










Fructooligosaccharides production by immobilized *Pichia pastoris* cells expressing *Schedonorus arundinaceus* sucrose:sucrose 1-fructosyltransferase

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Abstract: Fructooligosaccharides (FOSs)—fructose-based oligosaccharides—are typical prebiotics with health-promoting effects in humans and animals. The trisaccharide 1-kestotriose is the most attractive inulin-type FOS. We previously reported a recombinant sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) from *Schedonorus arundinaceus* (Sa) that efficiently converts sucrose into 1-kestotriose. In this study, *Pichia pastoris* PGFT6x-308 constitutively expressing nine copies of the Sa1-SST gene displayed fructosyltransferase activity in undisturbed biomass (49.8 U/ml) and culture supernatant (120.7 U/ml) in fed-batch fermentation (72 hr) with sugarcane molasses. Toluene permeabilization increased 2.3-fold the Sa1-SSTrec activity of whole cells entrapped in calcium-alginate beads. The reaction with refined or raw sugar (600 g/l) yielded 1-kestotriose and 1,1-kestotetraose in a ratio of 8:2 with their sum representing above 55% (wt/wt) of total carbohydrates. The FOSs yield decreased to 45% (wt/wt) when sugarcane syrup and molasses were used as cheaper sucrose sources. The beads retained 80% residual Sa1-SSTrec activity after a 30-day batchwise operation with refined cane sugar at 30°C and pH 5.5. The immobilized biocatalyst is attractive for the continuous production of short-chain FOSs, most particularly 1-kestotriose.

Keywords: Calcium alginate, Cell immobilization, Fructooligosaccharides, Fructosyltransferase, *Pichia pastoris*

Introduction

Fructooligosaccharides (FOSs)—fructose-based oligosaccharides—are prebiotic fibers widely utilized as functional food ingredients and in the pharmaceutical industry. FOSs ingestion improves the composition of the resident intestinal microbiota by stimulating selectively the growth of probiotics (bifidobacteria and lactobacilli), which results in the competitive exclusion of pathogens. Other associated benefits are the reduction of serum cholesterol, the increase in calcium and magnesium absorption, the prevention of colon cancer, and the production of B-vitamins. Additionally, FOSs are noncariogenic, low-calorie sugars of sweet taste and exhibit antioxidant activity by free radical scavenging (Cunha et al., 2019; Faria et al., 2021; Kaplan & Hutkins, 2000; Maiorano et al., 2020; Pereira & Gibson, 2002; Perna et al., 2018).

Commercially available FOSs contain a terminal α -D-glucose residue (G) and two to four fructosyl units (F) linearly connected by β -(2 \rightarrow 1) linkages. Among inulin-type FOSs, the trisaccharide 1-kestotriose (1-kestose, GF₂) has a superior bifidus-stimulating effect and tastes sweeter than the tetrasaccharide 1,1-kestotetraose (nystose, GF₃) and the pentasaccharide 1,1,1-kestopentaose (fructosyl-nystose, GF₄) (Mendlik et al., 2012; Suzuki et al., 2006). With these attributes, 1-kestotriose when supplemented with a more intense low-calorie sweetener (e.g.,

stevia) is adequate for replacing table sugar in prebiotic diabetic recipes that reduce the risk of obesity (Mabel et al., 2008).

Current industrial systems for inulin-type FOSs production are based on the use of β -fructofuranosidases (EC 3.2.1.26) and β -fructosyltransferases (EC 2.4.1.9) from few species of filamentous fungi, mainly from the genera *Aspergillus* and *Aureobasidium*. The reaction needs to be conducted at high sucrose concentrations (600–700 g/l) to restrict substrate hydrolysis. Under this condition, the fungal enzymes produce a mixture of GF₂, GF₃, and GF₄, with their sum representing around 55% (wt/wt) of total carbohydrates (Cunha et al., 2019; Fernandez et al., 2007; Sánchez-Martínez et al., 2020).

FOS-synthesizing enzymes of different origins have been produced in transgenic yeasts (Hernández et al., 2018; Rehm et al., 1998; Trujillo et al., 2001; Yang et al., 2016). Our group reported a recombinant sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) from the plant *Schedonorus arundinaceus* (Sa) with catalytic properties highly attractive for the production of short-chain inulin-type FOSs (Hernández et al., 2018). The enzyme produced in *Pichia pastoris* (Sa1-SSTrec) yielded 1-kestotriose and 1,1-kestotetraose in an invariable ratio of 9:1 and overall FOSs content above 55% (wt/wt) from sucrose at the wide concentration range of 100–800 g/l. Fructose release to water (sucrose hydrolysis) was negligible even at the lowest

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substrate concentration (Hernández et al., 2018). The *P. pastoris* multicopy clone PGFT6x-308 secreted Sa1-SSTrec as a glycoprotein into the periplasmic space (38%) and the extracellular medium (62%) in fed-batch fermentation using cane sugar for cell growth (Hernández et al., 2018). The yeast host holds the GRAS (generally recognized as safe) status and lacks endogenous sucrose- or fructan-transforming enzymes. Immobilization of whole PGFT6x-308 cells retaining N-glycosylated Sa1-SSTrec in the periplasmic space may result in an invertase-free biocatalyst appropriate for reuse in a cost-effective continuous process that efficiently converts sucrose into 1-kestotriose.

