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ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 49 (2007) 43–49
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Screening of yeast strains for transfructosylating activity

Francisco Maugeri *, Saartje Hernalsteens

Department of Food Engineering, University of Campinas, 13083-970 Campinas, SP, Brazil

Received 30 September 2006; received in revised form 31 May 2007; accepted 2 August 2007
Available online 7 August 2007

Abstract

Fructooligosaccharides (FOSs) are functional food ingredients with prebiotic properties, and a recent increase in the use of oligosaccharides in the food industry has led to the search for "new" microorganisms and enzymes for the production of oligosaccharides. This paper focuses on the screening of yeasts obtained from fruits and flowers (from Brazilian tropical forests), and capable of secreting extra-cellular enzymes with high fructosyl transferase activity (FTA). The screening and isolation procedures resulted in four potentially interesting yeast strains: *Candida* sp. (LEB-I3), *Rhodotorula* sp. (LEB-U5.), *Cryptococcus* sp. (LEB-V2) and *Rhodotorula* sp. LEB-V10. All were able to produce more then 100 g l^{-1} of FOS from a 500 g l^{-1} sucrose solution, but only the last one, (LEB-V10), showed no hydrolytic activity with respect to the FOS produced, giving a continuous increase in FOS content up to the end of the reaction, when it was about 50% of the total carbohydrates.

Keywords: Fructooligosaccharides; Prebiotics; Fructosyl transferase; Yeasts; Screening

1. Introduction

The concept of functional food represents a food, or food ingredient, with positive effects on host health and/or well being beyond its nutritive value. In more recent years, the concept has become more directed towards food additives that may exert a positive effect on the gut microbiota composition, mainly probiotics and prebiotics. A probiotic is a live microbial feed supplement that exerts beneficial effects for the host, promoting a good microbiological balance in the intestine, and a prebiotic is a non-digestible food component that could stimulate selectively the growth and/or activity of a limited number of colonic bacteria (probiotic bacteria) [1].

Fructooligosaccharides (FOSs) are functional food ingredients, showing prebiotic properties. In the literature FOSs is generally accepted as a common name only for fructose oligomers composed of 1-kestose (GF2), nystose (GF3) and 1^F-fructofuranosyl nystose (GF4), as shown in Fig. 1. The reaction mechanism depends on the source of the enzyme, but most of the microbial enzymes may catalyse the reactions of a readily reversible primary step and a subsequent irreversible step (Eq. (1)):

$$\begin{aligned} &\text{F\_R} + \text{E} \leftrightarrow \text{F\_E} + \text{R} \\ &\text{F\_E} + \text{acceptor} \rightarrow \text{F\_acceptor} + \text{E} \end{aligned} \quad (1)$$

where F is fructose, E is fructosyltransferase and R represents a carbonyl of an aldose. According to this mechanism, one molecule of sucrose serves as a donor and another acts as an acceptor for GF2 synthesis, releasing one molecule of glucose, for the production of GF3, the GF2 acts as an acceptor [2].

FOSs can be produced by either invertase (β-fructofuranosidase) or transferases (β-D-fructosyltransferase) [3] and many reports can be found in the literature on the production of FOS by fungi such as *Aspergillus niger* [4], *Aspergillus japonicus* [5] and *Aureobasidium sp*. [6]. However, few reports can be found on its production by extra cellular yeast enzymes, although studies do exist on *Kluyveromyces* sp. and its inulinase enzyme [7] and on *Rhodotorula* sp., referring to the production of gluco- and galactooligosaccharides [8]. Vranešić et al. [9], Risso [10] and Treichel [11] have done a great number of studies on inulinase and FOS production by *Kluyveromyces* sp., but despite all their efforts and the high enzyme production achieved (1300 U ml^{-1}), the maximum yield in FOS was about 12% in aqueous media and 18% in organic solvents.

* Corresponding author. Tel.: +55 21 19 35214034; fax: +55 21 19 35214027.
E-mail address: maugeri@fea.unicamp.br (F. Maugeri).

