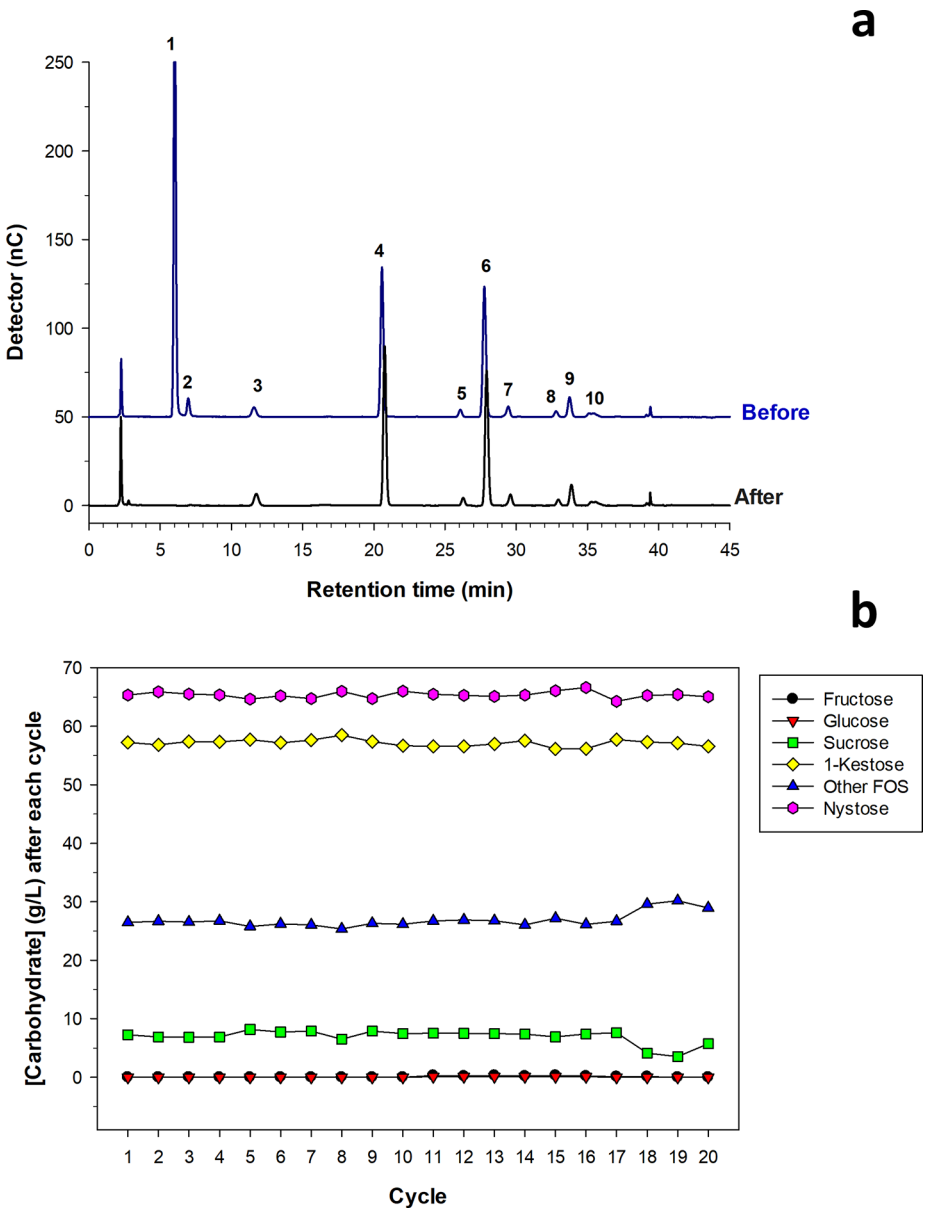

Figure 3. (a) HPAEC-PAD analysis of a FOS syrup synthesized by β-fructofuranosidase from *A. aculeatus* before (black line) and after (blue line) the treatment with *K. phaffii* immobilized cells. Peaks assignation: (1) D-glucose; (2) D-fructose; (3) sucrose; (4) 1-kestose; (5) neokestose; (6) nystose; (7) neonystose; (8) unknown; (9) ^{1}F-fructosylnystose; (10) ^{1}F-fructosyl-fructosylnystose. (b) Sugar concentration after each cycle of treatment with *K. phaffii* immobilized in alginate beads.

