Synthesis of Isomaltooligosaccharides by *Saccharomyces cerevisiae* Cells Expressing *Aspergillus niger* α-Glucosidase

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**ABSTRACT:** The α-glucosidase encoded by the aglA gene of *Aspergillus niger* is a secreted enzyme belonging to family 31 of glycoside hydrolases. This enzyme has a retaining mechanism of action and displays transglycosylating activity that makes it amenable to be used for the synthesis of isomaltooligosaccharides (IMOs). We have expressed the aglA gene in *Saccharomyces cerevisiae* under control of a galactose-inducible promoter. Recombinant yeast cells expressing the aglA gene produced extracellular α-glucosidase activity about half of which appeared cell bound whereas the other half was released into the culture medium. With maltose as the substrate, panose is the main transglycosylation product after 8 h of incubation, whereas isomaltose is predominant after 24 h. Isomaltose also becomes predominant at shorter times if a mixture of maltose and glucose is used instead of maltose. To facilitate IMO production, we have designed a procedure by which yeast cells can be used directly as the catalytic agent. For this purpose, we expressed in *S. cerevisiae* gene constructs in which the aglA gene is fused to glycosylphosphatidylinositol anchor sequences, from the yeast SED1 gene, that determine the covalent binding of the hybrid protein to the cell membrane. The resulting hybrid enzymes were stably attached to the cell surface. The cells from cultures of recombinant yeast strains expressing aglA-SED1 constructions can be used to produce IMOs in successive batches.

**INTRODUCTION**

Oligosaccharides are important compounds for the food and pharmaceutical industries because of their growing use as prebiotics and antioxidants and for drug delivery.1 A relevant type of oligosaccharides are isomaltooligosaccharides (IMOs), roughly defined from a chemical point of view as short glucose oligomers (between two and nine units) containing α-(1→6), α-(1→3), or α-(1→2) linkages. Examples are isomaltose, panose, isomaltotriose, nigerosylglucose, and kojibiosylglucose, among others. While α-(1→4) linkages that constitute the backbone of starch and glycogen molecules are readily digested by intestinal enzymes, α-(1→6) linkages, as well as α-(1→3) and α-(1→2), are not so easily hydrolyzed, one of the characteristics that confer to these compounds their recognized status of prebiotics.2,3

Enzymatic synthesis has become a preferred procedure for oligosaccharide production over alternative chemical synthesis or polysaccharide hydrolysis. Transferases and retaining glycoside hydrolases which are naturally endowed with transferase activity and do not require activated substrates are generally used.4 In the specific case of IMO synthesis, the enzymes used are dextrantransferases or alternansaccharases from lactic acid bacteria, or fungal transferases and α-glucosidases.5–7 The use of glycoside hydrolases, belonging to family GH31, for IMO synthesis is possible because these are retaining enzymes, whose catalytic mechanism allows them to act as transferases. The reactions mediated by these enzymes comprise two consecutive steps. In the first one, a nucleophilic residue (Asp or Glu) attacks a glycosidic bond, forming a covalent bond with the split glycoside. In the second step, another (acid/base) catalytic residue (again Asp or Glu) mediates the transference of the glycosyl moiety to an acceptor molecule, most commonly water, in which case hydrolysis is accomplished. If a molecule other than water, frequently the glycoside substrate itself, acts as the acceptor, the result of the reaction is a transglycosylation. The structure of a glycoside hydrolase enzyme, in particular its catalytic center, determines its transglycosylating activity, its preference for specific acceptors, and therefore the pattern of transglycosylation products that it may produce.7–11

IMO synthesizing capability has been reported for the α-glucosidase produced by *Aspergillus niger*. Panose and isomaltose are the main transglycosylation products when maltose is used as the substrate.12–15 Both glycosides show prebiotic properties16,17 and can be used as low calorie sweeteners.6,18 Therefore, the development of efficient procedures for large scale synthesis of IMOs is a significant biotechnological goal. The cost of IMO production at the industrial level is largely determined by enzyme production, recovery and purification. A simpler and cheaper alternative relies on the direct use of the producer microorganism in a “one pot” process.19 To apply this concept to the production of IMOs, we have constructed *Saccharomyces cerevisiae* strains...
that display *A. niger* α-glucosidase covalently attached to the cell surface. Our results show that the engineered yeast provides an efficient procedure for the production of panose and isomaltose with high yield.