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doi:10.1016/j.molcatb.2007.08.001

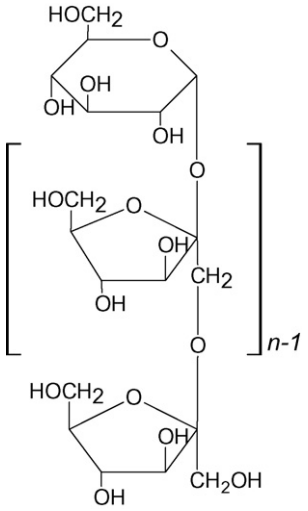

Fig. 1. Chemical structure of fructooligosaccharides. (GF_2: $n = 2$, kestose; GF_3: $n = 3$, nystose; GF_4: $n = 4$, 1^F-fructofuranosyl nystose).

The search for "new" enzymes for oligosaccharide production, using either microbial screening or molecular engineering, became necessary as a result of the increasing number of applications of oligosaccharides in the cosmetic, agrochemical, pharmaceutical and food industries.

The present study reports on the screening for high transfructosilating enzymes in yeasts strains isolated from fruits and flowers obtained from tropical Brazilian biomass. Tropical environments (forests) are one of the greatest sources of all kinds of living organisms, including yeasts, unicellular fungi with a ubiquitous distribution throughout almost all ecosystems. Brazil shows four regions of great biodiversity: the Amazon Forest; the Atlantic Forest (stretches along the Brazilian coast, showing an extremely diverse and unique mixture of vegetation); the Cerrado (tropical savanna eco-region) and the Pantanal (the world's largest wetland). Since Brazil is also a sucrose producing country (sugar-cane), and considering the increasing demand for FOS as a functional food, it would be interesting to find alternative processes for the production of fructooligosaccharides.

2. Experimental

2.1. Sample collection and yeast isolation

Fresh fruits and flowers were collected from the Amazon and Atlantic Forests and the Cerrado and Pantanal areas of Brazil. The samples collected were inoculated into 125 ml Erlenmeyer flasks containing 25 ml of a standard medium consisting of 2% yeast extract, 5% sucrose, 1% $NaNO_3$, 0.01% $MgSO_4{\cdot}7H_2O$, 0.1% K_2HPO_4 (pH 5.5) and incubated at 25 °C in a reciprocal shaker (150 rpm) for 72 h [5].

The cultures were streaked onto plate media (PDA, WLN and Inulin agar), supplemented with nalidixic acid and ampicilin (50 ppm, pharmaceutical grades) for bacterial inhibition.

The WLN agar (Wallerstein Laboratory nutrient agar) is a complex medium that provides easy differentiation of colonies by yeast morphology. It consists of (per litre): 4 g yeast extract; 5 g bactocasitone; 50 g dextrose; 550 mg KH_2PO_4; 125 mg KCl; 250 mg $MgSO_4{\cdot}7H_2O$; 2.5 mg $FeCl_3$; 2.5 mg $MnSO_4$; 22 mg Bromocresol green and 20 g agar.

Inulin agar medium was used to screen for inulinase producing yeasts, and showed the following composition (per litre): 20 g inulin (Raftline-Orafti); 1 g yeast extract; 3 g $NaNO_3$; 500 mg $MgSO_4{\cdot}7H_2O$; 200 mg $MnSO_4$; 1 g K_2HPO_4 and 20 g agar.

The pH values of the media were 5.0, and the cultures were grown on the agar plates at 25 °C for 48–72 h. Individual colonies were transferred to PDA plates to avoid contamination and the strains were maintained at 4 °C in GYMP-agar slants (2% glucose; 0.5% yeast extract; 1% malt extract; 0.2% KH_2PO_4 and 2% agar).

2.2. Primary screening: enzyme activity determination

Loopfuls of cells (from each strain), sub cultured on GYMP agar slants, were inoculated into 10 ml of standard medium in test tubes and cultivated at 25 °C for 24 h. After adequate development of the cultures, they were transferred into 500 ml flasks (with baffles) containing a further 100 ml of the same medium, and cultivated aerobically at 25 °C for 72 h in a reciprocal shaker (150 rpm).

Samples were collected after 24, 48 and 72 h, the cells harvested by centrifugation at $6000 \times g$ (10 min, 10 °C), and the cell-free supernatants used to screen for transfructosylating activity.

2.3. Secondary screening: oligosaccharide production

Strains shown to be potential producers of fructofuranosidase activity (FA) and transfructosylating activity (FTA) underwent a secondary screening procedure, with monitoring of the oligosaccharide production.