We assayed the alginate-entrapped *K. phaffii* cells to remove monosaccharides in a FOS mixture obtained with the commercial enzyme Pectinex Ultra SP-L as described in Materials and Methods. Apart from glucose (62.3 g/L), fructose (5.5 g/L) and sucrose (7.3 g/L), the mixture contained ^{1}F-FOS as main products: 1-kestose (57.2 g/L), 1-nystose (65.3 g/L), and higher oligosaccharides (mostly ^{1}F-fructosylnystose) (17.9 g/L). It also contained minor amounts of neoFOS (neokestose, neonystose, and so forth) (8.5 g/L) with $\beta(2 \rightarrow 6)$ linkages between fructose and the glucosyl moiety. As shown in Figure 3a, when the FOS syrup was treated with *K. phaffii* beads, the disappearance of D-glucose and D-fructose was complete without affecting the concentration of sucrose and FOS, both of the ^{1}F- and ^{6}G- type.

We determined the operational stability of these immobilized biocatalysts to remove both monosaccharides. Figure 3b shows the concentration of the main sugars in the mixture after each cycle with *K. phaffii* immobilized cells. After 20 cycles, the concentration of FOS remained constant, thus showing the capacity of these biocatalysts to remove the undesired D-glucose and D-fructose.

Elimination of D-Glucose in IMOS Syrup. When using α-glucosidases for the preparation of IMOS, a significant amount of glucose and digestible carbohydrates with low degree of polymerization (DP) are formed. These can be removed by membrane techniques, chromatography, and fermentation with *Saccharomyces cerevisiae*.[40,41]

In the present work, IMOS syrup was prepared using α-glucosidase GAM1 from *Schwanniomyces occidentalis* and 200 g/L maltose as substrate in 3 h. The synthesized syrup contained a high concentration of D-glucose (57.2 g/L), residual maltose (8.0 g/L), and several products with $\alpha(1 \rightarrow$

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4), $\alpha(1 \rightarrow 6)$, or mixed linkages: isomaltose (26.3 g/L), isomaltotriose (9.8 g/L), panose (32.9 g/L), maltotriose (14.6 g/L), and isomaltotetraose (2.4 g/L), apart from kojibiose (2.1 g/L) and other oligosaccharides (46.8 g/L). The syrup was incubated with alginate-entrapped *K. phaffii* beads. Samples were taken every 15 min until the peak of glucose disappeared completely. As shown in Figure 4, the remaining glucose was consumed in 3 h 15 min without an appreciable decrease in the amount of the rest of carbohydrates and without formation of undesired byproducts.

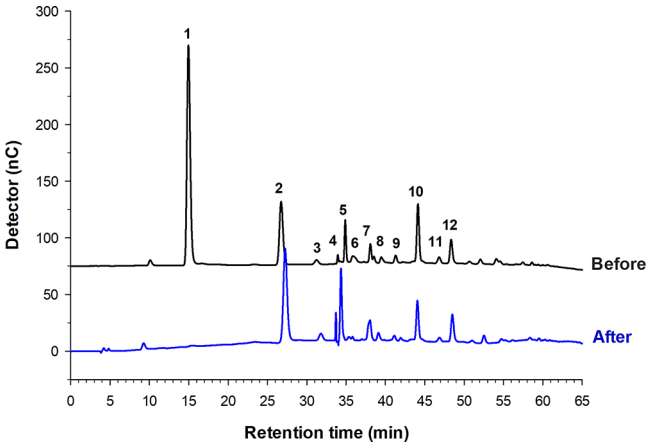

Figure 4. HPAEC-PAD analysis before (black line) and after (blue line) the treatment with *K. phaffii* immobilized cells of IMOS syrup synthesized by α-glucosidase GAM1 from *Schwanniomyces occidentalis*. Peaks assignation: (1) glucose; (2) isomaltose; (3) kojibiose; (5) isomaltotriose; (7) maltose; (8) isomaltotetraose; (10) panose; (12) maltotriose; (4,6,9,11) unknown.