## RESULTS

**Expression of *A. niger* *aglA* Gene in *S. cerevisiae*.** *S. cerevisiae* BY4741, used as the host strain, is isogenic to the wild type S288C. It carries in its genome functional genes encoding maltose permease (*AGT1* and *MAL31*) and intracellular α-glucosidase (*MAL12* and *MAL32*) but lacks a gene (*MAL13* or *MAL33*) encoding the activator protein required for the expression of the permease and the glucosidase. Consequently, strain BY4741 is phenotypically maltose negative, a desirable characteristic because it avoids interference with the analysis of heterologous enzyme activity.

Galactose induced cultures of strain BY4741 transformed with plasmid pSSP-AG harboring the *aglA* gene showed α-glucosidase activity, ca. 1.4 mU/mL, almost equally distributed between the supernatant fraction (53%) and cell-bound (47%). A control culture of the same strain transformed with the cloning vector, assayed as a control, showed no detectable enzyme activity. Production of secreted α-glucosidase by the *aglA* yeast transformant could be visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a diffused protein band of high molecular mass (above 240 kDa), indicating a high degree of glycosylation (Figure 1, lane C). The protein mass decreased to ca. 135 kDa, after treatment with Endo H (Figure 1, lane D). This indicates effective albeit not complete deglycosylation because the expected mass of the protein monomer, as deduced from the amino acid sequence, is 106 kDa.

**Analysis of IMO Formation.** Figure 2 shows the products recovered from the incubation of *S. cerevisiae* cells, expressing the *aglA* gene with 30% maltose as the substrate. After 8 h incubation, the only reaction product present at a significant concentration was panose. This trisaccharide is formed by transglycosylation between two maltose molecules, one of them acting as the glucosyl donor and the other as the acceptor. The glucosyl residue from the donor maltose is transferred to the acceptor, forming an α-(1→6) linkage. It is noteworthy that the absence of glucose, despite the fact that panose and glucose are produced at equimolar concentrations in the transglycosylation reaction, indicates that the monosaccharide is consumed by the yeast during this initial period. After 24 h incubation, the predominant transglycosylation product is isomaltose, with lower but significant amounts of panose and isomaltotriose. At this stage, maltose is detectable although in residual amounts. Glucose accumulates, likely as the cells are no longer metabolically active after such an extended incubation period at 50 °C. Other unidentified peaks eluting at higher retention times likely correspond to α-(1→6) IMOs of higher degrees of polymerization.

Time course of IMO production at different concentrations of maltose was monitored. Figure 3A shows that panose production increases rapidly, at similar rates for different maltose concentrations, up to a maximum (400 mM after 20 h for 60% maltose), and then decreases. As it could be expected, maltose kinetics showed an initial linear decay with similar slopes for different substrate concentrations (Figure 3B). Isomaltose and glucose formation was only detectable using the higher maltose concentrations at long incubation times (Figure 3C,D). After an initial delay, both products were progressively accumulated. The fact that isomaltose was only formed upon glucose accumulation suggests that isomaltose is formed by transglycosylation with glucose as the acceptor molecule and not by a possible alternative mechanism (i.e. isomerization). Moreover, such a delay in isomaltose synthesis compared to panose production was not observed when using a purified enzyme, in which glucose is not sequestered from the medium.12,13

Figure 4 shows a model of *A. niger* α-glucosidase with isomaltose and panose docked at the active site of *A. niger* α-glucosidase. The model was adjusted to superimpose the orientation of the catalytic residues (Asp490 and Asp660, putative nucleophile and acid/base catalytic residues) with the corresponding residues of sugar beet α-glucosidase (PDB id 3WEO). We assume that the disposition of the catalytic residues must be the same in both proteins because they catalyze the same reaction, and the degree of sequence identity (37%) becomes nearly 80% if only the residues likely involved in direct interaction with the substrate are considered. The model shows the formation of isomaltose and panose and suggests a site for the coupling of the acceptor molecule (glucose or maltose).

![Figure 1: SDS-PAGE analysis of proteins recovered from the culture supernatant of *S. cerevisiae* BY4741 (pMCAG1) (C,D) and a control of the same strain transformed with the cloning vector (A,B) and with (B,D) or without (A,C) treatment with Endo H glycosidase. The arrows point differential bands present in the pSSP-AG-transformed strain, which produces extracellular α-glucosidase activity.](Image 100x346 to 261x439)
Synthesis of Panose or Isomaltose by Transglycosylation as a Function of the Glucosyl Acceptor.

