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Screening of whole yeast free-cells and optimization of pH and temperature for fructooligosaccharides production

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ABSTRACT. Fructooligosaccharides are catalyzed by β–fructofuranosidase enzyme, produced by many microorganisms. However, in order to achieve a more profitable, low time-consuming process with lower cost, researchers have sought alternatives. This study aimed to select and identify yeasts able to produce fructooligosaccharides and evaluate the influence of pH and temperature on their synthesis. Yeast suspensions, solutions of 500 g L^{-1} sucrose and three values of pH (4.5, 5.5, and 6.5) and temperature (40, 50, and 60ºC) were tested. Yeast species were identified by molecular techniques. Among 141 yeast isolates from grapes, 65 were able to synthesize fructooligosaccharides. The maximum concentration of fructooligosaccharides was 4.8% (w v^{-1}), and *Saccharomyces cerevisiae* 222 produced 1-kestose and nystose.

Keywords: prebiotics, biotransformation, identification, PCR.

Seleção de células inteiras de levedura e otimização dos parâmetros pH e temperatura para a produção de fruto-oligossacarídeos

RESUMO. Fruto-oligossacarídeos são catalisados pelas enzimas β–fructofuranosidase, produzida por muitos micro-organismos. No entanto, para obter processos mais rentáveis, de menor custo e tempo, pesquisadores têm procurado alternativas. Este trabalho tem objetivo de selecionar e identificar leveduras capazes de produzir fruto-oligossacarídeos e avaliar a influência do pH e da temperatura na sua síntese. Suspensões de leveduras, soluções de sacarose de 500 g L^{-1} e três valores de pH (4,5, 5,5 e 6,5) e de temperaturas de (40, 50 e 60ºC) foram utilizados. As espécies de leveduras foram identificadas por técnicas moleculares. De 141 isolados de leveduras de uvas, 65 foram capazes de sintetizar fruto-oligossacarídeos. A concentração máxima de fruto-oligossacarídeos foi de 4,8% (p v^{-1}), e a levedura *Saccharomyces cerevisiae* 222 produziu 1-cestose e nistose.

Palavras-chave: prebióticos, biotransformação, identificação, PCR.

Introduction

In the last few years, it has been noted a growth in consumer interest in healthier and nutritional food. This trend increased the search for fructooligosaccharides, functional ingredients that stimulate the metabolism and cellular division of the beneficial bacteria of the digestive tract and improve the hosts' health and well-being (Roberfroid et al., 2008, Nobre, Suvarov, & Weireld, 2014). In 2015, the U.S. market for prebiotics was estimated at $225.1 million and the European market at $1.17 billion. Nowadays, the world demand for prebiotics is estimated to be around 1,670.000 tons and fructooligosaccharides contributed to 10% of natural sweeteners (Singh, Singh, & Kennedy, 2016).

Fructooligosaccharides are constituted of one molecule of sucrose and one or more fructose units bond by β-(2→1) or β-(2→6) glycosidic bonds. These carbohydrates are degraded by anaerobic bacteria in the gut into acetate, propionate and butyrate, which reduce colon pH, improve the absorption of minerals ions, such as iron, calcium and magnesium, enhance the growth of colon microbiota and improve the immunological and anti-inflammatory response (Cummings & Macfarlane, 2007, MacDonald, Cochrane, Wopereis, & Loveridge, 2011, Quigley, 2011).

The industrial production of fructooligosaccharide uses β–fructofuranosidase enzyme to catalyze the hydrolysis of sucrose into glucose and fructose, and promote the link of a fructose moiety to another sucrose molecule (Flores-Maltos et al., 2016). This enzyme is found in a great number of filamentous fungi, yeasts and bacteria (Oku, Tokunaga, & Hosoya, 1984, Maugeri & Hernalsteens, 2007, Gutiérrez-Alonso, Fernández-Arrojo, Plou, & Fernández-Lobato, 2009, Linde et al., 2009, Ning et al., 2012, Tian & Karboune, 2012).