Cell immobilization processes often use insoluble calcium alginate as the entrapping matrix. The method is rapid, nontoxic, inexpensive, versatile, eco-friendly, and recommended for applications in the food and pharmaceutical industries (Smidsrød & Skja, 1990). In bioprocess, Ca-alginate entrapment of enzymes and cells offers relevant advantages, such as greater resistance to external aggressions, increased possibility of enzyme recovery and straightforward enzyme/product separation, reusable biocatalysts with operational stability, and reduction in operating costs (Gonçalves et al., 2019).

Several authors have successfully entrapped enzymes or fungal cells in Ca-alginate gels for continuous FOSs production from sucrose (Cheng et al., 1996; Fernandez-Arrojo et al., 2013; Hayashi et al., 1994; Park et al., 2005; Ganaie et al., 2014; Gonçalves et al., 2020). There are no previous reports on immobilized yeast cells expressing a plant fructosyltransferase. Ca-alginate entrapment of PGFT6x-308 cells may be advantageous over immobilization of the free enzyme. The whole-cell biocatalyst may prevent Sa1-SST from leaking out of the beads, but it may cause operational troubles if the confined yeast remains alive and fermentative metabolism of glucose releases the undesired products ethanol, acetate, and CO₂.

Immobilized microorganisms are often treated with a membrane-permeabilizing agent in order to reduce diffusional barriers and accelerate biocatalytic reactions (He et al., 2015; Panesar et al., 2007, 2011; Park et al., 1994; Yun et al., 1994). Permeabilization treatments using toluene, a hydrophobic solvent, at appropriate levels dramatically decreased the viability of bacteria and yeasts without causing cell lysis (De Smet et al., 1978; Jackson & DeMoss, 1965; Murakami et al., 1980).

In this work, Ca-alginate entrapment of whole *P. pastoris* PGFT6x-308 cells was followed by a membrane-permeabilization treatment. The toluene-treated beads showed enhanced specific activity and a dramatic decrease in cell viability. The inactivation of the fermentative metabolism of glucose, a major product of the Sa1-SST reaction, in the permeabilized yeast avoided the undesired formation of ethanol, acetate, and CO₂. The whole-cell biocatalyst was assayed for the production of short-chain FOSs, mainly 1-kestotriose, in repetitive cycles using different sucrose sources.

Materials and Methods

Reagents

Sucrose, glucose, fructose, 1-kestotriose, and 1,1-kestotetraose were purchased from Sigma Aldrich (USA). Toluene (≥99.5%) and sodium alginate (source *Laminaria hyperborea*) were purchased from BDH Chemicals (England). Refined sugar, raw sugar, sugarcane syrup, and molasses were collected at the sugar mill “Melanio Hernández,” Tuinucú, Sancti Spiritus, Cuba.

Strain

P. pastoris PGFT6x-308 constitutively expressing nine copies of the 1-SST gene from *S. arundinaceus* (Hernández et al., 2018) was maintained on agar plates containing YPG (Yeast extract 10 g/l, peptone 20 g/l, and glucose 20 g/l) with hygromycin (100 μg/ml) at 30°C.

Culture Conditions

One liter of MM-molasses medium for batch cultures of *P. pastoris* PGFT6x-308 contained 22 g NH₄SO₄, 18.2 g K₂HPO₄, 7.5 g MgSO₄ 7H₂O, 0.5 g CaCl₂ 2H₂O, 5 g yeast extract, and sugarcane molasses at 5°Bx. Vitamins and trace elements were prepared and used as recommended by d’Anjou and Daugulis (2001). The inoculum was cultured in a 500-ml shake flask with 100 ml of MM-molasses medium using a Minित्रon shaker (Infors, Switzerland) at 30°C with 250 rpm for 24 hr.

Fed-batch cultures of *P. pastoris* PGFT6x-308 were performed in a 5-l fermenter (Infors, Switzerland) with the temperature kept at 30°C and pH 5.5 controlled by the automatic addition of 40% (wt/vol) H₃PO₄ and 25% (wt/vol) NH₃OH. The air flow rate was 3 l/min and the dissolved oxygen was maintained above 20% by increasing automatically the agitation speed from 500 rpm to 900 rpm. After 20 hr of batch cultivation, the air flow rate was adjusted to 6 l/min and the feeding phase was accomplished by the automatic addition of the carbon source at a rate range of 6–8 ml/l/hr according to the carbon demand, as monitored by the level of dissolved oxygen. The feeding solution was sugarcane molasses at 45°Bx. After 72 hr of cultivation, the cells were harvested by centrifugation (4°C, 3000 rpm).

Cell Immobilization

Whole cells (10, 20, 30, or 40 g, wet biomass) and 2 g of sodium alginate were suspended in water to a final volume of 100 ml and stirred thoroughly (100 rpm) at 25°C to ensure a homogenous distribution before extrusion in the CaCl₂ solution. The alginate/cell mixtures were dropped through a fine needle into 1 l of 37 mM CaCl₂ solution with constant stirring (100 rpm) using an impeller, type marine propeller, to avoid droplet aggregation. Gelation time was 1 hr and then the CaCl₂ solution was discarded. The spherical alginate beads (diameter 2–3 mm) were hardened overnight in 67 mM CaCl₂ at 4°C and stored in 1.46 M sucrose in 50 mM sodium acetate buffer (pH 5.5) at 4°C before use.

Permeabilization of Immobilized Cells

Beads (5 g, wet weight) were added to 10 ml of sodium acetate buffer (100 mM, pH 5.5) containing toluene at final concentrations of 3, 5, and 10% (vol/vol). The flasks were shaken at 100 rpm, 30°C for 1 hr to provide adequate contact between the entrapped cells and the solvent floating on the surface of the aqueous solution. After the membrane-permeabilization treatment, beads were recovered by filtration, washed twice with sodium acetate buffer (100 mM, pH 5.5) to remove residual toluene and stored in 1.46 M sucrose in 50 mM sodium acetate buffer (pH 5.5) at 4°C. Beads and solutions were assayed for sucrose-transforming activity.