The reaction was performed at 50 °C in 50 mM sodium acetate buffer (pH 5.0) containing 60% (w/v) sucrose with an enzyme: reaction medium (v/v) ratio of 1:10. Samples were taken at appropriate times during the reaction, inactivated in boiling water and the oligosaccharide composition analysed by ion chromatography (HPLC-PAD).

2.4. Strain genus identification procedures

The genera were identified by the methodology described in "Key to specimen and genera" by Kregger-van-Rij [12].

2.5. Prebiotic effect

The growth of *Bifidobacterium longum* (BL-04, Rhodia) using the FOS produced as the carbon source, was studied by preparing a modified MRS medium containing 0.05% L-cysteine and about 0.20% carbohydrate (Glucose or FOS). Strain development was monitored from the optical density at 595 nm (OD) and by carbohydrate consumption (HPLC-PAD). Growth on glucose was considered as the positive control and growth on MRS with no carbohydrate source (basal MRS) as the negative control.

2.6. Enzyme properties

As a guide for future studies on the enzyme kinetics and mechanisms some properties of the extra-cellular enzymes were studied. Thus, the enzyme activities (initial reaction rate) with substrates of 2% and 50% sucrose and 2% inulin in 50 mM sodium acetate buffer (pH 4.5) at 50 °C were measured. Oligosaccharides were produced by incubating the enzyme (1 FTA/ml) in 0.5%, 5% and 50% sucrose (sodium acetate buffer 50 mM, pH 4.5), at 50 °C for 72 h.

The enzyme was concentrated by precipitation from a cell free culture broth, using ethanol at 4 °C to the required concentration. Precipitation with 70% ethanol and a two-step fractional precipitation (first adding ethanol to 50% saturation, centrifuging, and then adding more ethanol up to 70%) were tested. The cell-free supernatant and the enzyme rich precipitate were collected by centrifugation (6000 × *g*, 4 °C, 10 min). Both the enzyme activities and protein concentration were analysed.

2.7. Enzyme activity assay

The reaction medium to determine enzyme activity consisted of 50% sucrose in 50 mM sodium acetate buffer (pH 4.5) and a 10% adequately diluted enzyme suspension, incubated at 50 °C. Samples were collected at constant time intervals for 30 min and assayed for glucose (glucose-oxidase commercial kit) and reducing sugars (Somogi-Nelson method).

Sucrose conversion by fructofuranosidase yields glucose and fructose. However, in the presence of fructosyl transferase activity, part of the fructose is built into a fructan polymer, so it is possible to measure both the hydrolytic and transfructosylating activities from the amounts of glucose and reducing sugars released into the reaction media [5]. The activities can be determined from the equations below (Eq. (2)) after measuring the amounts of glucose (G) and reducing sugars (R) in the reaction media (F = fructose, F′ = transferred fructose):

$$\begin{aligned} R &= G + F \Leftrightarrow F = R - G \\ F' &= G - F \Leftrightarrow F' = 2G - R \end{aligned} \quad (2)$$

One unit of fructofuranosidase activity (FA) is defined as the amount of enzyme required to hydrolyse 1 μmol of sucrose per minute. One unit of transfructosylating activity (FTA) is defined as the amount of enzyme required to transfer one μmol of fructose per minute.

2.8. Chromatographic analysis for FOS

Identification and quantification of the oligosaccharides was achieved by ion exchange chromatography with a pulsed amperometric detector (HPLC-PAD). Chromatography was performed on a Carbopac PA-100 column at 22–24 °C, using a GP50 gradient pump, ED40 electrochemical detector and the software PEAKNET, all from DIONEX (USA). The sugars were eluted in 50 mM sodium hydroxide with a linear gradient of sodium acetate (0–200 mM), at a flow rate of 1 ml min^{-1}.