Environmental microorganisms are a good source of metabolites, such as fructooligosaccharides, due to their exposure to different stressing factors that alters the biochemical metabolism in order to survive the adverse conditions (Hohmann, 2002). The aim of this work was to investigate the synthesis of fructooligosaccharides by yeasts isolated from grape peel and to determine the optimal pH and temperature to produce these prebiotics.

Material and methods

Microorganisms

The 141 yeast strains investigated were isolated from *Vitis labrusca* grapes from vineyards located in the state of Paraná, Brazil. The strains were stored at -20°C until use.

Cells cultivation

The cell growth was conducted in 50 mL Erlenmeyer flasks containing 10 mL YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, initial pH value 5.5±0.2). The medium was sterilized at 121°C, 1 atm, using adequate time. After inoculation, the flasks were kept in orbital shaker at 150-rpm and 28°C for 24 hours.

To increase cell number, yeast cells were transferred to 1000 mL Erlenmeyer flasks containing 200 mL YPD medium. The flasks were kept in orbital shaker under the same conditions. Hence, the yeast cells were separated from the media by centrifugation at 7000 *xg* for 5 min. The cells were suspended with a certain amount of sterilized distilled water, quantified by dry weight and maintained at 4°C until the moment of use.

Screening of wild yeasts for fructooligosaccharides producing enzymes

An amount of 28 mg dried cells mL^{-1} of each yeast suspension was, individually, inoculated into 10 mL of 500 g L^{-1} sucrose solution (initial pH value 4.5±0.2). The experiment was conducted with four flasks for each strain of indigenous yeasts, in triplicate. After inoculation, the Erlenmeyer flasks were kept in orbital shaker at 50°C and 150 rpm agitation for a total of 12 hours.

Every three hours, a flask of each isolated yeast was withdrawn and maintained at 90°C for 10 min to inactivate the enzymes. The content of each flask was centrifuged to separate the cells from the supernatant and supernatants were used to investigate the formation of the fructooligosaccharides and monosaccharides by thin layer chromatography.

Evaluation of pH and temperature for fructooligosaccharides synthesis

An amount of 28 mg dried cells mL^{-1} of each yeast suspension was used. The 500 g L^{-1} sucrose solution was adjusted to different pH values: 4.5 and 5.5, 100 mmol L^{-1} acetate buffer, and pH 6.5, 100 mmol L^{-1} phosphate buffer. Each experiment conducted at the three pH values was evaluated at the temperatures of 40, 50 and 60°C.

Analytical methods

Thin layer chromatography

Each sample of the screening experiment was tested by thin layer chromatography. Glass plates measuring 20 x 20 cm with a Silica Gel 60 layer of 300 µm were used. A mobile phase of butanol: acetic acid: water (5:4:1) and the visualization solution was 2% sulphuric acid in methanol were employed. An aliquot of 4 µL of the samples and of the analytical standards were applied on the silica plate. Solutions of glucose, fructose, sucrose, 1-kestose, and nystose (all from Sigma Aldrich) at the concentration of 1 mg mL^{-1} each, were used as analytical standards to determine the retention factor of each substance.

Carbohydrate quantification by high pressure liquid chromatography

The analysis was performed on a Varian ProStar HPLC (Varian Inc., Walnut Creek, CA, USA) comprising an index refractor detector, Varian, model 350. Chromatographic separation was performed in a Supelcosil LC-NH_2 column (5 μm; 250 x 4.6 mm) through an isocratic gradient, using as mobile phase a mixture of acetonitrile: water (80: 20) at a flow rate of 1 mL min^{-1}.

Target compounds (glucose, fructose, sucrose, 1-kestose and nystose) were identified according to retention times and quantified by interpolation in analytical curves of the external standards. The carbohydrates glucose and fructose had an analytical curve developed between the concentrations of 0.10 and 10.0 g L^{-1}; sucrose, 0.10 and 20.0 g L^{-1}; and, 1-kestose and nystose, 0.10 and 5.0 g L^{-1}. The equations and coefficients of determination are listed in Table 1. The samples were filtered through a 0.20 µm filter before the injection and a 20 µL injection volume was used.

Table 1. Analytical curves and coefficient of determination for the substances analyzed by HPLC.