To determine cell viability, beads were smashed in a liquid YPG medium and aliquots (0.1 ml) of sequential dilutions were plated on YPG agar. The number of colony-forming units (cfu) per gram of wet bead was determined on plates incubated at 30°C for 48 hr. Beads (5 g, wet weight) were incubated in 10 ml of sucrose solution (100 g/l) at 30°C for 12 hr to detect the release of CO₂ as evidence of cell viability.

Enzyme Assay

Free or immobilized cells were reacted with 1.75 M sucrose in 100 mM sodium acetate buffer (pH 5.5) in a shaking water bath at 30°C with agitation (50 rpm). After 2 hr, the reaction was stopped by heating 20 min at 80°C. Glucose released to the solution was quantified by the dinitrosalicylic acid (DNSA) colorimetric method (Miller, 1959). One unit of Sa1-SSTrec is defined as the amount of enzyme releasing 1 μ mol of glucose per minute at initial rates under the reaction conditions described above.

For evaluating the effect of toluene on the stability of free Sa1-SSTrec, the enzyme recovered from the culture supernatant of sucrose-grown *P. pastoris* PGFT6x-308 (Hernández et al., 2018) was preincubated with the organic solvent (0, 3, 5, and 10% vol/vol) in 100 mM sodium acetate buffer (pH 5.5) at 30°C. The residual Sa1-SSTrec activity after 30- and 60-min preincubations was measured with 1.75 M sucrose in 100 mM sodium acetate buffer (pH 5.5).

For assaying pH stability, toluene-treated beads (1 g, wet weight) were preincubated with 60% (wt/vol) sucrose in 100 mM sodium acetate/phosphate buffers of varying pH (4–8) at 30°C. For assaying thermal stability, the entrapped cells (1 g, wet weight) were preincubated with 60% (wt/vol) sucrose in 100 mM sodium acetate buffer (pH 5.5) at temperatures of 30, 40, 50, and 60°C. The residual Sa1-SSTrec activity of beads collected at time intervals was measured with 1.75 M sucrose in 100 mM sodium acetate buffer (pH 5.5).

FOSs Production and Operational Stability Assay

Beads (1 g, wet weight) were incubated in a 10-ml solution of the sucrose source (white refined sugar, raw sugar, sugarcane syrup, or molasses) adjusted to 50°Bx (around 600 g/l) in 100 mM sodium acetate buffer pH 5.5, at 30°C for 48 hr with agitation (100 rpm) in a stirred tank reactor. Samples were collected at regular intervals.

To evaluate the operational stability of the biocatalyst, 15 repetitive batches of 48 hr were performed under the conditions mentioned above. At the end of each cycle, the alginate beads were recovered by filtration and a fresh substrate solution was added. After 30 days, the beads were collected and assayed for Sa1-SSTrec activity. The residual activity was established by comparing with the activity of the beads stored in a sucrose solution (1.46 M) at 4°C.

Carbohydrate Analysis

Qualitative FOSs analysis was performed by thin layer chromatography (TLC) on TLC Silica gel 60 F254 plates (Merck, Germany) using acetone 95% (vol/vol) as a mobile phase. Detection was achieved by spraying with a saturated solution of 1-butanol 80% (vol/vol), urea 3% (wt/vol), H₃PO₄ 6% (vol/vol), and absolute ethanol 4% (vol/vol) and heating for 5 min at 120°C.

Quantitative sugar analysis was performed by HPLC using an Aminex HPX-42C column (0.78 × 30 cm, BIORAD) equipped with a refractive index detector. The column temperature was kept at 85°C. Water was used as a mobile phase at a flow rate of 0.5 ml/min. Samples were appropriately diluted before injection. Solutions (20 mg/ml) of fructose, glucose, sucrose, 1-kestotriose, and 1,1-kestotetraose were used as standards. The program package ezData (www.chemilab.net) was used for sugars visualization.

Statistical Analysis

The statistical package for social sciences (SPSS) 15.0 was used for the data analyses. The data are presented as means \pm standard deviation. The level of significance used in this study was $p < 0.05$.

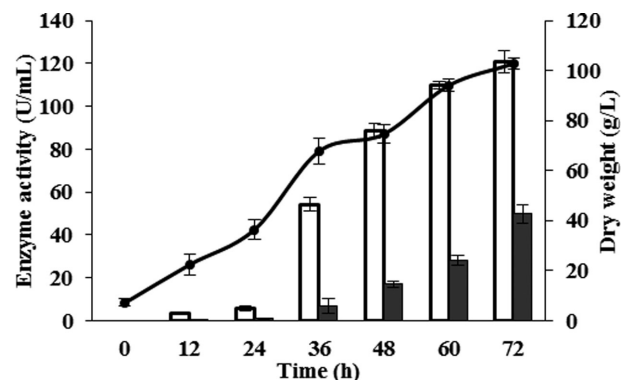


Fig. 1. Performance of *Pichia pastoris* PGFT6x-308 in fed-batch fermentation using molasses as a carbon source. Periplasmic (black bars) and extracellular (white bars) Sa1-SSTrec activities were measured in intact cells and the culture supernatant, respectively. Biomass yield (line) is expressed as dry weight.

