


Efficient production of isomelezitose by a glucosyltransferase activity in *Metschnikowia reukaufii* cell extracts

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Summary

***Metschnikowia reukaufii* is a widespread yeast able to grow in the plants' floral nectaries, an environment of extreme conditions with sucrose concentrations exceeding 400 g l⁻¹, which led us into the search for enzymatic activities involved in this sugar use/transformation. New oligosaccharides were produced by transglucosylation processes employing *M. reukaufii* cell extracts in overload-sucrose reactions. These products were purified and structurally characterized by MS-ESI and NMR techniques. The reaction mixture included new sugars showing a great variety of glycosidic bonds including α -(1→1), α -(1→3) and α -(1→6) linkages. The main product synthesized was the trisaccharide isomelezitose, whose maximum concentration reached 81 g l⁻¹, the highest amount reported for any unmodified enzyme or microbial extract. In addition, 51 g l⁻¹ of the disaccharide trehalulose was also produced. Both sugars**

show potential nutraceutical and prebiotic properties. Interestingly, the sugar mixture obtained in the biosynthetic reactions also contained oligosaccharides such as esculose, a rare trisaccharide with no previous NMR structure elucidation, as well as erlose, melezitose and theanderose. All the sugars produced are naturally found in honey. These compounds are of biotechnological interest due to their potential food, cosmeceutical and pharmaceutical applications.

Introduction

Bioactive oligosaccharides are highly demanded products mainly for food and pharmaceutical industries, and their biotechnological production has increased in Asia, Europe and USA markets (Goffin *et al.*, 2011; Sorndech *et al.*, 2018). This sort of carbohydrates include lactulose, lactosucrose, soybean-oligosaccharides, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and xylo-oligosaccharides (XOS), as well as gluco-oligosaccharides (GlcOS) containing α -(1→6) (isomalto-oligosaccharides, IMOS), α -(1→4), α -(1→2) and α -(1→3) linkages (Crittenden and Playne, 1996; Kim *et al.*, 2014). In addition to its potential prebiotic effects, which would promote the colonic beneficial bacteria growth/activity (mainly *Lactobacilli* sp. and *Bifidobacteria* sp.), several physicochemical and biological properties would make them of interest for food, and biotechnological applications (Remaud-Simeon *et al.*, 2000; Singla and Chakkaravarthi, 2017; Singh *et al.*, 2017). For instance, the consumption of bioactive oligosaccharides is associated with an increase in calcium and magnesium absorption, the prevention of colon cancer and cardiovascular diseases, or even with the immune system improvement (Patel and Goyal, 2012; Florowska *et al.*, 2016). Besides, oligosaccharides are frequently included in food and beverages as non-cariogenic ingredients, sugar substitutes or bulking agents (Crittenden and Playne, 1996; Kim *et al.*, 2014). In addition, the gelling properties of certain bioactive carbohydrates allow their use as low-caloric fat replacement that maintain the spreadable texture of creamy products like yoghurts and sauces, or due to its moisture retention capacity increase foodstuff shelf life (Al-Sheraji *et al.*, 2013).

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Bioactive oligosaccharides can be produced by direct isolation from natural sources (plants, milk and honey), chemical processes and biotechnological procedures employing biocatalysts. The enzymatic methods can include the controlled hydrolysis of polysaccharides or the synthesis of oligomers from mono- and di-saccharides, processes with high substrate specificity, regio- and stereo- selectivity (Díez-Municio *et al.*, 2014). Enzymes involved in the bioactive carbohydrate synthesis are glycosidases (EC 3.2) or glycosyltransferases (EC 2.4), that catalyse the hydrolysis of glycosidic bonds of oligo- and polysaccharides or the glycosyl group-transfer from a donor to an acceptor molecule respectively (Plou *et al.*, 2007). It was extensively reported that the hydrolytic activity of glycosidases, such as α / β -glucosidases or β -fructofuranosidases, could be shifted towards synthesis (transglycosylating activity) using a high substrate concentration. Thus, the α -glucosidase from the yeast *Xanthophyllomyces dendrorhous* produced IMOS (mainly panose) using 200–525 g l⁻¹ maltose (Fernández-Arrojo *et al.*, 2007; Gutierrez-Alonso *et al.*, 2016) and β -fructofuranosidases from *Aspergillus awamori* or yeast such as *Schwanniomyces occidentalis* and *Saccharomyces cerevisiae* synthesized FOS using different sucrose-rich by-products from a variety of palm tree dates (the first) or 600 g l⁻¹ sucrose (the last two) (Álvaro-Benito *et al.*, 2007; Lafraya *et al.*, 2011; Smaali *et al.*, 2012). In fact, sucrose is a relatively cheap and renewable raw material that has already been widely employed in the synthesis of bioactive oligosaccharides (Monsan and Ouarné, 2009), and among them several hetero-oligosaccharides. As examples, novel hetero-FOS were obtained using sucrose as fructosyl donor, different sugar acceptors and β -fructofuranosidases from microorganisms such as *X. dendrorhous* (Gimeno-Pérez *et al.*, 2014) or *S. occidentalis* (Piedrabuena *et al.*, 2016). In addition, the α -glucosidase from *Bacillus licheniformis* produced the trisaccharide theanderose by transglycosylation of sucrose, while glucansucrases GtfA and Gtf180 from *Lactobacillus reuteri* generated different lactose derivatives by transferring the glucose unit of sucrose to lactose (Nimpiboon *et al.*, 2011; Pham *et al.*, 2017).

Metschnikowia reukaufii is a widespread budding yeast (*Ascomycota*, *Saccharomycetales*) able to grow in the floral nectaries, an environment of extreme conditions with sucrose concentrations exceeding 400 g l⁻¹. It is the predominant microorganism in nectaries of plants such as *Helleborus foetidus* (Pozo *et al.*, 2011; Belisle *et al.*, 2012; Herrera, 2014; Dhami *et al.*, 2016). Although the ecological function of this yeast is relatively unknown, several studies relate it to the nectar sugar composition, the synthesis of volatile compounds or even with the nectaries temperature increases, all factors that may have incidence on the pollinators behaviour

(Herrera and Pozo, 2010; Canto *et al.*, 2015; Yang *et al.*, 2019).

In this work, we have analysed an enzymatic activity associated to *M. reukaufii* cell extracts able to hydrolyse sucrose and generate a mixture of oligosaccharides, which were purified and structurally characterized using mass spectrometry (MS-ESI) and nuclear magnetic resonance (NMR) techniques. The mixture of sugars obtained is found naturally in honey, and includes several molecules of biotechnological interest whose production profiles were also evaluated.