Carbohydrate	Analytical curve	Coefficient of determination
Fructose	y = 654926x-58077	0.9960
Glucose	y = 639942x-34,251	0.9992
Sucrose	y = 758439x+296170	0.9964
1-Kestose	y = 750048x+23655	0.9975
Nystose	y = 657976x+45129	0.9967

Taxonomic identification of yeasts

MALDI-TOF mass spectrometry

For MALDI-TOF MS analysis, yeasts were grown on YPD solid medium (pH 5.5) at 28ºC, for 48 hours. Sample preparation, mass spectra acquisition and data analysis were conducted as described elsewhere (Agustini, Silva, Bloch, Bonfim, & Silva, 2014). MALDI-TOF MS analyses were performed on a MicroFlex LRF mass spectrometer (Bruker Daltonics, Bremen, Germany). To identify a microorganism, the spectrum acquired was loaded with the MALDI Biotyper Software and analyzed by use of the standard pattern-matching algorithm, which compared the spectrum acquired with those present in the manufacturer library (Biotyper version 3.0.1) and in the Supplementary database (Agustini et al., 2014). According to manufacturer protocols, the results of the pattern-matching process are expressed as log score values, which ranged from 0 to 3. Score values >1.7 indicate identification beyond the genus level, and score values >2.0 indicate identification to the species level. Scores <1.7 were interpreted as no identification.

PCR-RFLP technique

DNA extractions were carried out using the freeze-thawing process described by other authors (Silva, Bernardi, Schaker, Menegotto, & Valente, 2012). The primer pairs used were ITS1 and ITS4. PCR mix and the amplification conditions followed Agustini et al. (2014). For Restriction Fragment Length Polymorphism Analysis (RFLP), the endonuclease used was *Hae* III (Promega, USA) employing temperature and incubation time as recommended by the manufacturer. PCR products were resolved in 1% agarose gel electrophoresis, while restriction fragments were resolved in 3% agarose gel electrophoresis. The gels were stained with ethidium bromide and the stained DNA was visualized under UV light on the Eagle Eye Image II. The size of fragments was estimated by comparisons with a 100-bp DNA ladder (Invitrogen, Brazil).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and to Student's t-test for independent variables. Statistica for Windows version 8.0 (StatSoft Inc., Tulsa, OK, USA) was used for all analyses.

Results and discussion

Screening of indigenous yeasts for fructooligosaccharides producing enzymes

The screening of wild yeasts for fructooligosaccharides production was made through thin layer chromatography. The thin layer chromatography plaques demonstrated four distinct spots. The retention factors of monosaccharides (glucose and fructose) was 0.6, sucrose, 0.5, trisaccharide (similar to 1-kestose standard solution), 0.4, and tetrasaccharide (similar to nystose standard solution), 0.3. From the 141 indigenous yeast tested, 65 synthesized trisaccharide and/or tetrasaccharide in the screening experiment. Among them, 60 strains formed the fructooligosaccharides after three hours of reaction, four, after six hours and only one strain after nine hours of reaction. After 12 hours of reaction, the number of strains producing monosaccharides increased.

The optimal reaction time for most of the yeasts was estimated at six hours. The samples from the yeasts that were positively identified as a possible prebiotic producer were analyzed by HPLC/RID. The trisaccharide was identified as 1-kestose, and the tetrasaccharide, as nystose.

Evaluation of pH and temperature for fructooligosaccharides production

Indigenous yeast identification

To evaluate the influence of pH and temperature on fructooligosaccharide synthesis, eight indigenous yeasts were randomly chosen and numbered, namely 34, 67, 80, 97, 205, 222, 225, and 283. These yeasts were identified by molecular techniques and by MALDI-TOF MS using the Biotyper database (Agustini et al., 2014). All the strains were identified as *Saccharomyces cerevisiae*. Their amplicon and restriction fragments were in accordance with others studies, as shown in Figure 1 (Guillamón, Sabaté, Barrio, Cano, & Querol, 1998, Fernández-Espinar, Esteve-Zarzoso, Querol, & Barrio, 2000, Pham et al., 2011).