Results and Discussions

Performance of Recombinant *P. pastoris* PGFT6x-308 in Fed-batch Fermentation With Molasses as Carbon Source

In a previous report, *P. pastoris* PGFT6x-308 constitutively expressing nine copies of the Sa1-SST was found to secrete the recombinant enzyme (Sa1-SSTrec), a fully active glycoprotein, to the cell periplasm and the extracellular medium in fed-batch fermentations using either glycerol or cane sugar as the carbon source (Hernández et al., 2018). The acquired ability of *P. pastoris* PGFT6x-308 to utilize sucrose prompted us to evaluate the use of sugarcane molasses as a cheaper substrate for cell growth.

Fig. 1 shows the behavior of *P. pastoris* PGFT6x-308 in a representative fed-batch fermentation using molasses as a carbon source. Cell density and Sa1-SSTrec production increased continuously over time (Fig. 1). After 72 hr of culture, Sa1-SSTrec activity was detected both in undisrupted biomass (49.8 U/ml of culture, equivalent to 0.48 U/mg of dry cell) and in extracellular medium (120.7 U/ml). The overall Sa1-SST activity (170.5 U/ml) was about 5.3- and 1.7-fold higher than the values reported for the carbon sources glycerol and cane sugar, respectively (Hernández et al., 2018). Previous cell fractionation experiments demonstrated that Sa1-SSTrec is not attached to membranes but secreted into the periplasmic space, where the substrate sucrose readily enters by diffusion (Hernández et al., 2018). The replacement of glycerol or cane sugar by molasses had no effect on the biomass yield, which remained above 100 g/l (dry weight). The volumetric productivity of *P. pastoris* PGFT6x-308 at the end of cell growth in the molasses-containing medium was 2368.0 U/l/hr.

Reducing sugars (glucose and fructose) and sucrose are the major carbohydrates in sugarcane molasses. Additional glucose is released from sucrose by the action of periplasmic and extracellular Sa1-SSTrec during cell growth. The continuous availability of the monosaccharide may have reinforced the transgene transcription. The GAP promoter, although constitutive, is known to behave stronger in glucose-grown cells (Waterham et al., 1997; Cereghino & Cregg, 2000).

Entrapment of *P. pastoris* PGFT6x-308 Cells in Calcium-Alginate Beads

Whole cells of *P. pastoris* PGFT6x-308 were entrapped in calcium-alginate gel using different biomass concentrations (100–400 g/l, wet weight) (Fig. 2). The highest recovery of Sa1-SSTrec

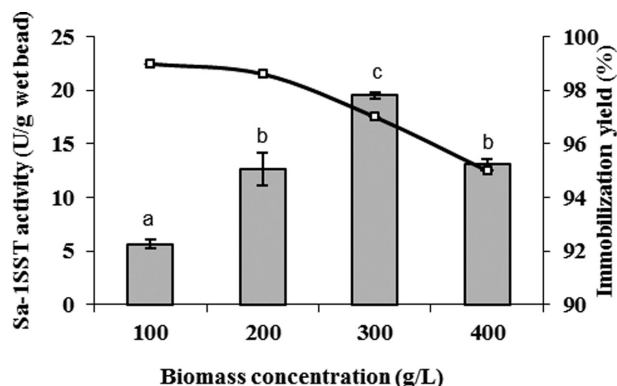


Fig. 2. Effect of biomass loading on cell entrapment efficiency (line) and Sa1-SSTrec activity (bars). Immobilization yield expresses the percentage of initial Sa1-SSTrec activity which was not recovered in the entrapped cells but remained in the CaCl₂ solution. Data are averages of three experiments with standard deviation. Different letters show significant differences between enzyme activities per bead for each biomass loading (Student–Newman–Keuls test, $\alpha = 0.05$).

activity was 98.9%, achieved in the beads prepared with the lowest biomass concentration (100 g/l). The immobilization yield decreased to 98, 97, and 95% in the treatment using 200, 300, and 400 g/l of wet biomass, respectively. Similarly, the immobilization yield dropped slightly with the increase in the biomass in the Ca-alginate entrapment process of heat-killed *P. pastoris* cells carrying a recombinant thermostable invertase in the periplasmic space (Martínez et al., 2014). We do not exclude the possibility that in both cases vigorous stirring may have caused some degree of mechanical damage to the cells releasing the soluble periplasmic enzymes to the external medium prior to bead formation.

The inferior Sa-1SSTrec activity (5.67 U/g) in the beads formed using the least concentrated biomass suspension (100 g/l) can be attributed to a suboptimal number of entrapped cells. The specific activity of the beads reached the maximum value (19.51 U/g wet bead) when biomass loading was 300 g/l. The treatment with the most concentrated cells (400 g/l) resulted in heavier beads of lower specific activity (13.11 U/g), suggesting a restricted internal diffusion of the substrate sucrose. Similarly, high biomass/alginate ratios failed to increase the activity of Ca-alginate entrapped cells of *Saccharomyces cerevisiae* (Polakovič et al., 2001) or *P. pastoris* with the recombinant periplasmic invertase (Martínez et al., 2014).

Scanning electron microscopy (SEM) studies are necessary to confirm the homogenous distribution of whole *P. pastoris* PGFT6x-308 cells inside the alginate spheres (diameter 2–3 mm) as well as to explore the 3D surface structure of the beads. SEM examinations of entrapped whole cells from different microorganisms have allowed a detailed characterization of the internal and external morphology of Ca-alginate beads (Van Neerven et al., 1990; Duarte et al., 2013; Gonçalves et al., 2020).