Results and discussion

Analysis of the standard sucrose-splitting activity in Metschnikowia reukaufii cultures

Initially, the potential hydrolytic activity on sucrose of *M. reukaufii* was assessed using a solid rich medium for yeast including sucrose and bromothymol blue (BTB), a pH indicator (yellow, greenish and blue in acidic, neutral and basic solutions respectively). *Schwanniomyces occidentalis* and *Pichia pastoris* were used as positive and negative controls in this assay due to their proven or absent capacity to hydrolyse sucrose respectively (Álvaro-Benito *et al.*, 2007; Gimeno-Pérez *et al.*, 2015). As expected, all yeast species were able to grow on this rich medium. Thus, *S. occidentalis* turned the colour of YEP(S-BTB) plates from greenish to yellow due to the acidic compounds formed as a result of sucrose utilization, whereas *P. pastoris* turned it into dark blue because of the use of peptone and ammonium excretion. As proof of its sucrose-splitting activity, the medium where *M. reukaufii* grew was also yellow (Fig. 1A).

Metschnikowia reukaufii was grown in YEP liquid media supplemented with different carbon sources as described in the Experimental procedures section and the standard sucrose-splitting activity was evaluated in both, extracellular and cell-associated fractions. Hydrolytic activity towards sucrose was detected in the cellular fractions of yeast grown in media containing sucrose, inulin, maltose, raffinose and lactose (Fig. 1B), but not in the extracellular fractions in any of the analysed conditions. The maximum activity detected (~ 4.2 U ml⁻¹) was obtained using sucrose-based culture, the major component in the nectar sugar mixture of *Helleborus foetidus* (Pozo *et al.*, 2011), and was expressed at the beginning of the microorganism stationary growth phase (OD₆₆₀ ~ 3.6 ; ~ 4 h; Fig. 1C). As expected, no activity was detected when using a glucose-based medium, a very common process known as glucose (catabolic) repression, since it is widely documented that glucose represses the expression of a large number of genes involved among others, in the use of alternative carbon sources and gluconeogenesis (Trumbly, 1992; Kayikci and Nielsen, 2015).

Analysis of the transglycosylating activity in *M. reukaufii* cell extracts

The search and characterization of new enzymatic activities involved in the production of oligosaccharides with possible bioactive properties is an interesting biotechnological challenge. Therefore, the potential transferase activity of the *M. reukaufii* cell extracts, which were able to hydrolyse sucrose, was assessed using a high this sugar concentration (500 g l^{-1}) and the composition of the reaction mixtures analysed by HPLC.

A representative HPLC-ELSD chromatographic profile of the sugar mixture (72 h of reaction) is shown in Fig. 2A. In addition to the expected signals corresponding to sucrose (peak 3) and its hydrolysis products, fructose (peak 1) and glucose (peak 2), new signals (peak 4 to 8) were detected in the chromatograms. Interestingly, the amount of glucose detected was significantly lower than that of fructose in the HPLC-ELSD chromatograms analysed, suggesting that a potential glucosyltransferase activity was transferring the glucosyl moiety from sucrose (glucosyl donor) to another molecule (glucosyl acceptor). As expected, only the sucrose signal was detected at time 0 reactions (Fig. 2A) and in a control reaction lacking the enzymatic extract (data not shown). Besides, only sucrose was detected when using enzymatic extracts obtained from yeast cells grown in a glucose-based medium (data not shown), very probably due to the absence of activity caused by the glucose repression effect already mentioned.

Identification and structural characterization of the transfer products

The new oligosaccharides synthesized in overload-sucrose reaction (peaks 5 to 8, Fig. 2A) were purified by semi-preparative HPLC. Although it was attempted, it was not possible to purify peak 4 due to its proximity to the sucrose signal. The purity of each collected fraction was analysed by HPAEC-PAD given the higher sensitivity of this system compared to that of HPLC-ELSD. Figure 2B shows the HPAEC-PAD chromatographic profile of the reaction mixture including sucrose as single substrate after 72 h and each one of the purified product profiles. Although a high degree of purity was achieved in all the analysed fractions, sugars corresponding to the purified peaks 5 and 7 show traces of sucrose and the compound responsible of the peak 8 in the HPLC-ELSD chromatogram (Fig. 2A) respectively.

The molecular weight of the purified products was determined by MS-ESI using a hybrid QTOF analyser. The mass spectra of the purified compound responsible for peak 5 showed a major signal in positive mode at m/z 365.11 corresponding to a disaccharide- $[\text{Na}]^+$ form

(Fig. S1), while those for peaks 6, 7 and 8 at m/z 527.16 to trisaccharides- $[\text{Na}]^+$ (Figs S2, S3 and S4). A standard NMR analysis permitted to deduce the structure of these new oligosaccharides. The compound in the purified fraction-peak 5 was identified as trehalulose: $[\alpha\text{-D-Glc-(1}\rightarrow\text{1)-}\beta\text{-D-Fru}]$. The results from the 2D $^1\text{H-}^{13}\text{C}$ spectra (Fig. 3A) allowed the identification of two signal sets corresponding to the most abundant tautomeric forms, in which the β -fructosyl ring exists as a pyranosyl (major form) and furanosyl (minor form) mixture: $\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\beta\text{-D-Frup}$ and $\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\beta\text{-D-Fruf}$ respectively. Although the α -fructosyl tautomers of trehalulose were not detected, they should be present in the α/β equilibrium as described by Lichtenthaler and Rönninger (1990).