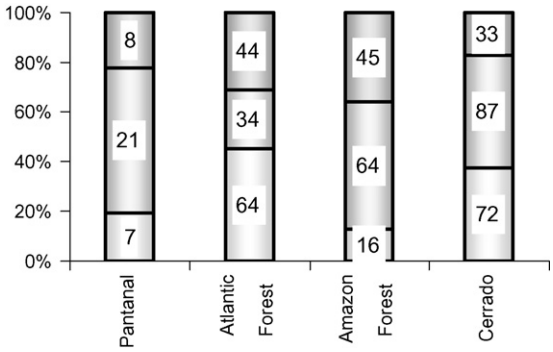

Fig. 2. Data from the isolation and primary screening procedures. (■) Strains with FTA activity; (■) strains with FA activity; (□) strains without activity over sucrose.

3. Results and discussion

3.1. Yeast selection

The isolation procedure resulted in 495 yeast strains. The first step in the selection of the interesting yeast strains was the determination of their extra-cellular fructofuranosidase and transferase activities (on sucrose). About 25% of the isolated strains had some kind of activity on sucrose (*Primary screening*), resulting in 130 strains for further testing. Fig. 2 shows the distribution of the yeast strains within the collection areas and the results of the primary screening procedure.

After the quantitative analysis of the enzyme activity and the qualitative analysis of the oligosaccharide production (*Secondary screening*), four strains showing potential for FOS production were selected. After the qualitative analysis of oligosaccharide production, a synthesis reaction was carried out to determine the FOS content. All the enzymes studied produced fructo-oligosaccharide 1-kestose (GF2). On the other hand, the nystose (GF3) and fructofuranosyl-nystose (GF4) contents were only important in the syntheses performed by the LEB-V10 and LEB-U5 enzymes.

The genera of these microorganisms were, in sequence, *Candida* (LEB-I3 strain), *Rhodotorula* (LEB-U5 and LEB-V10 strains) and *Cryptococcus* (LEB-V2 strain).

3.2. Enzyme production and concentration

The production assays were performed in shaken flasks and the results obtained for the FA and FTA activities of the selected strains during fermentation shown in Fig. 3. The strains I3 and V10 showed remarkable FTA activities and, interestingly, low FA activities.

The enzymes were recovered from the culture broth and concentrated by adding ethanol to the required concentration, as shown on Table 1. Attempts at fractional precipitation gave poor results, since the loss of activity was considerable, although low protein concentrations were achieved. In the case of single step precipitation with 70% ethanol saturation, the protein content was almost the same as in the crude broth, but the loss of enzyme activity was significantly decreased, except for the enzyme from I3, where yields of only about 30% were recovered.

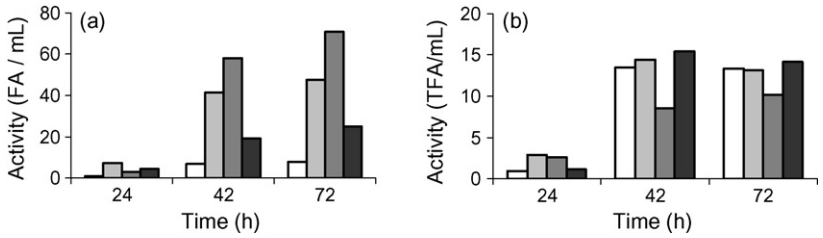

Fig. 3. Enzyme production by different strains during fermentation: (a) FA and (b) FTA activity ((□) LEB-I3; (▨) LEB-U5; (▩) LEB-V2; (■) LEB-V10).

Table 1
Enzyme recovery from the cell-free broth

Enzyme	Concentration step	Enzyme (FTA)	Protein (mg)	Specific activity (FTA/mg)	Purification factor	Yield (%)
	Crude broth	480	1178	0.4	1.0	100
I3	One step[a]	140 ± 4	927 ± 3	0.2	0.4	29
	Two steps[b]	55 ± 5	48 ± 2	1.1	2.8	11
	Crude broth	510	654	0.8	1.0	100
U5	One step[a]	484 ± 5	614 ± 4	0.8	1.0	95
	Two steps[b]	91 ± 8	26 ± 1	3.5	4.6	18
	Crude broth	450	1354	0.3	1.0	100
V2	One step[a]	414 ± 4	1298 ± 4	0.3	1.0	92
	Two steps[b]	45 ± 6	105 ± 5	0.4	1.3	10
	Crude broth	910	1561	0.6	1.0	100
V10	One step[a]	546 ± 6	1445 ± 3	0.4	0.6	60
	Two steps[b]	119 ± 7	214 ± 5	0.6	1.0	13

[a] Final ethanol concentration of 70%.
[b] First step: final ethanol concentration of 40% and second step: ethanol up to 70%.