Permeabilization of Immobilized *P. pastoris* PGFT6x-308 Cells

The spherical Ca-alginate beads (diameter 2–3 mm) with specific sucrase activity of 19.51 U/g were incubated with toluene at three different concentrations. Sucrose conversion by the whole-cell biocatalyst was increased 2.2-, 2.3-, and 1.5-fold after the treatment with 3, 5, and 10% (vol/vol) of toluene, respectively (Fig. 3a). The membrane-permeabilization treatment enhanced the diffusion of the substrate sucrose into the cell increasing the catalytic action of the recombinant enzyme in the periplasmic space. Sa1-SSTrec is known to behave more active with the increase of sucrose concentration (Hernández et al., 2018). The

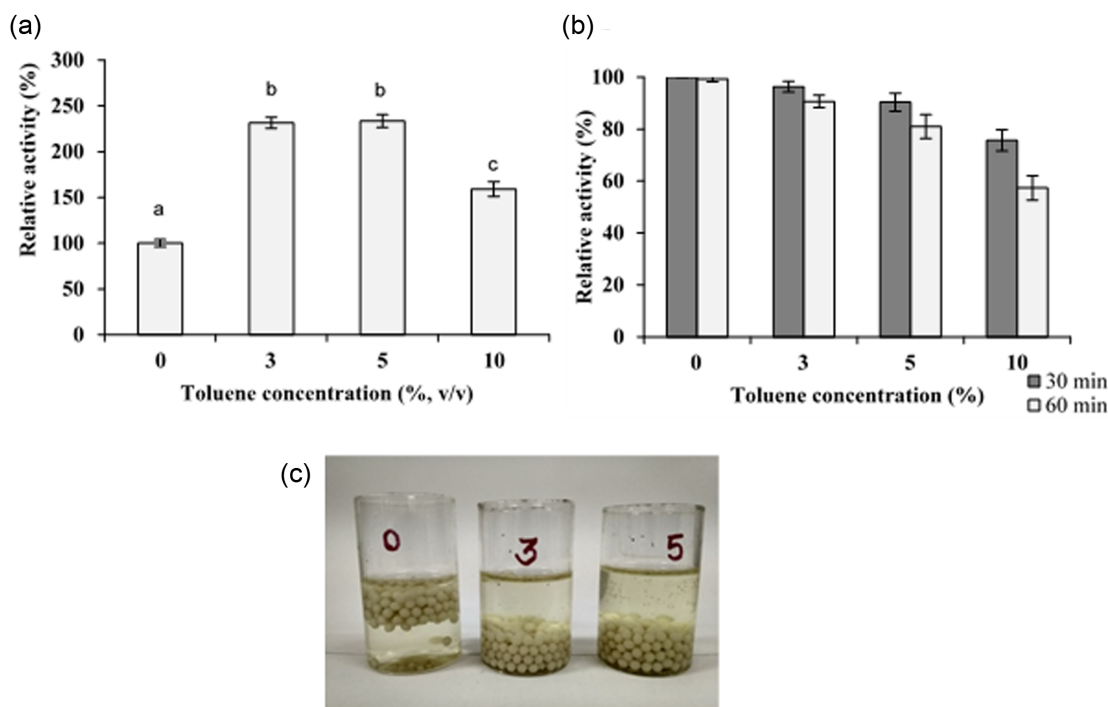


Fig. 3. Permeabilization of immobilized *Pichia pastoris* PGFT6x-308 cells. (a) Effect of toluene concentration on the Sa1-SSTrec activity of the calcium-alginate beads. (b) Effect of toluene on the stability of free Sa1-SSTrec. (c) Reduction of CO₂ formation by toluene-permeabilized cells during transformation of sucrose (1.75 M) at 30°C. Cut glasses contain beads previously incubated with 0, 3, or 5% (vol/vol) toluene. Data are the means of triplicate measurements \pm standard deviation. Different letters are significant differences between relative activity at the three concentrations with respect to the control (Student–Newman–Keuls test, $\alpha = 0.05$).

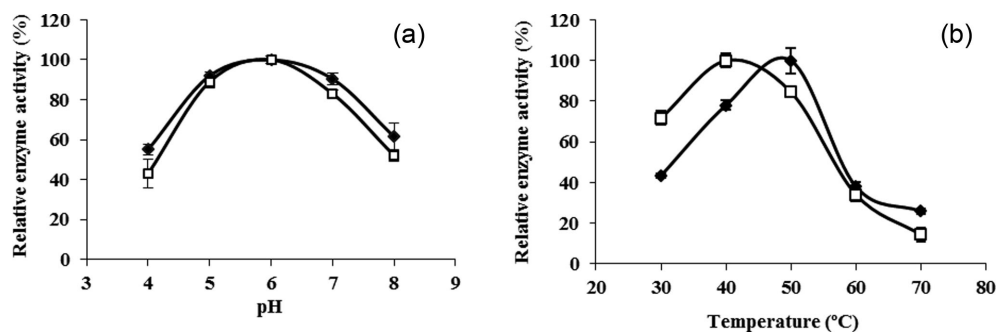


Fig. 4. Effect of pH (a) and temperature (b) on the initial rates of Sa1-SSTrec activity in free (□) and immobilized (◆) cells. Values are means of three experiments with standard deviation.