In the purified fraction corresponding to peak 6, three different trisaccharides were characterized from the combination of the signals from COSY, TOCSY, NOESY, HSQC and HMBC experiments. The major components were identified as erlose $[\alpha\text{-D-Glcp-(1}\rightarrow\text{4)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{2)-}\beta\text{-D-Fruf}]$ (Fig. S5A) and melezitose $[\alpha\text{-D-Glcp-(1}\rightarrow\text{3)-}\beta\text{-D-Fruf-(2}\rightarrow\text{1)-}\alpha\text{-D-Glcp}]$ (Fig. S5B). The data obtained for these compounds were reasonably consistent with those previously reported by Wei *et al.* (1997) and Antenuis *et al.* (1975) respectively. Despite its low concentration, the third component of the mixture was also analysed. The presence of two α -Glc moieties (G1 and G2) was deduced from the measurement of the J_{H1H2} couplings (4 Hz). The analysis of the standard COSY, HSQC (Fig. 4A), Heteronuclear Multiple Bond Correlation (HMBC) and HSQC-TOCSY (Fig. 4B) experiments showed the existence of $[\alpha\text{-Glc-(1}\rightarrow\text{2)-}\beta\text{-Fru}]$ and $[\alpha\text{-Glc-(1}\rightarrow\text{x)-Fru/Glc}]$ for G1 and G2 respectively. Moreover, the analysis of the NOESY experiments (Fig. 4C, left) permitted to deduce the existence of a G1-H1/F-H1 cross peak highlighting the spatial proximity of these residues, strongly suggesting the existence of an $\alpha\text{-G1-(1}\rightarrow\text{2)-}\beta\text{-Fru}$ fragment. One glycosylated carbon was detected at 80 ppm (Fig. 4B, red). This carbon was part of the G1 residue, as deduced from HSQC-TOCSY experiment (Fig. 4B, black). Both anomeric G1-H1 and G2-H1 protons showed correlation in the HMBC spectra with this carbon (Fig. 4B, blue), thus demonstrating that G2 was linked to G1. This G2-G1 connectivity led to different trisaccharide molecules. The $\alpha\text{-G2-(1}\rightarrow\text{2)-}\alpha\text{-G1-(1}\rightarrow\text{2)-}\beta\text{-Fru}$ trisaccharide has been previously described (Fischer *et al.*, 2006), but no matching with the reported NMR data was found (Table S1). The $\alpha\text{-G2-(1}\rightarrow\text{3)-}\alpha\text{-G1-(1}\rightarrow\text{2)-}\beta\text{-Fru}$ trisaccharide has also been described (Chiba *et al.*, 1984), but no NMR data were published. Our NMR data for the major product fit well with those described for the $\alpha\text{-G2-(1}\rightarrow\text{4)-}\alpha\text{-G1-(1}\rightarrow\text{2)-}\beta\text{-Fru}$ (Erlöse) (Wei *et al.*, 1997). Finally, the NMR data reported for the $\alpha\text{-G2-(1}\rightarrow\text{6)-}\alpha\text{-G1-(1}\rightarrow\text{2)-}\beta\text{-Fru}$ trisaccharide

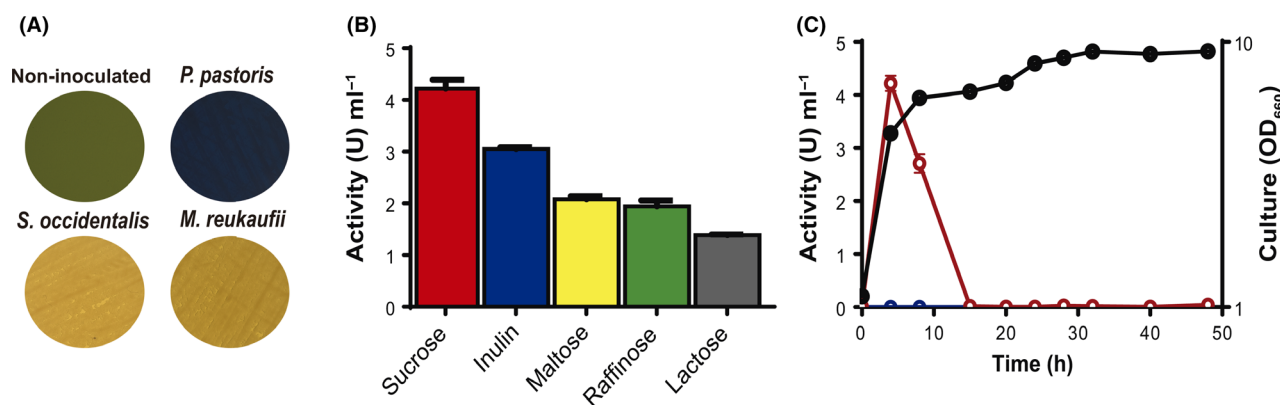


Fig. 1. Analysis of the standard hydrolytic activity on sucrose expressed in *M. reukaufii* cultures.

A. Activity assay in YEP(S-BTB) medium. Plate inoculated with *M. reukaufii* cells (yellow) is shown. *S. occidentalis* (yellow) and *P. pastoris* (dark blue) or non-inoculated (greenish) plates were used as positive and negative controls respectively.

B. Maximum activity levels detected in YEP cultures containing the referred sugars, all 20 g l⁻¹.

C. Time-course of standard activity. Inocula from *M. reukaufii* were grown (black circles) in 250 ml flasks containing 25 ml of YEP(S) medium and samples were withdrawn at the indicated times. OD₆₆₀ measurements differing < 1% were obtained for all cultures analysed. The extracellular (blue circles) and cell-associated (red circles) activity were determined using sucrose as substrate. Each point represents the average of three independent measurements with its standard deviation.

(Theanderose) do not match with those deduced for our molecule (Ruiz-Aceituno *et al.*, 2017). Therefore, the acquired evidences, after elimination of the previously described products, suggest that the third component of peak 6 of the HPLC-ELSD chromatograms corresponds to esculose, α -G2-(1→3)- α -G1-(1→2)- β -Fru. Although few signals are unassigned due to the large overlapping with those belonging to major component erlose (the Fru residue), the assigned signals and the previously described data provide solid evidences for this interpretation. As far as we know, this is the first time that the structural characterization of this trisaccharide is described in a detailed way.

Two trisaccharides were identified in the purified fraction 7, isomelezitose [α -D-Glcp-(1→6)- β -D-Fruf-(2→1)- α -D-Glcp] (Fig. 3B) and theanderose [α -D-Glcp-(1→6)- α -D-Glcp-(1→2)- β -D-Fruf] (Fig. S6). The signal pattern obtained for theanderose and isomelezitose spectra were coincident to those described by Ruiz-Aceituno *et al.* (2017) and Shi *et al.* (2016) respectively. Moreover, isomelezitose was the only compound detected in the purified fraction from the peak 8 of the HPLC-ELSD chromatograms (Figs 2B and 3B).