Elimination of D-Glucose/D-Fructose in Hetero-GlcOS Syrup. An α-glucosidase from the yeast *Metschnikowia reukaufii* catalyzes the synthesis of a series of hetero-GlcOS naturally present in honey, mainly isomelezitose and trehalulose.[11] Since sucrose is the glucosyl donor of these reactions and the enzyme exhibits some hydrolytic activity, significant amounts of D-glucose and D-fructose are present in the final syrup.

We scaled-up the synthetic process to 25 mL using 500 g/L of sucrose as a substrate and recombinant α-glucosidase from *M. reukaufii* as a biocatalyst.[36] Once the concentration of hetero-GlcOS reached a maximum, the reaction was stopped and then incubated with *K. phaffii* beads. Aliquots were harvested at different times and sugars quantified by HPAEC-PAD (Figure 5a) as described.[11] D-glucose (97.5 g/L) and D-fructose (142 g/L) disappeared completely in approximately 24−28 h (Figure 5b), whereas the concentration of sucrose remained unchanged (thus increasing slightly its percentage). D-glucose was removed by *K. phaffii* faster than D-fructose. The removal of monosaccharides in this type of mixtures may facilitate the purification of bioactive hetero-GlcOS like the synthesized herein. Thus, the percentage of hetero-GlcOS increased from 44.8 to 80.2% at the end of the treatment (Figure 5a). This experiment demonstrates that the use of immobilized *K. phaffii* cells can be easily scaled-up without loss of efficiency.

Elimination of D-Glucose in the Presence of D-Galactose. Application to GOS Syrups. A solution containing 50 g/L of D-glucose and 50 g/L of D-galactose