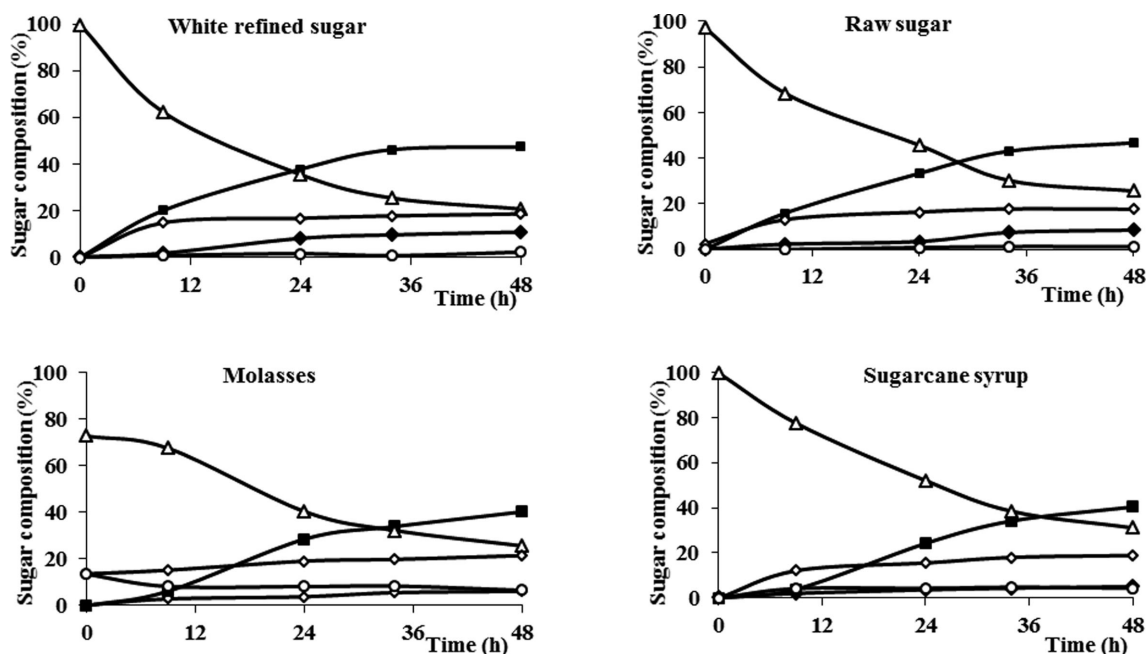


Fig. 5. Fructooligosaccharides production by the immobilized whole-cell biocatalyst reacting with different sucrose sources in a stirred tank reactor. Fructose (○), glucose (◇), sucrose (Δ), 1-kestotriose (■), and 1,1-kestotetraose (◆). Raw data are included as a supplementary table.

activity decay observed when the toluene concentration was raised from 5 to 10% (vol/vol) may be attributed to the enzyme sensibility to the organic solvent. Preincubation of free Sa1-SSTrec with toluene in 100 mM sodium acetate buffer (pH 5.5) at 30°C for 1 hr decreased the enzyme stability in direct proportion to the solvent concentration (Fig. 3b). In another experiment, the stirring (100 rpm) of a whole-cell suspension in the presence of 10% (vol/vol) toluene at pH 5.5 and 30°C for 1 hr did not result in the release of *N*-glycosylated periplasmic Sa1-SSTrec to the external medium.

Beads that were not exposed to toluene (control beads) produced CO₂ bubbles and floated up during incubation with 1.75 M sucrose at 30°C. Instead, the toluene-treated beads hardly released gas and sank to the bottom of the flask (Fig. 3c). Cell viability decreased from 10⁹ in the control beads to 10³ cfu/g of wet beads after the treatment with toluene at the optimal concentration of 5% (vol/vol). Toluene permeabilization elicited cell death, which is technically advantageous for biocatalyst operation. The reaction of Sa1-SSTrec on sucrose releases glucose, which is metabolized by living PGFT6x-308 cells producing the contaminants ethanol and acetate in the FOSs syrup. The glucose fermentation process generates CO₂. The formation of CO₂ bubbles by immobi-

lized yeast increases the internal pressure of packed-bed columns which then tend to crack (Najafpour et al., 2004).

Toluene at the assayed concentrations (3, 5, and 10% vol/vol) did not provoke the leakage of periplasmic Sa1-SSTrec out of the Ca-alginate matrix, considering that no sucrose-transforming activity was detected in the solutions after beads recovery. We assume that the permeabilization treatment killed the yeast without causing cell disruption. Early microscopic examinations revealed that nonviable bacterial and yeast cells treated with toluene remain essentially intact (Jackson & DeMoss, 1965; De Smet et al., 1978; Murakami et al., 1980).

Effect of pH and Temperature on Sa1-SSTrec Activity and Stability in Immobilized Cells

The effect of pH on the initial rates of Sa1-SSTrec reaction on sucrose (1.75 M) in free and immobilized *P. pastoris* PGFT6x-308 cells was evaluated in the range of 4–8 at 30°C (Fig. 4a). Free cells and toluene-treated beads showed similar pH-activity curves with the highest values at pH 6.0. The use of calcium alginate as the cell entrapment matrix caused no changes in the net charge of the biocatalyst.

Table 1. Carbohydrate Composition, Fructooligosaccharide (FOS) Yield and Productivity of the Biocatalyst Reaction With Different Sucrose Sources

Sucrose source	Refined sugar	Raw sugar	Molasses	Sugarcane syrup
Carbohydrate composition (% wt/wt)				
Glucose (G)	18.7	17.7	21.4	18.9
Fructose (F)	2.3	1.3	6.6	4.4
Sucrose (GF)	20.8	25.7	25.6	31.1
1-Kestose (GF ₂)	47.3	46.8	40.2	40.4
Nystose (GF ₃)	10.9	8.6	6.2	5.1
Y _{FOS/SUC} ^a	58.2	55.4	46.4	45.5
Y _{FOS/BC} ^b	3.5	3.3	2.8	2.7
Y _{kestosa/FOS} ^c	81.2	84.5	86.7	88.7
Productivity ^d	7.3	6.9	5.7	5.8

^aYield was calculated as FOS produced (g)/initial sucrose concentration (g) × 100.