The structural characterization of the synthesized sugars indicates that the transglycosylating activity of the *M. reukaufii* cell extracts was able to transfer the glucosyl moiety of sucrose principally to the 6-OH of the β -fructofuranosyl residue of sucrose and to the 1-OH position of the free fructose (released in the reaction), yielding isomelezitose and trehalulose respectively. In addition, the hydroxyls 3-OH of the β -fructofuranosyl residue, and the 3-OH, 4-OH and 6-OH

of the α -glucopyranosyl unit of sucrose were also glucosylated, but to a lesser extent, producing melezitose, esculose, erlose and theanderose respectively. This biosynthetic behaviour had been previously described for some glycosidases, specifically α -glucosidases, which were not only capable of transferring the glucosyl moiety to the more reactive primary hydroxyl groups of acceptor sugars, but also to the secondary ones of several acceptor molecules (Plou *et al.*, 2007). In fact, the synthesis of isomelezitose was first described using a partially purified α -glucosidase from *S. cerevisiae*, but this was the only trisaccharide detected in the reactions when using sucrose as substrate (Chiba *et al.*, 1979). Afterwards, three sugars, theanderose (6^G- α -D-Glucosyl-sucrose), isomelezitose (6^F- α -D-Glucosyl-sucrose) and 4^F- α -D-Glucosyl-sucrose were also produced using the *Bacillus* sp. SAM1606 α -glucosidase in similar conditions (Inohara-Ochiai *et al.*, 2000). However, and differing from what has been previously reported, a mixture of oligosaccharides containing a great diversity of glycosidic bonds was obtained, with isomelezitose as the major product, in the transfer reactions mediated by the enzymatic extract of *M. reukaufii*. Although it seems plausible that an α -glucosidase activity produces all these compounds, given the varied type of molecules formed, the possibility that not a single enzyme could be implicated in the transglucosylation of sucrose cannot be eliminated by now, which requires further protein characterization that is in progress. In any case, a schematic view of the reactions catalysed by the *M. reukaufii* cell extract and 500 g l⁻¹ sucrose is represented in Fig. 5.

Effect of sucrose concentration on the transfer products' level

The transglucosylating activity of the *M. reukaufii* extract was evaluated using different sucrose concentrations and the main transfer products in the reaction mixtures, isomelezitose and trehalulose (Fig. 5), were quantified after 72 h (Fig. 6A). As expected, the concentration of these two compounds increased as the sucrose did from 100 to 500 g l⁻¹. This behaviour has been previously described for many microbial glycosidases, where the substrate concentration and the transglucosylating/hydrolysing ratio increased in parallel. As examples, the transfer activity of the β -fructofuranosidase from *X. dendrorhous* improved by 1.5-fold when the sucrose concentration increased from 420 to 600 g l⁻¹ (Linde *et al.*, 2012), and that of the α -glucosidase from *Aspergillus niger* did in 1.8-fold when the maltose concentration went from 100 to 300 g l⁻¹ (Duan *et al.*, 1995). By contrast, when using the *M. reukaufii* extract and the initial sucrose concentration increased from 500 to 700 g l⁻¹, the amounts of the majority transfer products were either reduced significantly (~50%, from 50 to 24 g l⁻¹) or slightly increased (~16%, from 54 to 63 g l⁻¹) for trehalulose and isomelezitose respectively. Thus, the highest total amount of the major transfer products was achieved at 500 g l⁻¹ sucrose (Fig. 6A). Accordingly, this sucrose concentration was used to analyse the evolution of sugars in the reaction mixture for a total of 120 h. Figure 6B shows the production profiles of trehalulose and isomelezitose. The final carbohydrate mixture contained 81 and 51 g l⁻¹ of isomelezitose and trehalulose, which represented 16.1% and 10% (w/w) of total sugars in the mixture respectively. At this point, 120 g l⁻¹ of sucrose (24% of total sugars) was detected. Besides, the concentration of erlose plus melezitose and theanderose reached 8.6 and 5.6 g l⁻¹, which represented 1.7% and 1.1% of total sugars in the reaction mixture respectively (data not shown).

The α -glucosidases mentioned above, responsible for the synthesis of oligosaccharides such as isomelezitose or theanderose, were all structurally included in the family 13 of the glycosyl hydrolases (GH13; carbohydrate-active enzymes database: CAZy) (Lombard *et al.*, 2014). Therefore, it seems plausible that if more than one protein were responsible for the transglucosylating activity detected in this work they would also be included in this family.

As previously mentioned, several microbial α -glucosidases has been already used to produce isomelezitose, a rare trisaccharide found in honey, which shows potential nutraceutical and prebiotic properties (Görl *et al.*, 2012). However, and as far as we know, the obtained yields when using native enzymes or cellular extracts

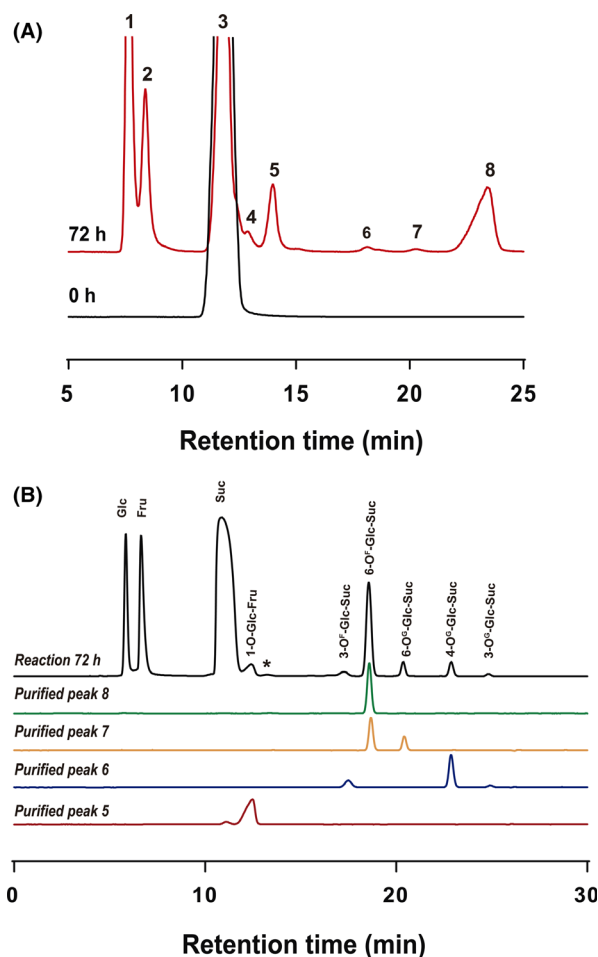


Fig. 2. Representative HPLC chromatograms of the reaction mediated by the *M. reukaufii* extract on sucrose 500 g l⁻¹.

A. HPLC-ELSD analysis. Chromatograms correspond to the sugar mixtures at 0 (black line) and 72 h (red line) of reaction. Similar product profiles were obtained by using longer reaction times. Peaks assignment: (1) fructose, (2) glucose, (3) sucrose, (4–8) new signals, initially unknown compounds.