3.3. Enzyme properties

In order to assess what type of enzyme we were dealing with, fructofuranosidase or fructosyl transferase, a series of experiments were carried out involving sucrose conversion and FOS production. Considering the enzyme activities with 2% and 50% sucrose and with 2% inulin 2 (Table 2), and analysing for oligosaccharide production (Figs. 4–7), it was possible to determine important enzyme characteristics. As can be seen in Table 2, all the enzymes showed higher fructofuranosidase activities with 2% sucrose and some inhibitory effects could be noticed with 50% sucrose.

Hidaka et al. [13] showed that the greater the ratio between the fructosyl transferase and hydrolytic activities, the greater the production of FOS, and only enzymes with high ratios between the activities were able to produce FOS at low sucrose concentration (5.0% and 0.5%). They also showed that enzymes with lower ratios between the activities, had hydrolytic activity for the FOS, and consequently all the fructooligosaccharide produced during the first 20 h of reaction was hydrolysed by the end of the reaction period. At low sucrose concentrations, transfructosylating activity is more common in the transferase type enzymes, although fructofuranosidase enzymes can also produce FOS under these conditions if there is an adequate transglycosilation mechanism present (requiring an efficient accepting molecule).

The U5 enzyme maintained almost the same fructofuranosidase activity at both sucrose concentrations, but showed higher transfructosylating activity at 50% sucrose. On the other hand, the V10 enzyme showed low FTA activity on 2% sucrose, but at 50% sucrose concentrations the FTA activity was almost eight times the FA activity, so although this enzyme shows some fructofuranosidase characteristics, it may have a very particular kinetic behaviour.

Other valuable information was that no enzyme showed remarkable inulin hydrolysing activity, so they could not be characterized as inulinase enzymes. At 50% sucrose concentration, the V2 enzyme showed high transfructosylating activity,

Table 2
Enzyme activities with different substrates

Enzymes	50% Sucrose			2% Sucrose			2% Inulin
	FTA	FA	FTA/FA	FTA	FA	FTA/FA	U_F
I3	16.5 ± 0.8	2.6 ± 0.5	6.3	3.0 ± 0.5	4.7 ± 0.4	0.6	0.6 ± 0.2
U5	6.4 ± 0.7	2.8 ± 1.2	2.3	2.6 ± 1.4	2.9 ± 1.5	0.9	0.5 ± 0.3
V2	13.6 ± 1.0	3.8 ± 1.4	3.6	7.8 ± 1.7	8.2 ± 1.4	1.0	0.3 ± 0.2
V10	6.9 ± 0.5	0.9 ± 0.4	7.7	1.7 ± 0.2	2.9 ± 0.2	0.6	0.3 ± 0.1

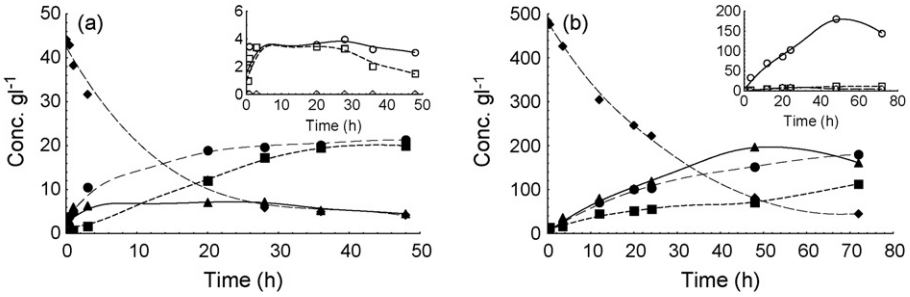

Fig. 4. FOS production by the LEB-I3 enzyme (a) 5% sucrose and (b) 50% sucrose. (♦) Sucrose, (■) fructose, (●) glucose, (▲) FOS, (◯) GF2, (□) GF3 and (◊) GF4.