^bYield was calculated from FOS produced (g)/biocatalyst (g).

^cYield was calculated from 1-kestose produced (g)/FOS produced (g) × 100.

^dProductivity was calculated from FOS produced (g/l)/time of reaction (hr).

The influence of temperature (30–70°C) on the initial rates of Sa1-SSTrec reaction on sucrose (1.75 M) in free and immobilized cells was evaluated at pH 5.5 (Fig. 4b). A 10-degree higher optimal temperature (50°C) was observed for the immobilized cells. It is plausible to think that the increase of the reaction temperature reduced the viscosity of the concentrated sucrose solution (1.75 M) enhancing the substrate diffusion into the beads. Similarly, mycelial fructosyltransferases from *Aspergillus flavus* NCCCI 2364 and *Aspergillus oryzae* IPT-301 displayed a higher optimal temperature after cell entrapment in calcium-alginate beads (Ganaie et al., 2014; Gonçalves et al., 2020).

The effect of pH (4–8) and temperature (30–60°C) on Sa1-SSTrec stability in nonviable entrapped cells was assayed at a sucrose concentration of 600 g/l to mimic the operational conditions of

FOSs synthesis. No significant variations in the enzyme stability were observed for the pH range 5–7 with remaining activities of 97 and 66% after preincubation for 2 and 30 days at 30°C, respectively. The half-life values of Sa1-SSTrec in the toluene-treated beads preincubated at 30, 40, and 50°C were 37 days, 8 days, and 18 hr, respectively. The enzyme was rapidly inactivated in the beads exposed to 60°C. The half-life of Sa1-SSTrec in the beads at 30°C is comparable to the values reported for immobilized fungal fructosyltransferases, which otherwise are often operated at 50°C due to their higher thermal stability (Chien et al., 2001; Nishizawa et al., 2001; Gonçalves et al., 2020).

Use of Different Sucrose Sources in FOSs Production by Immobilized Cells

Toluene-treated beads were incubated with refined sugar, raw sugar, sugarcane syrup, and molasses. The reactions were conducted at pH 5.5 and 30°C, and the product composition was monitored for 48 hr (Fig. 5). In all sucrose sources, 1-kestotriose (GF₂) was the main product, and 1,1-kestotetraose (GF₃) appeared as the reactions progressed. At the end of the four reactions, the ratio of 1-kestotriose and 1,1-kestotetraose was above 8:2. The overall FOSs yield in the reactions with refined or raw sugar represented above 55% (wt/wt) of total carbohydrates, but it decreased to 45% (wt/wt) with sugarcane syrup and molasses (Table 1). The highest FOSs productivity (7.3 g/l/hr) was achieved when using refined sugar as the substrate. On the other hand, the use of an inexpensive sucrose source as molasses appears particularly attractive for applications that do not require FOSs purification, for instance, animal feed.

Reuse of Immobilized Cells in Semicontinuous FOSs Production

Semicontinuous FOSs production was performed in stirred tanks at 30°C using refined sugar, raw sugar, sugarcane syrup, and