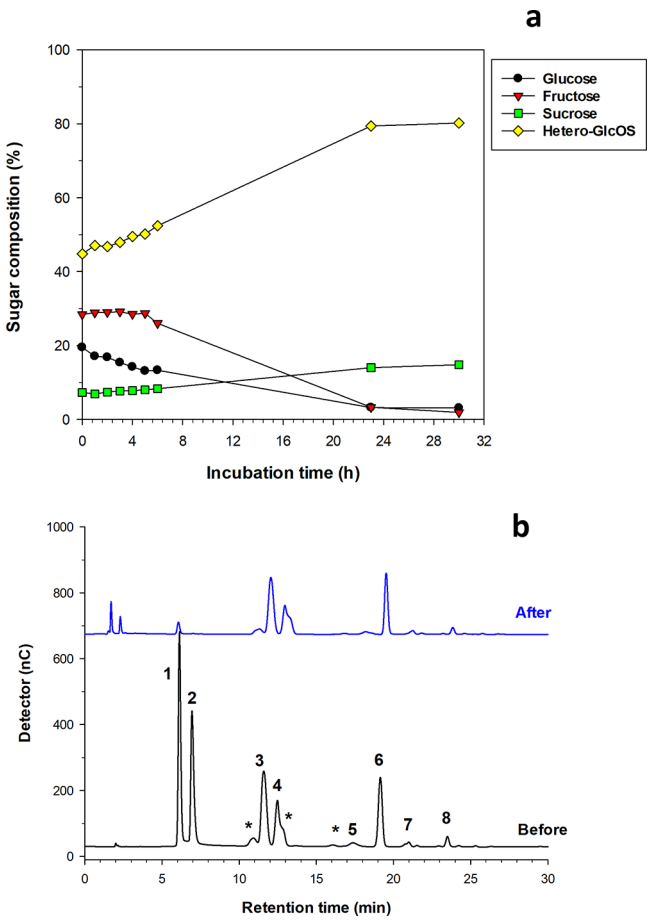

Figure 5. (a) Evolution of sugar composition (weight percentage of glucose, fructose, sucrose and total hetero-GlcOS) upon treatment with *K. phaffii* beads. (b) HPAEC-PAD analysis before (black line) and after (blue line) the treatment with *K. phaffii* immobilized cells of hetero-GlcOS synthesized by α-glucosidase from *Metschnikowia reukaufii*. Peak assignation: (1) D-glucose; (2) D-fructose; (3) sucrose; (4) trehalulose; (5) melezitose; (6) isomelezitose; (7) theanderose; (8) erlose; (*) unknown products.

was mixed with alginate-entrapped *K. phaffii* cells (approximately 0.75 g of *K. phaffii* beads per milliliters of syrup) and the mixture incubated at 30 °C for 2 h 30 min. After each reaction cycle, the beads were separated, and the concentration of sugars was analyzed as described. We performed 20 reaction cycles, and the results are represented in Figure 6. The removal of D-glucose was very satisfactory during the 20 cycles. However, at cycle 14 a small peak of glucose which corresponded to 0.9 g/L of this sugar, was detected. The highest peak of D-glucose that we obtained was in cycle 20, representing 8.2% of initial glucose (4.1 g/L). No significant decrease in D-galactose concentration was detected during the 20 cycles, which indicated a high selectivity of the yeast to remove D-glucose in this type of mixtures.

We produced GOS with β-galactosidase from *Bifidobacterium bifidum* using a concentrated (400 g/L) lactose solution to favor transglycosylation (see Materials and Methods). At the point of maximum GOS formation, the remaining D-glucose, quantified by HPAEC-PAD, was 150 g/L. The other components in the syrup were galactose (103 g/L), lactose (37.4 g/L), and a series of GOS among which stand out 3′-galactosyl-lactose (17.7 g/L), allolactose (16.2 g/L), 3-O-β-galactosyl-glucose (9.0 g/L), and 3-galactobiose (8.2 g/L).

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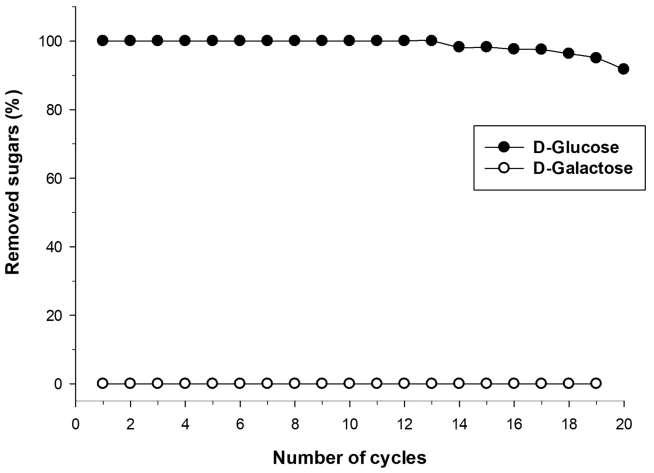

Figure 6. Reuse of *Komagataella phaffii* immobilized in alginate beads for the elimination of D-glucose (50 g/L) in the presence of D-galactose (50 g/L).