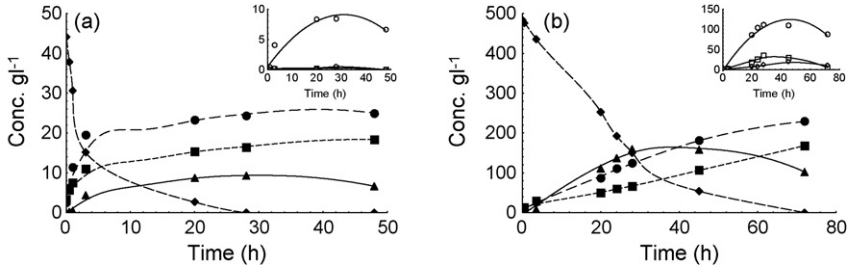

Fig. 5. FOS production by the LEB-U5 enzyme (a) 5% sucrose and (b) 50% sucrose. (♦) Sucrose, (■) fructose, (●) glucose, (▲) FOS, (◯) GF2, (□) GF3 and (◊) GF4.

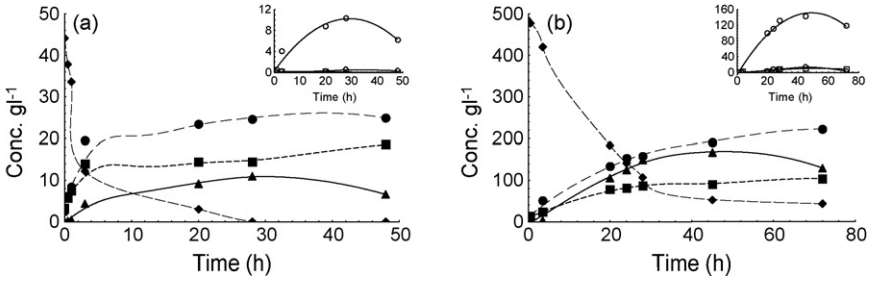

Fig. 6. FOS production by the LEB-V2 enzyme (a) 5% sucrose and (b) 50% sucrose. (♦) Sucrose, (■) fructose, (●) glucose, (▲) FOS, (◯) GF2, (□) GF3 and (◊) GF4.

but at low sucrose concentration, the two kinds of activity were equilibrated.

During the synthesis of fructooligosaccharides from 0.5%, 5% and 50% sucrose solutions, the changes in carbohydrate composition were followed for 72 h of reaction (Figs. 4–7). As the reaction progressed, and sucrose was converted into FOS, the glucose and fructose concentrations increased.

All four enzymes produced FOS (from 5% and 50% sucrose solution), but the enzymes I3, U5 and V2 showed a remarkable degree of hydrolysis of the FOS produced, after 24 h of reaction for enzyme I3, U5 and V2. The FOS content, considering the synthesis from 50% sucrose, reached almost 40% of the total carbohydrates within 48 h for enzyme I3, and the enzymes U5 and V2 showed the same behaviour with respect to FOS production, reaching maximum concentrations of 30% and 22%, respectively, after 24 h. Observing the oligosaccharide contents during the course of the reactions, the enzyme V10 was the only one that showed a continuous production of FOS, with

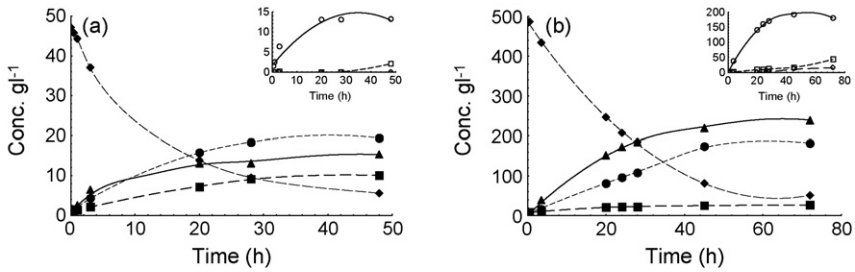

Fig. 7. FOS production by the LEB-V10 enzyme (a) 5% sucrose and (b) 50% sucrose. (♦) Sucrose, (■) fructose, (●) glucose, (▲) FOS, (◯) GF2, (□) GF3 and (◊) GF4.

considerable amounts of GF3 and GF4, reaching maximum concentrations of about 35%, after 24 h of reaction and 45%, after 48 h.