B. HPAEC-PAD analysis of carbohydrate fractions purified by semi-preparative HPLC. Chromatographic profiles of the 72 h reaction mixture (black line) and the referenced purified peaks (red, blue, yellow and green lines) are shown. Peaks assignment using the corresponding standards: Glc: glucose; Fru: fructose; Suc: sucrose. New products assignment after MS-ESI and NMR analysis of the purified peaks are indicated: (*) unknown compound.

were lower than those reported in this work. Thus, employing the partially purified α -glucosidase from *S. cerevisiae* and 100 g l⁻¹ sucrose, the isomelezitose production did not exceed 0.4% of total sugars in the reaction mixture (Chiba *et al.*, 1979), and using a *Bacillus* sp. α -glucosidase and 600 g l⁻¹ sucrose reached about 8% of total sugars (Inohara-Ochiai *et al.*, 2000). Interestingly, the proportion of isomelezitose, referred to total sugars produced by transglucosylation in the reaction mixture, increased by site-directed mutagenesis of this enzyme, but its transferase activity was also

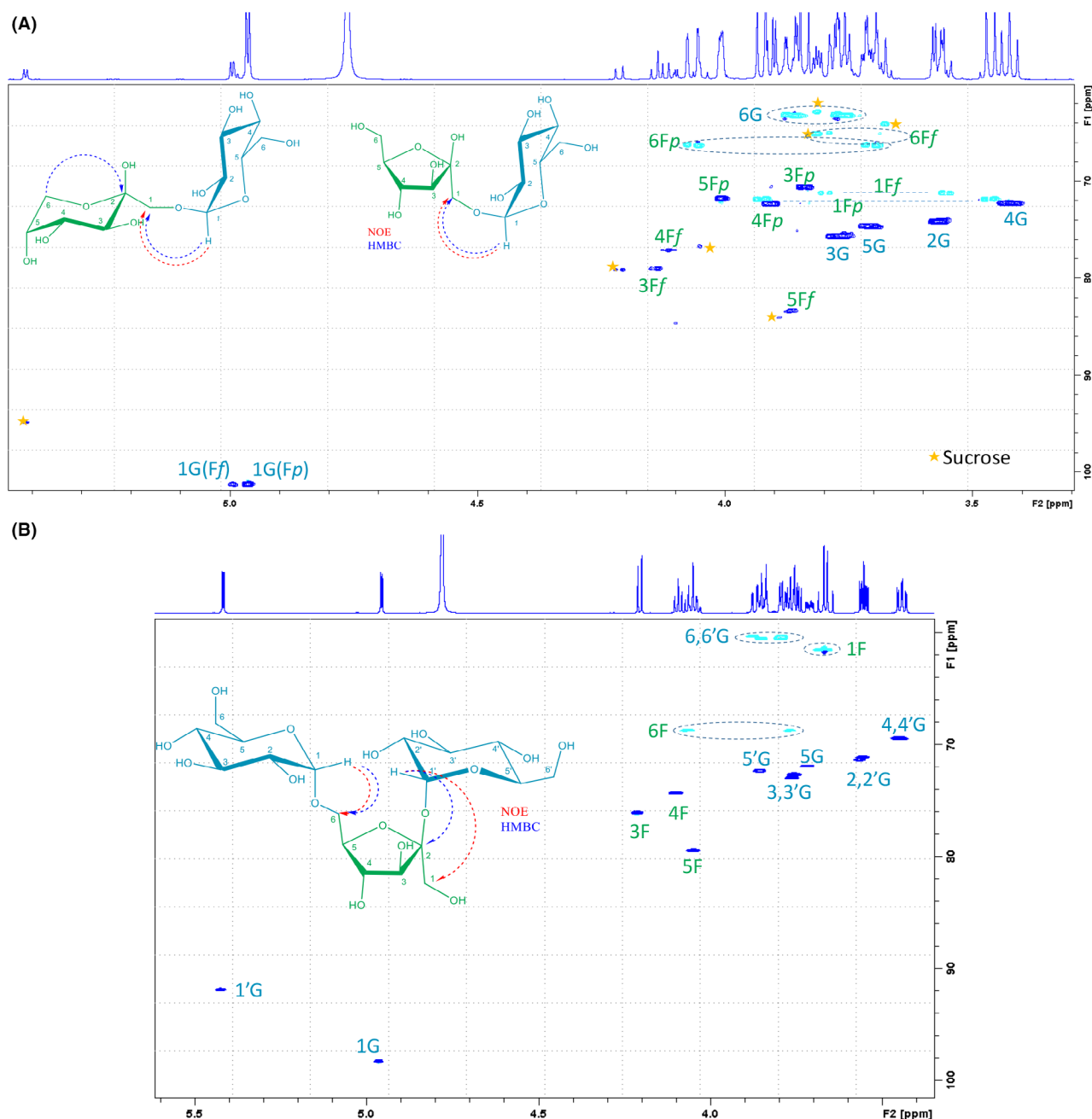


Fig. 3. NMR spectra (600 MHz, D₂O, 300 K) for the compounds separated as peaks 5 and 8.

A. ¹H-¹³C HSQC-edited spectra with signal assignment for the compound present in peak 5 (trehalulose). Signals corresponding to sucrose are depicted with an orange star (★).

B. ¹H-¹³C HSQC-edited spectra with signal assignment for the compound present in peak 8 (isomelezitose). The key NOE or HMBC correlations that confirmed the different linkages are indicated for each molecule.

reduced and finally isomelezitose constituted only about 3% of total sugars (Okada *et al.*, 2002). In this context, Munir and Vogel (1999) patented the synthesis of isomelezitose using immobilized cells of *Protaminobacter* and 50°C with yields close to 11% of total sugars. Besides, Görl *et al.* (2012) transformed a sucrose isomerase into an isomelezitose synthase, obtaining a

protein variant by mutation that yielded 70% of isomelezitose using 100 g l⁻¹ sucrose, but this amount was calculated based on sucrose consumed (~ 32%) instead of the total sucrose added to the reaction mixture, which means to produce about 22.4 g l⁻¹ of this trisaccharide. More recently, Côté *et al.* (2017) obtained an engineered glucansucrase from the bacteria *Leuconostoc*