Because the amount of glucose was so high, it took approximately 5 h 30 min to remove it completely from the reaction using the *K. phaffii* immobilized cells. Figure 7

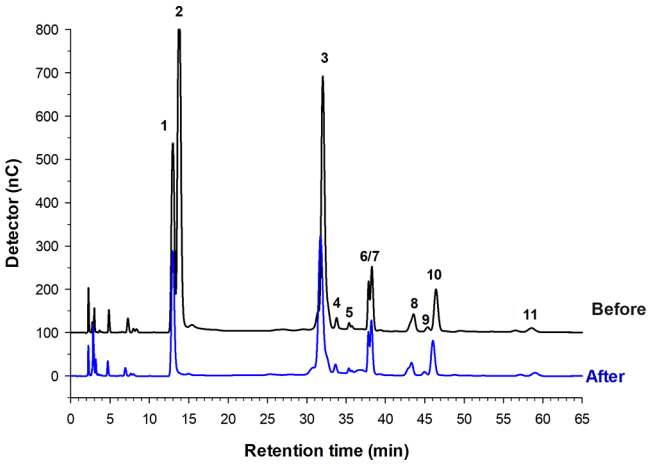

Figure 7. HPAEC-PAD analysis before (black line) and after (blue line) the treatment with *K. phaffii* beads of a GOS syrup synthesized by β-galactosidase from *B. bifidum* under optimal conditions. Peaks assignation: (1) galactose; (2) glucose; (3) lactose; (4) 3-galactobiose; (5) 4-galactobiose; (6) 3-O-β-galactosyl-glucose; (7) unidentified; (8) Gal-β(1 → 3)-Gal-β(1 → 3)-Glc; (9) 3′-galactosyl-allolactose; (10) 3′-galactosyl-lactose; (11) 3′-O-β-(3-galactobiosyl)-lactose.

illustrates the comparison of chromatograms before and after the treatment with *K. phaffii* entrapped in alginate beads. As shown, D-galactose, D-lactose, and GOS remained unaltered after the treatment with immobilized cells of the yeast.

DISCUSSION

Komagataella phaffii is able to use D-glucose, glycerol, and methanol as carbon sources but not D-galactose.[42,43] Avila-Fernandez et al. reported that *K. phaffii* cells could be used to remove D-glucose and D-fructose selectively in FOS syrups obtained by agave fructan hydrolysis.[26] In a recent work aimed to synthesize the functional sweetener D-tagatose, we demonstrated that *K. phaffii* was able to eliminate D-glucose with high selectivity in the presence of D-galactose.[28]

The objective of the present work was to determine if *K. phaffii* cells could be immobilized (and reused) to remove D-glucose and/or D-fructose in different sugar mixtures, namely those related with the synthesis of prebiotic oligosaccharides. Industrial benefits of cell immobilization include lower costs of downstream processing because cells can be easily recovered and recycled.[43,44] In addition, immobilization can protect cells against shear damage in the reactor.[45]

Surface binding and entrapment techniques are the most commonly used strategies to immobilize cells. Entrapped cells are confined within a protective matrix structure reducing cell release. In this context, polysaccharides such as alginate or carrageenan are excellent matrix materials due to their biocompatibility and acceptability in food applications. For the above reasons, we decided to immobilize *K. phaffii* cells by entrapment in calcium alginate beads.

We first analyzed the operational stability of immobilized *K. phaffii* cells for D-glucose/D-fructose removal. The results were very satisfactory for at least 20 cycles of 2 h 15 min, without the appearance of intermediate metabolites. Furthermore, we applied these biocatalysts to remove D-glucose and D-fructose in a typical reaction mixture of FOS. These oligosaccharides can be obtained by controlled hydrolysis of inulin or enzymatic transfructosylation of sucrose catalyzed by β-fructofuranosidases. FOS are composed of various fructosyl units linked to the sucrose skeleton by different glycosidic bonds depending on the source of the enzyme.[34] Our purpose was to eliminate the maximum amount of monosaccharides without affecting the bioactive carbohydrates and evaluate the operational stability of the immobilized biocatalysts.