Effect of pH and temperature on fructooligosaccharide production

The eight yeasts selected (34, 67, 80, 97, 205, 222, 225, 283) had different production of 1-kestose ($p < 0.05$) at the pH value of 4.5, at the temperatures 40, 50 and 60ºC and, also, at pH values 5.5 and 6.5, at the temperatures 40 and 60ºC.

At the temperature of 40ºC (Table 2), there was no detection of 1-kestose production by yeast 34 at pH value 6.5, and yeasts 67 and 283 at pH 4.5. At

this temperature, yeast 205 produced the trisaccharide, differently from the other strains. The maximum production of 1-kestose by the yeasts 205 and 80 was at pH 4.5, reaching a concentration of 6.34±1.11 g L^{-1} and 16.53±3.34 g L^{-1}, respectively. For both yeasts, it was noted that as pH values increase (pH 5.5 and 6.5), the concentration of 1-kestose decreases. The best conditions for yeast strains 225 and 283 to produce 1-kestose was pH value 5.5 and 40ºC temperature, achieving similar concentrations (14.78±1.02 and 13.25±4.05 g L^{-1}, respectively). At this temperature, the highest level for 1-kestose was achieved by yeast 97 at pH value 6.5 (23.25±1.88 g L^{-1}).

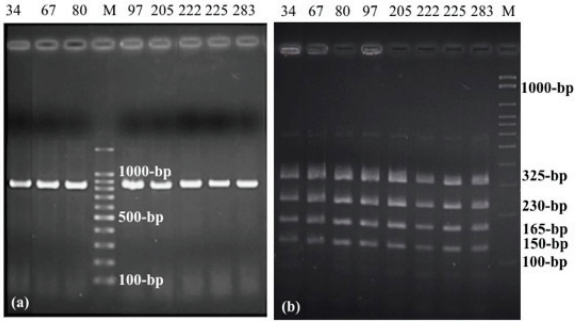

Figure 1. Gel electrophoresis of the PCR/RFLP results of the indigenous yeasts 34, 67, 80, 97, 205, 222, 225, and 283. (a) Amplicon sizes using ITS1 and ITS4 primers. (b) Restriction profile using the endonuclease *Hae* III.

The indigenous yeast 67 produced the highest concentration of 1-kestose at 50ºC and pH 4.5, reaching 20.39±2.90 g L^{-1}. At these conditions, yeast 67 had the lowest monosaccharide concentration, 57.25%, compared to 79.8 and 71.35% obtained under the same temperature and pH values of 5.5 and 6.5. A higher transfructosylation activity can be assumed at pH value 4.5. On the other hand, at pH value 5.5, a higher hydrolytic activity is demonstrated due to a lower 1-kestose concentration and a greater monosaccharide concentration.

At the temperature 50ºC, no statistical difference was detected between the 1-kestose production by the wild yeasts tested at pH values 5.5 ($p = 0.149$) and 6.5 ($p = 0.068$). And, the maximum concentration was reached by the yeast 97, 23.25±1.88 g L^{-1}, at pH 5.5.

Finally, at 60ºC, the greater amount produced was at pH value 4.5 regarding the activity of yeast 225, reaching 23.47±1.88 g L^{-1} of 1-kestose. This strain presented lower production at higher pH values. The yeasts 80 and 205, at the temperature 40ºC, had a similar behavior.

At the temperatures 50 and 60ºC, pH value 6.5, yeast 222 produced the highest concentrations of 1-kestose, respectively, 10.69±5.59 and 10.54± 1.59 g L^{-1}. This strain was the most robust, once it was able to synthesize the trisaccharide 1-kestose in all the pH values and temperatures tested. Moreover, the yeast 222 was also the only one that, besides 1-kestose, also produced nystose under the reactional condition using pH value 4.5.

Table 2. Concentration of 1-kestose (g L^{-1}) obtained after six hours of reaction by the selected indigenous yeasts tested at the temperatures 40, 50 and 60ºC, and pH values of 4.5, 5.5 and 6.5.