At 0.5% sucrose solutions, all experiments led to the complete hydrolysis of sucrose, producing only glucose and fructose. The use of reaction media containing 5.0% sucrose yielded 14–25% FOS, showing the high fructosyl transferase activity. On basis of the analysis of the enzyme activities and the FOS production with different sucrose concentration, and considering that β-D-fructosyltransferase only possesses transfructosylating activity [5], the results suggest that although the high transfructosylating activity, all four enzymes should therefore be classified as β-fructofuranosidase.

At commercial scale, FOS production is made with immobilized cells from *A. niger* and *Aureobasidium pullulans* on 60–70% sucrose solution, resulting in 55–60% FOS yield after 24 h of reaction [14]. The comparison of our data and the literature best results, shows that although the process was not optimised and up-scaled, the FOS production and yield from these fours yeasts strains (particularly the enzyme from the strain LEB-V10), are quite similar to the commercial production.

According to the above results, more extensive works are needed to maximize FOSs yield and productivity using the enzymes from strains LEB-I3, LEB-U5 and LEB-V2, mainly because the hydrolytic activity, as described for the inulinase from *Kluyveromyces marxianus* enzyme [10], whose assessment showed that the use of both statistical procedures (experimental design) and organic media could increase the yield and/or the productivity in about 50%. However, the high hydrolytic activity, inherent to this enzyme, made it impossible to obtain very high yields.

Regarding the LEB-V10 enzyme, the long reaction time needed to reach the maximum yield was certainly due to the low enzyme activity used in the reaction. Nevertheless, in this case it is worth noticing that the FOSs are not hydrolysed along the whole process, leading to high concentrations of this product. Furthers studies of optimisation will probably lead to the increase of the yields and mainly productivity, by varying, e.g. temperature, pH, sucrose and enzyme concentrations.

3.4. Prebiotic effect

To study the prebiotic effect of the oligosaccharides synthesized, the growth of a probiotic bacterium, *B. longum* was tested (data not shown). After sterilization and adequate dilution, the carbohydrate composition of the FOS culture medium was (per litre): LEB I3–11.8 g sucrose; 1 g glucose; 1 g fructose; 11 g FOS; LEB *U5*–4 g glucose; 3.6 g fructose; 15.3 g FOS; LEB *V2*–8.3 g sucrose; 3.3 g glucose; 2.5 g fructose; 14.2 g FOS; LEB *V10*–4.9 g glucose; 4.3 g fructose; 16 g FOS.

Development of the probiotic bacterium on the glucose medium resulted in total consumption of the substrate after 60 h of fermentation. For the medium containing fructooligosaccharides from the strains LEB-I3, LEB-U5 and LEB-V2, the oligosaccharides were consumed before 30 h of fermentation. The LEB-V10 FOS consumption rate was slower than that of the other strains, however it had been completely vanished after 80 h of fermentation.

The FOSs produced by the above enzymes were shown to support (data not shown) the growth of a variety of probiotic bacteria, proving to exert an efficient prebiotic effect. According to those results glucose was shown to be the preferred substrate for the probiotic bacteria growth (higher cellular growth and substrate consumption), however FOS was equally a good substrate, as the conclusions reported by several researchers, who demonstrated that FOSs are a prebiotic ingredient trough in vivo and in vitro assessments, studying the development of probiotic bacteria and the metabolism effect of both pure and commercial FOS [15,16].

4. Conclusion

The results of this study showed what a great source of microorganisms are present in our environment, with the possibility of screening for all kinds of biotechnological uses. The screening and isolation procedures for extra cellular transfructosylating yeast activity (FTA) resulted in four yeast strains with great potentials for application. Although the enzymes from the strains LEB-I3 (*Candida* sp.), LEB-U5 (*Rhodotorula* sp.) and LEB-V2 (*Cryptococcus* sp.) showed high hydrolytic activity as well, a study of their biochemical characteristics might determine one condition where the FOS content was optimum. The efforts made to screen for high extra-cellular transfructosylating enzyme producing yeasts gave very promising results. The production of fructooligosaccharides by the LEB-V10 enzyme (another *Rhodotorula* sp.) was the most successful of the four, showing a continuous increase in FOS concentration up to the end of the synthesis reaction, when it was about 50% of the total carbohydrate content.

Acknowledgments

To FAPESP, CAPES and CNPq for their financial support.

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