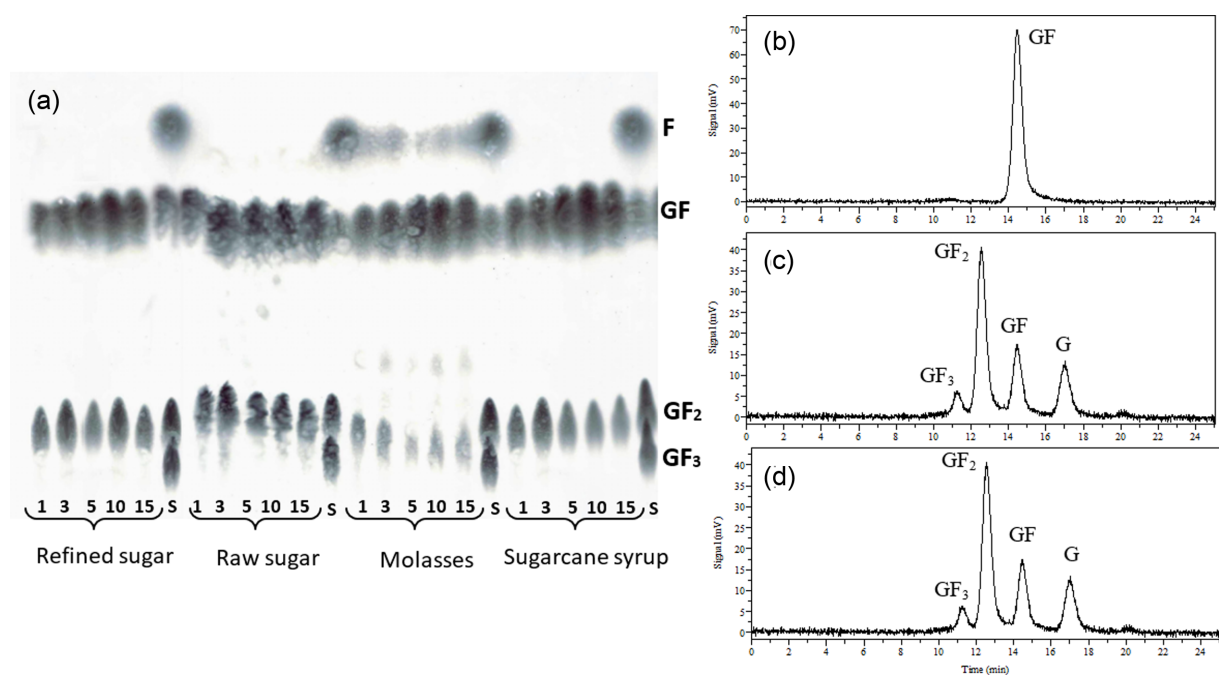


Fig. 6. Chromatographic analysis of fructooligosaccharides synthesized by immobilized cells from different sucrose sources in repetitive cycles. (a) TLC chromatograms of samples collected after batches 1, 3, 5, 10, and 15. S means mixture of standard sugars. (b–d) HPLC chromatograms. (b) Refined and raw sugar. (c) Refined sugar after the first batch. (d) Raw sugar after the first batch. GF₃, 1,1-kestotetraose; GF₂, 1-kestotriose; GF, sucrose; G, glucose; F, fructose.

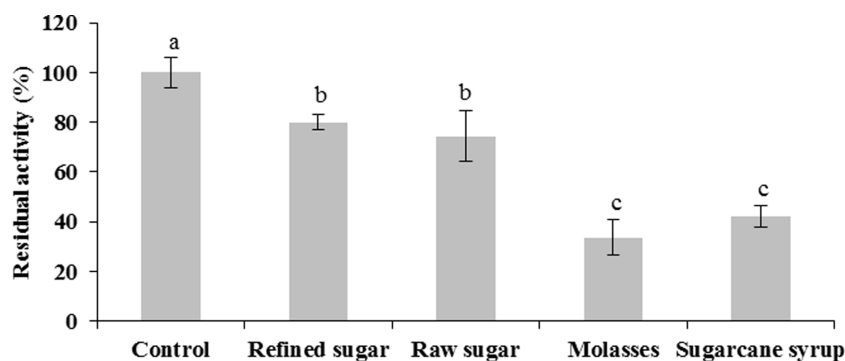


Fig. 7. Sa1-SSTrec stability in immobilized cells after 15 cycles of batchwise operation. Different letters show significant differences between residual activities in each sucrose source (Student–Newman–Keuls test, $\alpha = 0.05$).

molasses as substrates. The immobilized cells were reused in 15 repeated cycles of 48 hr, each for a total operation time of 30 days. TLC analysis of samples collected after cycles 1, 3, 5, 10, and 15 revealed the synthesis of 1-kestotriose (GF₂) in the four sucrose sources (Fig. 6). After 5 cycles, the 1-kestotriose yield tended to decrease, particularly when using sugarcane syrup and molasses as the sucrose source.

The stability of the biocatalyst was evaluated at the end of the 30-day batchwise operation at 30°C (Fig. 7). The residual activity of the beads incubated with sugarcane syrup and molasses was 41 and 32%, respectively. The continuous exposition with molasses softened and deformed the beads. Deterioration of the alginate matrix might have been caused by cations as manganese and zinc, with lower stabilizing power than calcium, as well as chelating agents as phosphates (Thu et al., 1996).

More attractively, the beads reacted with refined or raw sugar retained 75–80% of the initial Sa1-SSTrec activity. The high enzyme stability in the immobilized biocatalyst allows producing repetitive FOSs batches and offers the possibility to increase the residence time in the last cycles during prolonged reuse.

To our knowledge, this work is the first report of immobilized yeast cells expressing a plant fructosyltransferase. While Sa1-SSTrec recovered from the culture supernatant (free enzyme) of *P. pastoris* PGFT6x-308 is appropriate for sucrose conversion into 1-kestotriose in one-cycle batch reactions (Hernández et al., 2018), the stable performance of periplasmic Sa1-SSTrec in Ca-alginate entrapped cells during repetitive operation cycles makes the immobilized whole-cell biocatalyst attractive for continuous FOSs production. The reduction of diffusional barriers in the toluene-treated beads not only accelerated the Sa1-SSTrec reaction but also induced cell death avoiding the formation of undesired metabolic by-products (ethanol, acetate, and CO₂) during sucrose transformation. A heating treatment killed *P. pastoris* expressing a bacterial thermophilic invertase and enhanced sucrose hydrolysis in the cell periplasm (Martínez et al., 2014). Here, we show that membrane permeabilization is a valid option for the operational optimization of immobilized yeast cells expressing a mesophilic fructosyltransferase specialized in 1-kestotriose synthesis.

Conclusions

P. pastoris PGFT6x-308 cells constitutively expressing 1-SST from *S. arundinaceus* (Sa) were entrapped in calcium-alginate beads and exposed to a membrane-permeabilizing agent. In the toluene-treated beads, the enhancement of substrate and products diffusion through the outer membrane increased the catalytic action

of periplasmic Sa1-SSTrec. The immobilized whole-cell biocatalyst yielded 55% (wt/wt) FOSs, 1-kestotriose, and 1,1-kestotetraose in a ratio above 8:2, when reacted with refined or raw sugar (600 g/l) in a stirred tank reactor at 30°C and pH 5.5. The beads retained 75–80% of the initial Sa1-SSTrec activity after 15 repetitive cycles of FOSs production in a 30-day batchwise operation. The immobilized biocatalyst is suitable for reuse and cost-effective production of 1-kestotriose, the most attractive commercial FOS.

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Supplementary Material

Supplementary material is available online at JIMB (www.academic.oup.com/jimb).

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Conflict of Interest

The authors confirm that there is no conflict of interest regarding this article.

Data Availability

Experimental data are provided in the manuscript. Authors agree to provide any other data if requested.

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