Indigenous Yeast	1 – kestose (g L^{-1})/ mean ± standard deviation/ median/ (minimum-maximum)								
	Temperature 40ºC			Temperature 50ºC			Temperature 60ºC		
	pH 4.5	pH 5.5	pH 6.5	pH 4.5	pH 5.5	pH 6.5	pH 4.5	pH 5.5	pH 6.5
Saccharomyces cerevisiae 34	8.77 ±1.83$^{a, b}$ 9.69 (6.66-9.96)	2.43±1.03^{c} 2.78 (1.26-3.24)	0.00* -	9.54±0.31^{g} 9.59 (9.21-9.82)	3.05±2.62$^{c, h}$ 4.47 (0.02-4.66)	9.73±2.09$^{f, g}$ 9.08 (8.03-12.07)	0.23±0.04^{j} 0.21 (0.20-0.28)	0.00* -	0.00* -
Saccharomyces cerevisiae 67	0.00* -	6.29±1.11^{b} 6.31 (4.92 – 7.64)	4.06±2.30^{c} 3.14 (2.35-6.68)	20.39±2.90 19.87 (17.75-23.56)	0.00$^{\star c}$ -	6.87±3.83^{f} 8.47 (2.5 – 9.65)	0.71±0.47^{j} 0.92 (0.17 – 1.04)	12.17±4.59^{i} 12.37 (7.48-16.66)	0.71±0.47^{j} 0.92 (0.18 – 1.05)
Saccharomyces cerevisiae 80	16.53±3.34^{a} 15.26 (14.00-20.32)	6.29±1.37^{b} 6.31 (4.92 – 7.64)	4.58±1.82^{c} 4.39 (2.86-6.5)	10.45±1.15^{g} 10.04 (9.56 – 11.75)	0.00$^{\star c}$ -	7.86±1.14^{f} 7.77 (6.78-9.05)	8.76±2.13^{i} 9.94 (6.3 – 10.04)	0.00* -	10.25±3.26^{i} 9.05 (7.77-13.94)
Saccharomyces cerevisiae 97	19.00±1.91^{a} 19.43 (16.92-20.66)	5.34±1.53^{b} 5.66 (3.68 – 6.69)	23.25±1.88 22.75 (21.68 – 25.53)	9.76±3.17^{g} 10.53 (6.27-12.48)	0.00$^{\star c}$ -	8.09±2.88$^{f, g}$ 8.55 (5.07 – 10.66)	0.00* -	0.00* -	4.54±2.88^{i} 3.07 (2.7 – 7.86)
Saccharomyces cerevisiae 205	6.34±1.11$^{b, d}$ 6.30 (5.25 – 7.47)	3.75±0.38^{c} 3.05 (0.21-4.02)	0.42±0.03^{c} 0.4 (0.4- 1.8)	0.00* -	0.00$^{\star c}$ -	0.00$^{\star f}$ -	0.00* -	0.00* -	0.00* -
Saccharomyces cerevisiae 222	3.19±1.18$^{c, d}$ 2.62 (2.4 – 4.55)	2.32±0.83^{c} 1.87 (1.82 – 3.28)	5.82±2.56^{d} 4.98 (3.78 – 8.70)	3.89±2.56$^{g, h}$ 4.15 (1.22 – 6.31)	2.45±1.47$^{c, h, i}$ 2.60 (0.92 – 3.84)	10.69±5.59$^{f, g}$ 9.75 (5.63 – 16.69)	6.52±2.33^{i} 7.83 (3.83 – 7.91)	9.38±1.76^{i} 10.12 (7.38 – 10.65)	10.54±1.59^{i} 9.80 (9.46 – 12.38)
Saccharomyces cerevisiae 225	9.94±1.93^{a} 9.52 (8.26 – 12.05)	14.78±1.02^{a} 14.70 (13.8 – 15.84)	12.69±2.83^{a} 14.19 (9.43 – 14.46)	10.86±1.73^{g} 10.39 (9.44 – 12.80)	0.00$^{\star c}$ -	8.22±1.95^{f} 7.55 (6.68 – 10.42)	23.47±1.88 24.09 (21.35 – 24.97)	4.70±2.03^{i} 5.44 (2.12 – 6.55)	0.08±0.04 0.06 (0.05 – 0.13)
Saccharomyces cerevisiae 283	0.00★ -	13.25±4.05^{a} 13.72 (8.99 – 17.05)	11.36±3.21^{a} 10.56 (8.63-14.90)	6.53±1.99^{g}	0.00$^{\star c}$	0.54±0.27$^{f, i}$	0.00* -	0.00* -	1.02±0.80^{j} 0.95 (0.25 – 1.85)