As it has been mentioned before, the results reported in Figure 3 indicate that both maltose and glucose can act as glucosyl acceptors in the transglycosylation reaction. Thus, when maltose is used as the substrate, at shorter reaction times, maltose is the only available...
acceptor yielding panose as the transglycosylation product. As the reaction progresses, the glucose concentration increases, becoming available as the acceptor to produce isomaltose. To confirm that isomaltose is formed by transglycosylation with glucose as the acceptor, and not by the direct isomerization of maltose, we assayed the production of IMOs, at different initial ratios of glucose to maltose (Figure 6). For this analysis, yeast transformants expressing AglA_Sed1B were preincubated at 50 °C during 24 h to avoid the interference of glucose consumption by cell metabolism. Because this α-glucosidase is stably attached to the cell surface and remains active (Figure 5B), pretreated cells with an attenuated metabolism could be used as the enzyme source. Compared to the previous experiment (Figure 3), cell pretreatment at 50 °C allowed, after 5 h of incubation with maltose, a higher accumulation of glucose (ca. 200 mM) and a higher yield of isomaltose (ca. 60 mM), even in the absence of exogenously added glucose. An increase in the glucose concentration is not expected to enhance the isomaltose production if this disaccharide is formed by isomerization of maltose. Therefore, positive correlation between isomaltose production and initial glucose concentration (compare Figures 6A and 3C) indicates a transglycosylation mechanism. Using pretreated cells, after 1 h incubation with maltose, the ratio of isomaltose to panose was 0.2 and the ratio of glucose to maltose had the same value. When the initial ratio of glucose to maltose

![Figure 4.](image1)

**Figure 4.** (A) Model of the catalytic site of *A. niger* α-glucosidase. Catalytic residues D660 (putative nucleophile) and D490 (putative acid/base catalyst) are depicted in blue. (B) Complex with isomaltose. (C) Complex with panose. The glucose at the reducing end is colored in pink. Putative hydrogen bonds with this residue are highlighted with dashed lines.

![Figure 5.](image2)

**Figure 5.** Enzyme activity of yeast cells expressing wild type or Sed1 hybrid versions of *A. niger* α-glucosidase. (A) Analysis of α-glucosidase activity, secreted into the culture medium (orange bars) or cell-associated (green bars). (B) Initial or remaining cell-associated α-glucosidase activity after several 24 h periods of incubation of the cells at 50 °C and pH 5.5. Initial activity (green bars), 1 × 24 h (yellow bars), 2 × 24 h (brown bars), 3 × 24 h (turquoise bars), and 6 × 24 h (gray). Error bars correspond to standard deviation of triplicates.

![Figure 6.](image3)

**Figure 6.** Kinetics of isomaltose (A) and panose (B) production. The reactions were carried out by incubation of pretreated cells (at 50 °C, pH 5.5 for 24 h) of transformant yeast strain (AglA-Sed1B) with different amounts of maltose and glucose: 300 mM maltose (blue squares), 300 mM maltose and 300 mM of glucose (orange circles), and 300 mM of maltose and 1.2 M of glucose (green triangles).
was increased (Figure 6), the panose yield decreased while isomaltose increased. In both cases, the ratio of isomaltose to panose after 1 h incubation was equivalent to that of glucose to maltose. These results suggest that glucose and maltose compete for the same binding site in the enzyme, acting as acceptors of the glucosyl residue, to synthesize isomaltose and panose, respectively. However, both products showed different kinetics at longer incubation times. Although panose reached a maximum concentration before being degraded (Figures 3A and 6B), isomaltose was progressively accumulated (Figure 6A), indicating that panose is a better substrate for the enzyme.

**DISCUSSION**

Industrial production of IMOs is carried out by the combined action of hydrolytic and transferase enzymes on starch, which yields a complex mixture of α-glucosides. Although in general terms, IMOs are considered to have prebiotic and other health promoting properties, the heterogeneous and variable nature or commercial preparations makes it difficult to ascribe observed effects to specific compounds. The development of new procedures for the production of IMOs of defined composition, specifically, panose and isomaltose, is a significant biotechnological goal because of present and potential applications of these compounds. Our results provide a procedure for inexpensive, food-grade production of panose and isomaltose, using an engineered yeast strain that displays a cell wall-linked α-glucosidase. The production of panose or isomaltose can be modulated by the ratio of the acceptor sugars (maltose or glucose) present in the reaction.