We demonstrated that entrapped *K. phaffii* cells were able to remove D-glucose and D-fructose without affecting the concentration of FOS, both of the ^{1}F- and ^{6}G- type. This methodology is more simple than chromatography and nanofiltration, which require special equipment. The use of immobilized yeasts also offers several advantages compared with enzymatic strategies based on the use of glucose isomerase (GI)[20] or glucose oxidase (GOx). These enzymes generate byproducts (fructose by GI; D-gluconic acid and hydrogen peroxide by GOx), which must be eliminated from the reaction mixture. D-Gluconic acid also causes a decrease of the pH. The H_2O_2 generated by GOx is typically removed by an auxiliary enzyme, catalase.[13]

IMOS are glucose oligomers linked by α(1 → 6) glycosidic bonds, but commercial IMOs syrups also contain α(1 → 4) bonds.[46] In addition, IMOS may contain α(1 → 3) and/or α(1 → 2) branches.[47] IMOS can be synthesized by hydrolysis of dextran[48] or by transglycosylation using bacterial dextransucrases with sucrose as substrate[49] or fungal α-glucosidases with maltose as substrate.[50] IMOS are oligosaccharides with low digestibility and exhibit prebiotic and anticariogenic properties, and lead the market of functional carbohydrates in some Asian countries.[46] The presence of glucose is undesirable in IMOS. We demonstrated that this monosaccharide can be selectively removed in IMOS syrups by entrapped *K. phaffii* cells.

Metschnikowia reukaufii is an ubiquitous budding yeast that colonizes floral nectaries.[51] Its α-glucosidase is capable to synthesize of a series of bioactive hetero-GlcOS, including isomelezitose and trehalulose. In this work, we scaled the biocatalytic process to 25 mL using 500 g/L of sucrose as the substrate and recombinant α-glucosidase from *M. reukaufii* as a biocatalyst. Our results indicated that glucose and fructose can be removed in the final reaction mixture by incubation with

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immobilized yeast cells. This fact can greatly help the purification process of hetero-GlcOS and other bioactive oligosaccharides.

The next step was to verify if *K. phaffii* beads could be reused to remove D-glucose in the presence of D-galactose. We previously demonstrated that free cells were able to selectively eliminate D-glucose.[28] The immobilized biocatalysts were very efficient for at least 20 cycles of 150 min without consumption of D-galactose. We applied this methodology to remove D-glucose in GOS syrups. Apart from their hydrolytic activity, β-galactosidases are able to catalyze transgalactosylation reactions in which lactose or other carbohydrates act as acceptor molecules of galactosyl units yielding di-, tri, tetra-, or even higher oligomers (GOS) that resemble HMOs and exhibit prebiotic properties.[9,37] Our results showed that the concentration of GOS, D-lactose, and D-galactose remained constant during the treatment with immobilized *K. phaffii*, while D-glucose completely disappeared.

In conclusion, *K. phaffii* alginate-entrapped beads are very efficient and selective to remove D-glucose and/or D-fructose in carbohydrate syrups. These monosaccharides are undesirable in the field of bioactive sugars. Compared with other methodologies, the use of immobilized yeasts is less expensive, easier to scale-up, more specific and easily reusable. We have demonstrated that this strategy can be employed for the elimination of D-glucose (and eventually D-fructose) in FOS, IMOS, hetero-GlcOS, and GOS syrups. In all cases, the monosaccharides disappeared without any significant decrease of the sugars of interest and without the formation of undesired byproducts.

The most attractive issue of *K. phaffii* beads is that they can be reused at least for 20 times with minimum loss of efficiency. The use of these immobilized biocatalysts can be expanded to other sugar syrups in which the presence of D-glucose is undesirable. The combination of *K. phaffii* cells with other microorganisms (e.g., *S. cerevisiae, Kluyveromyces marxianus* or *Zymomonas mobilis*) could render sugar syrups free of digestible carbohydrates.

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Notes

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ABBREVIATIONS USED

FOS; fructooligosaccharides; GOS; galactooligosaccharides; IMOS; isomaltooligosaccharides; HMOS; human milk oligosaccharides; MWCO; molecular weight cutoff; YEPD; yeast extract peptone dextrose; HPAEC-PAD; high-performance anion-exchange chromatography with pulsed amperometric detection; GlcOS; glucooligosaccharides.

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