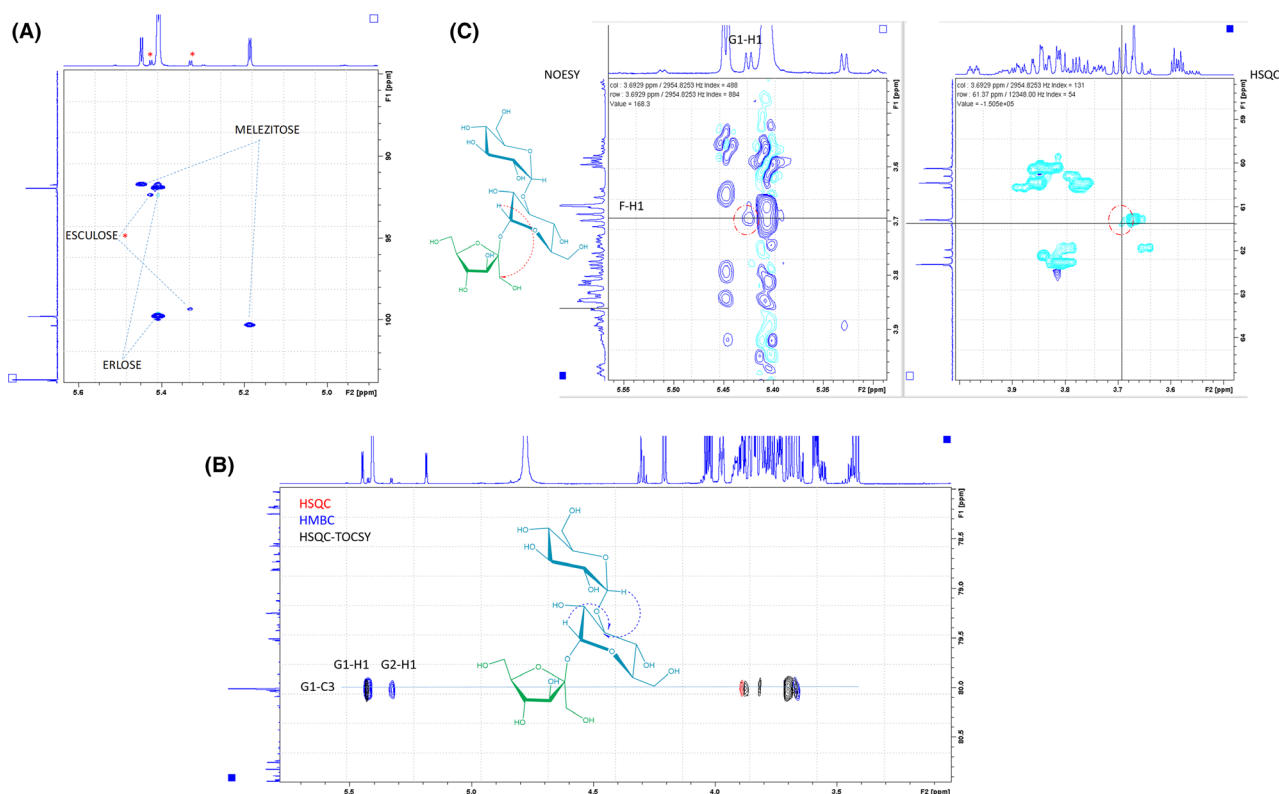


Fig. 4. NMR spectra (600 MHz, D₂O, 300 K) for the minor component from the purified peak 6. A. Key region of the HSQC experiment. The minor product signals are marked with a red dot and show the existence of [α -Glc-(1 \rightarrow 2)- β -Fru] and [α -Glc-(1 \rightarrow x)-Fru/Glc] type residues for G1 and G2. B. Superimposition of partial HSQC (red), HMBC (blue) and HSQC-TOCSY (black) spectra, for the ¹³C region of 80 ppm, demonstrating the G2-G1 connectivity in a non-ambiguous manner. C. The key NOE that evidences the spatial proximity of the G1-H1/F-H1 pair.

mesenteroides that produced isomelezitose very efficiently using $\sim 800 \text{ g l}^{-1}$ sucrose. Compared to the wild type enzyme (DsrI), which only synthesized traces of isomelezitose, the mutated variant (Dsr1-L441E) converted 57% of sucrose in this trisaccharide. Regardless of this excellent result, to our knowledge, the yield of isomelezitose obtained with the *M. reukaufii* extract (81 g l^{-1} , 16.1% of total sugars in the mixture) is the highest reported for any microbial wild type enzyme, enzymatic extract or cellular system.

In conclusion, this study presents an enzymatic activity from *M. reukaufii* cell extracts producing a mixture of oligosaccharides with very different glycosidic bonds and biotechnological interest. Among them, isomelezitose, a potential nutraceutical and prebiotic sugar (Görl *et al.*, 2012) and trehalulose, a disaccharide with approximately 60% of the sucrose sweetness and low rate of monosaccharide release into blood, an interestingly property for the development of new food products for diabetics (Wei *et al.*, 2013). In addition, erlose and theanderose were produced, two non-cariogenic sugars with low-caloric value that also could be used as alternative to sucrose sweetener (Daudé *et al.*, 2012) or as bifidogenic

substrate (Ruiz-Aceituno *et al.*, 2017) respectively. Because all sugars produced by the *M. reukaufii* extract are found naturally in honey (Daudé *et al.*, 2012), possibility that this microorganism could have some role in the final composition of this natural product could be considered. The relevance of our results makes further analysis necessary to characterize and tries to improve the biotechnological potential of the activity here analysed, as well as to address its functional improvement using molecular bioengineering.

Experimental procedures

Microorganisms and culture conditions

Metschnikowia reukaufii 1LL10 was isolated from *Helleborous foetidus* nectaries (Sierra de Cazorla, southeastern Spain) by Dr. Carlos M. Herrera [Estación Biológica de Doñana (CSIC), Sevilla, Spain]. Yeast was maintained at 4°C on YEP(D) solid media [10 g l^{-1} yeast extract (Laboratorios Conda, S.A., Madrid, Spain), 20 g l^{-1} peptone (Laboratorios Conda), 20 g l^{-1} glucose (Merck, Darmstadt, Germany), 20 g l^{-1} agar (Laboratorios Conda)]. YEP liquid media including 20 g l^{-1} of