A conclusion derived from our results is that the formation of isomaltose takes place by transglycosylation and not by intramolecular isomerization of maltose. The enzyme-bound glucosyl residue formed as a reaction intermediate can be transferred to the acceptor molecule, either glucose or maltose, to synthesize isomaltose or panose, respectively. This is a significant result because other retaining glycosyl hydrolases have isomerase activity. For example, trehalase synthesize catalyzes the isomerization of maltose to trehalose through an intramolecular isomerization of the substrate, whereas the enzyme is unable to incorporate exogenously added glucose. A conspicuous case is that of the LacZ α-galactosidase from *Escherichia coli*. This enzyme converts lactose into allolactose through an isomerization reaction, in which glucose and galactose, resulting from the hydrolysis of lactose, are bound again, through a different linkage, before leaving the active site. LacZ is also able to catalyze transglycosylation of a galactosyl moiety to exogenous glucose added at high concentrations. The mechanistic difference between isomerization and transglycosylation is important from a biotechnological point of view. If isomaltose synthesis was carried out by isomerization, isomaltose would be formed together with panose. However, our results show that in the absence of glucose, panose is the sole transglycosylation product. On the other hand, increasing the glucose concentration displaces the mechanism toward the production of isomaltose.

Panose and isomaltose yield at different ratios of glucose to maltose indicate that both maltose and glucose compete with efficiency similar to glucosyl acceptors. However, once formed, the fate of the two products is notably different. Although panose reaches a maximum and then declines, isomaltose keeps increasing (Figure 6). This result is explained by the higher affinity of AglA for panose as a substrate for hydrolysis, compared to isomaltose. Structural modeling of *A. niger* α-glucosidase with panose docked at the active site supports this observation.

As shown in Figure 4, the glucosyl residue at the reducing end of panose may form three putative hydrogen bonds to D225, S495, and H596. This would represent an additional stabilization for the formation of the enzyme—substrate complex, as compared to the isomaltose molecule, which is equivalent to panose except for this glucosyl end. These three residues are conserved in the α-glucosidase from *Schwanniomyces occidentalis*, which has a product profile similar to that of AglA. Interestingly, H596 is substituted by Ser in the α-glucosidase from *Xanthophyllomyces dendrorhous*, which synthesizes panose and maltotriose with similar efficiencies. This suggests that the substrate represented by these residues is involved in the product specificity of the enzyme.

In conclusion, this study presents a simple procedure to synthesize IMOs (specifically, panose and isomaltose) with high yield. The cell surface display system used here allows the implementation of a one-pot procedure by which yeast biomass can be used and recycled as the catalytic agent for IMO production.

**MATERIALS AND METHODS**

**Microbial Strains and Culture Media.** *Saccharomyces cerevisiae* BY4741 (MATa AGT1 MAL12 mal13Δ MAL13 MAL32 mal33Δ his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), used as the yeast host strain, is isogenic to the standard wild type S288C (http://www.yeastgenome.org). *A. niger* CECT 2775 was obtained from Colección Española de Cultivos Tipo (http://cect.org). Composition of complete (yeast extract peptone dextrose, YPD) and minimal (synthetic defined) media is described by Sherman.

**Cloning Procedures.** The *A. niger* *aglA* gene (GenBank: D45356.1) encodes an α-glucosidase (EC 3.2.1.20) belonging to family 31 of glycoside hydrolases. For cloning of this gene, *A. niger* was cultured in liquid YPD for 36 h at 26 °C. The mycelium was recovered by filtration, frozen, and disrupted by grinding in liquid nitrogen with a mortar and pestle. RNA was isolated using RNA PLUS (MP Biomedicals), based on a procedure described by Chomczynski and Sacchi. Reverse transcription was carried out with the SuperScript III First-Strand Kit (Invitrogen). The coding gene sequence, excluding its signal peptide, was amplified from the complimentary DNA using primers 2MC70 (forward): CTGTCATAGCGCGACGTACCTCATATTTAC (XbaI site underlined) and 2MC71 (reverse): TCACAGACCTTTTTACATTCCAATACCCAGTTTTCC (SphI site underlined) and 2MC71 (reverse): TCACAGACCTTTTTACATTCCAATACCCAGTTTTCC (SphI site underlined). The amplified DNA fragment was cloned in NheI/HindIII sites of plasmid pSSP-GOX, a modified version of the yeast expression vector pEMBLyx4, that carries the coding region of the STA1 gene signal peptide, under control of a galactose inducible promoter (CYC-GAL). The resulting plasmid (pSSP-AG) was used to transform *S. cerevisiae* BY4741. The same yeast strain was also transformed with vector pEMBLyx4 to be used as a negative control.