Note: *no detection under the established conditions; superscript numbers with the same letters are considered statistically similar, $p < 0.05$ for columns, or equivalent mean and standard deviation for rows.

In this pH value, at temperature of 40, 50 and 60ºC the concentrations of nystose produced were, respectively, 1.31±0.31, 14.89±0.39 and 4.19±1.97 g L^{-1}. The best condition for the production of fructooligosaccharides (1-kestose and nystose) for the yeast 222 was 50ºC and pH 4.5, resulting in a total concentration of 18.79±1.23 g L^{-1}. Regarding the concentration of monosaccharides formed during the reactions conducted at pH 4.5, it was observed a concentration of, approximately, 43.4% of the initial sucrose concentration, and concerning to pH values 5.5 and 6.5, the concentrations were 18.8 and 26.5%. These results suggest a higher hydrolysis rate at lower pH values. Authors have described the thermostability of fructosyl transferase enzyme from *Rhodotorula* sp. LEB-V10 yeast and verified a loss of activity up to 61ºC. They suggest that the enzyme instability at higher temperatures led to the loss of transfructosylation capacity (Aguiar-Oliveira & Maugeri, 2011).

Other authors have described an optimum temperature, between 50 and 65ºC, for *Aspergillus aculeatus* enzymes activity (Ghazi et al., 2007). Up to 65ºC the enzymes have lost their activities. The temperatures between 55 and 60ºC have also been chosen as the best temperature for the activity of fructosyltransferase and β-fructofuranosidase enzymes (Yun, 1996). In this study, temperatures of 50 and 60ºC, employing a pH value of 6.5, were considered the optimal parameters for 1-kestose production by the wild yeast 222.

The fructooligosaccharide concentration is also influenced by pH, due to changes in hydrolytic and transfructosylation activities, and in reaction speed (Fernandez et al., 2007). Authors have demonstrated that, for *Aspergillus oryzae,* the pH 5.0 stimulates the hydrolytic activity and the pH 8.0 increases the transfructosylation activity. It was suggested that, at pH 8.0, fructooligosaccharides molecules have a higher resistance to hydrolysis due to the hydroxyl radicals present in the medium. This enzyme, at alkaline pH, suffers conformational modifications, which benefits transfructosylation activity (Cruz, Cruz, Belini, Belote, & Vieira, 1998, Ning et al., 2010, Alvaro-Benito et al., 2007).

The *Saccharomyces cerevisiae* strains 97, 222, and 225, tested in 50% (w v^{-1}) sucrose solution, synthetized a maximum of 4.8% (w v^{-1}) fructooligosaccharides. This result is similar to 5% yield obtained from purified β-fructofuranosidase from baker yeast (*Saccharomyces cerevisiae*), in a 0.2 mol L^{-1} sucrose solution, pH 2.5, 50ºC (Farine et al., 2001). When using 6 U mL^{-1} purified β-fructofuranosidase from *Saccharomyces cerevisiae,* a yield of 10% was reached using 525 g L^{-1} sucrose solution, at 55ºC and pH value 5.5 (Khandekar, Palai, Agarwal, & Bhattacharya, 2014).

Conclusion

The screening of 141 indigenous yeast using whole cells instead of isolated enzymes was a demand for obtaining a low cost process. The eight indigenous strains of *Saccharomyces cerevisiae* selected for a detailed study on fructooligosaccharides production had showed that the optimal production conditions, regarding pH and temperature, are directly dependent on the strain used. Further studies are necessary to determine the influence of other reaction parameters, such as stirring, oxygen concentration and medium composition (ions concentration) to determine the best productivity for these yeasts.

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