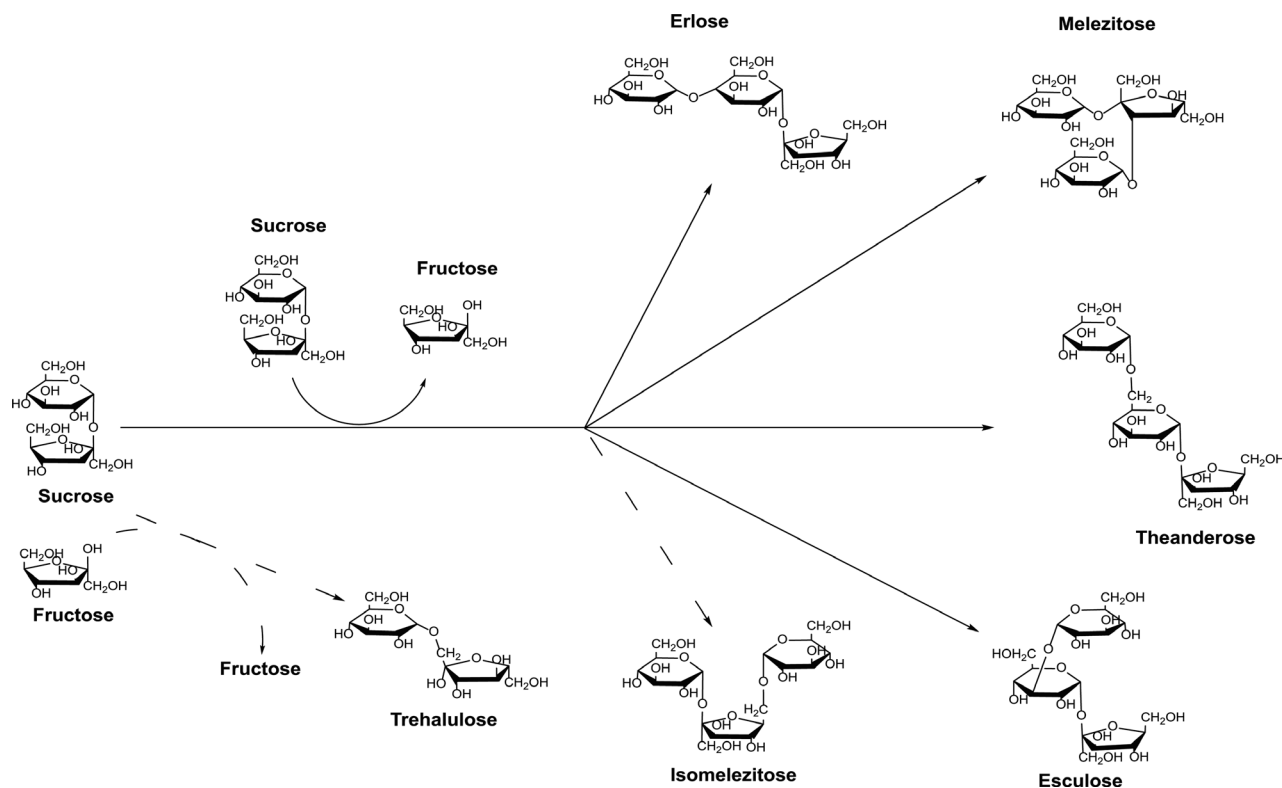


Fig. 5. Scheme of the transglucosylation process involving sucrose and *M. reukaufii* enzymatic extract. The formation of the main transglucosylation products is indicated with broken arrows.

glucose (D), sucrose (S), maltose (M), lactose (L), raffinose (R) or inulin (I) were used to analyse the hydrolytic activity profile on sucrose (also sucrose-splitting activity). Yeast was cultured at 30°C with orbital shaking (200 rpm) and growth was monitored spectrophotometrically at a wavelength of 660 nm (OD_{660}). Sucrose, maltose, lactose and raffinose were from Sigma-Aldrich (St. Louis, MO, USA) and inulin from Beneo Ibérica S.L. (Barcelona, Spain). The YEP(S-BTB) medium, used to analyse the hydrolytic activity on plates, was the same as YEP(D) but including 50 g l⁻¹ sucrose instead of glucose, supplemented with bromothymol blue 0.25 g l⁻¹ (BTB) and then adjusted to pH 6.5. In this assay, yeast *Schwanniomyces occidentalis* ATCC2322 and *Pichia pastoris* GS115 were used as positive and negative yeast controls respectively.

Obtention of enzymatic extracts and sucrose-splitting activity assay

M. reukaufii was cultivated in YEP(S) liquid medium during 4–8 h at 30°C, cells were harvested by centrifugation (6000 g for 15 min) and the soluble enzymatic extracts were obtained using YeastBuster™ protein extraction reagent (Novagen, San Diego, CA, USA) according to the manufacturer's specifications.

Hydrolytic standard activity on sucrose was determined by measuring the amount of reducing sugars (glucose or fructose) released from 60 g l⁻¹ sucrose in 50 mM sodium phosphate pH 6.0 using the 3,5-dinitrosalicylic acid (DNS) method adapted to a 96-well microplate scale (Ghazi *et al.*, 2005). Unless otherwise indicated, reactions mixtures (50 µl) including 5 µl of enzymatic extracts were incubated at 30°C. Glucose in the 0–3 mg ml⁻¹ range was used as calibration curve. Reactions without enzymatic extracts or sucrose were used as negative controls. One unit of standard hydrolytic activity (U) was defined as that catalysing the formation of 1 µmol of reducing sugar per minute under the above described conditions. Each reaction was performed at least in triplicate.

The hydrolytic on sucrose activity was detected in solid media including sucrose and a pH indicator (BTB), which shows greenish colour at pH 6.5. The use of sucrose would cause the acidification of the medium and the consequent change of the YEP(S-BTB) colour from greenish to yellow by the BTB protoning (pH < 6.5).

Transglycosylation reactions based on sucrose

The transglycosylation reactions were performed using 500 g l⁻¹ sucrose in 50 mM sodium phosphate pH 6.0,

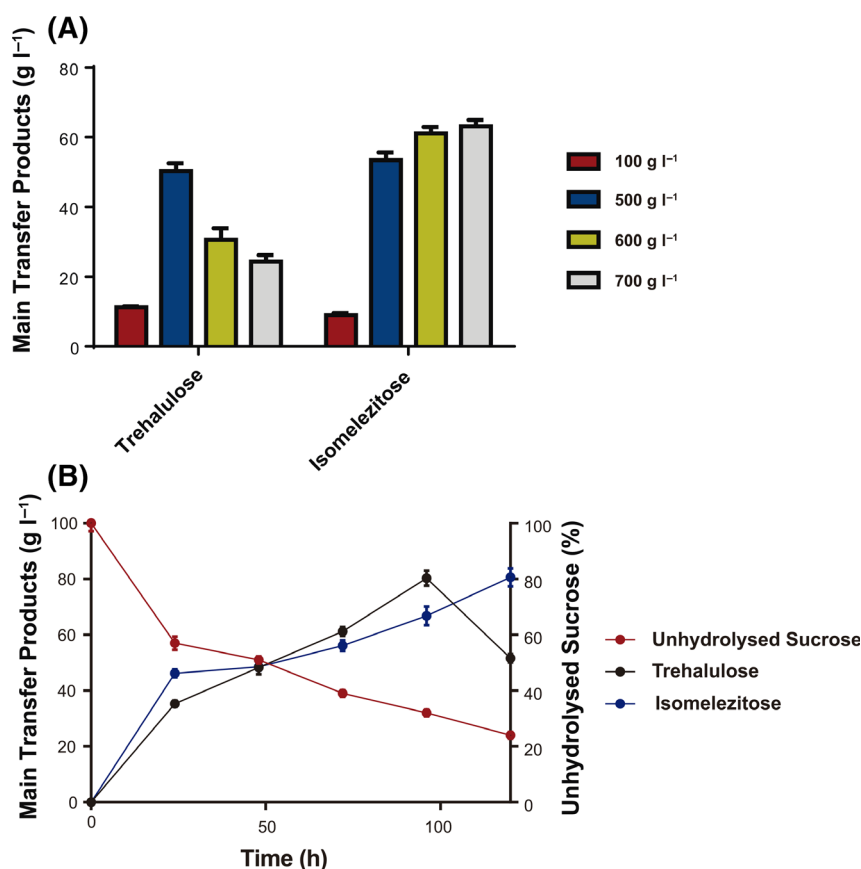


Fig. 6. Analysis of the main transglucosylation products synthesized by the *M. reukaufii* enzymatic extract.