Hybrid genes of *A. niger* *aglA* and *S. cerevisiae* *SED1* were constructed as follows. The coding sequence of the *aglA* gene version that includes a 5′ extension corresponding to the *S. cerevisiae* STA1 signal peptide was amplified with primers 2MC81 (forward): CCAAGATCCATGGTGAGCTTAAGACC (BamHI site underlined) and 2MC82 (reverse): CCTAATGCGTGGCGATTCAGATTCC (XbaI site underlined). The amplified DNA fragment was cloned in NheI/HindIII sites of plasmid pSSP-GOX, a modified version of the yeast expression vector pEMBLyx4, that carries the coding region of the STA1 gene signal peptide, under control of a galactose inducible promoter (CYC-GAL). The resulting plasmid (pSSP-AG) was used to transform *S. cerevisiae* BY4741. The same yeast strain was also transformed with vector pEMBLyx4 to be used as a negative control.

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**DISCLOSURE**

The authors declare no competing financial interests.
lacks the first 109 amino acids. For SED1_A synthesis, the gene, excluding the initial ATG, was amplified from S. cerevisiae genomic DNA with primers 2MC83 (forward): CCTAGTG-CATCGATGTTAAATATTGACTGCTCTTTGCTGC (SpII site underlined) and 2MC84 (reverse): CACAAAGCTTT-TATATAGTTAATAACATAGCAACACCAG (HindIII site underlined). SED1B was amplified using primers 2MC83 (forward): CCTAGTGCAATGCTGTCCTTCAACACTAAGTG- TAC (SpII site underlined) and 2MC84 (reverse): CA- CAAGCTTTATAGTAACATACGCAACACCAG (HindIII site underlined). The hybrid fusions of STAA-glA to either SEDIA or SEDIB were cloned at BamHI/HindIII sites of pEMBlx4, under the control of the CYC-GAL galactose inducible promoter (plasmids pSSP-AG_Sed1A and pSSP- AG_Sed1B, respectively).

Determination of α-Glucosidase Production and Activity. Enzyme (α-glucosidase) activity was measured with p-nitrophenyl-α-D-glucopyranoside (pNPG) as the substrate, in cells collected by centrifugation from galactose-induced cultures of transformed S. cerevisiae strains and in the supernatant of the cultures. Cultures were inoculated in liquid YPD medium at a cell density equivalent to A600 of ca. 0.5 and cultivated under agitation at 30 °C up to an A600 of around 2.0. At this point, galactose was added at 1% final concentration and the cultures were grown for an additional period of approximately 18 h. Cell and supernatant fractions of 1 mL aliquots from the induced cultures were separated by centrifugation. The cells were resuspended in 250 μL of citrate phosphate buffer pH 5.5. To 100 μL of sample (either supernatant or cell fraction), 150 μL of pNPG solution in citrate phosphate buffer pH 5.5 was added, making the final concentration of the substrate 1 mM. The reaction mixtures were incubated at 37 °C. The reactions were stopped at different time intervals by adding 500 μL of 1 M sodium carbonate. Released p-nitrophenol was measured spectrophotometrically at A400.

Extracellular α-glucosidase produced by S. cerevisiae was also analyzed by SDS-PAGE. The protein in the supernatant of the induced yeast cultures was concentrated about 65 times by centrifugation and heated at 95 °C for 10 min to stop the reaction. IMO synthesis was analyzed by ion exchange chromatography using high-performance liquid chromatography (HPLC) instrument equipped with CarbonPac PA100 columns and a pulsed amperometric detector (Dionex, Thermo Fisher Scientific) as previously described. Sugars were identified by their chromatographic retention times. The sugar concentration was calculated by interpolation of their peak areas. Glucose, isomalto-, isomaltotriose, maltose, panose, and maltooltriose (Sigma) were used as standards.

Protein Structure Analysis. A structural model of A. niger α-glucosidase was obtained using I-TASSER.37 The model was adjusted to superimpose the orientation of the putative catalytic residues (D490 and D660, nucleophile and acid/base, respectively) with the corresponding residues of the sugar beet α-glucosidase (PDB id 3WEO). The protein structure was analyzed with the PyMOL Molecular Graphics System (Schrödinger, LLC). Molecular docking of glucose, panose, and isomaltose was carried out with Maestro 11 (Schrödinger, LLC), taking as a reference the interaction of sugar beet α-glucosidase with acarbose.36,39

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Author Contributions

M.C.-V., J.M.-N. and J.P. designed the research, M.C.-V. and J.M.-N. performed the experimental work, M.C.-V., J.M.-N. and J.P. analyzed the data, and J.P. and J.M.-N. wrote the article.

Notes

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