A. Trehalulose and isomelezitose concentrations measured by HPLC-ELSD after 72 h of reaction using the referred initial sucrose amounts. Standard deviation values are shown.

B. Time-course reaction profile of the main transfer products using 500 g l⁻¹ sucrose. The evolution of unhydrolysed sucrose (red line), trehalulose (black line) and isomelezitose (blue line) are shown at the indicated reaction times with the corresponding standard deviations.

10 U of standard hydrolytic activity, at 30°C and 400 rpm in a Vortem 56 shaker (Labnet International, Woodbridge, NJ, USA). The final reaction volume was 1.5 ml. Samples of 100 µl were withdrawn at different reaction times (0–120 h), maintained at 100°C for 10 min to inactivate the enzyme, and store at –20°C. Sucrose in the range of 100–700 g l⁻¹ and 72 h of reaction time was used to analyse the effect of this sugar concentration on the transfer products level. For HPLC analysis, samples were conveniently diluted (1:5 or 1:10) in distilled water and filtered through 0.45 µm pore size nylon filters (Scharlau, S.L.; Sentmenat, Spain). Each reaction was performed in duplicate or triplicate.

HPLC-ELSD analysis

Sugars in the reaction mixtures were analysed and quantified by HPLC using a quaternary pump (Delta 600; Waters, Milford, MA, USA) coupled to a Kromasil-NH₂ column (250 × 4.6 mm, 5 µm) from Análisis Vínicos

S.L. (Tomelloso, Spain). Detection was performed using an evaporative light scattering detector (ELSD, mod. 1000; Polymer Laboratories, Ltd., Church Stretton, UK) equilibrated at 90°C. Column temperature was kept constant at 30°C. Analytes were eluted with a mixture acetonitrile/water 80:20, degassed with helium, at flow rate of 1.0 ml min⁻¹ for 30 min. The peaks corresponding to the transfer reaction products were purified and identified by MS-ESI and NMR techniques, as described in the following sections. All compounds were quantified on the base of peak areas using the most closely related commercial standard. Glucose, fructose, sucrose and melezitose monohydrate (all from Sigma) were used as standards. Data obtained were analysed using the Millennium 32 Software (Waters).

Purification of the transglucosylation products

Compounds formed during the transglucosylation reaction with 500 g l⁻¹ sucrose were purified by semi-

preparative HPLC using a system equipped with a quaternary pump (Delta 600; Waters) coupled to a Kromasil-NH₂ column (250 × 10 mm, 5 μm) from Análisis Vínicos S.L. (Tomelloso, Spain). A three-way flow splitter (Accurate, LC Packings) and the referred ELSD system equilibrated at 90°C was used and acetonitrile/water 90:10 (v: v), degassed with helium, was employed as mobile phase at 5.5 ml min⁻¹ for 55 min. Then, the percentage of acetonitrile was reduced to 80% and kept for 15 min. Finally, it was raised to 90% and maintained for five additional minutes. The column temperature was kept at 30°C. After collecting the different transglycosylation products, the mobile phase was eliminated by rotary evaporation in a R-210 rotavapor (Buchi, Essen, Germany).

The purity of the compounds collected in the semi-preparative HPLC was analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS3000 system and a CarboPack PA-1 column (4 × 250 mm) connected to a PA-1 guard column. An electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode was used. The initial mobile phase was 100 mM NaOH for 8 min. Then, a gradient from 100 to 88% 100 mM NaOH and from 0 to 12% 100 mM NaOH/600 mM sodium acetate was performed in 22 min. This last mobile phase composition was kept for six more minutes and then changed to 50% 100 mM NaOH and 50% 100 mM NaOH/600 mM sodium acetate. The flow rate was maintained at 1 ml min⁻¹ during the analysis. Eluents were degassed by flushing with helium, and peaks were analysed using Chromeleon software.

Characterization of the transglycosylation products

The molecular weight of the purified oligosaccharides was assessed using a mass spectrometer with hybrid QTOF analyser (model QSTAR, Pulsar i, AB Sciex). The samples were analysed by direct infusion and ionized by electrospray (with methanol containing 1% of sodium iodide as ionizing phase) in positive reflector mode.

The structure of the synthesized oligosaccharides was determined using a combination of 1D (¹H, ¹³C{¹H}), 1D-selective TOCSY, NOESY and/or ROESY) and 2D (COSY, HSQC, HSQC-TOCSY, HMBC, NOESY) NMR techniques. The spectra of the samples, dissolved in D₂O (ca. 7 mM), were recorded on a Bruker IVDr 600 spectrometer equipped with a BBI probe with gradients in the Z-axis, at a temperature of 300 K. Chemical shifts were expressed in parts per million (ppm). TSP-d₄ signal was used as internal reference (0 ppm). Standard Bruker pulse sequences were employed. For the HSQC and HMBC experiments, values of 5 ppm and 1 K points, for the ¹H dimension, and 70 ppm and 512 points for the

¹³C dimension, were used. For the homonuclear COSY and NOESY experiments, 3 ppm windows were used with a 1 K × 384–512 point matrix. For the NOESY the mixing time was set to 500 ms.

Acknowledgements

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Conflict of interests

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. MS-ESI Spectrum of purified peak 5.

Fig. S2. MS-ESI Spectrum of purified peak 6.

Fig. S3. MS-ESI Spectrum of purified peak 8.

Fig. S4. NMR Spectra (600 Hz, D₂O, 300 K) for the compounds separated as peak 6. ¹H-¹³C HSQC-edited spectra with signal assignment for erlose (A) and melezitose (B).

Fig. S5. NMR Spectra (600 Hz, D₂O, 300 K) for the minor compound of purified peak 7 (theanderose). Signal corresponding to isomelezitose are depicted with a red star (*).

Table S1. NMR data deduced for the minor component of the purified peak 6 (esculose) and those previously described for α -G2-(1→2)- α -G1 fragment.