**Running Head: Screening and optimization of parameters for FOS**

**Whole free-cells yeasts screening and optimization of pH and temperature parameters for fructooligosaccharides production**
**Seleção de células inteiras de levedura e otimização dos parâmetros pH e temperatura para a produção de fruto-oligossacarídeos**

***Abstract***

Fructooligosaccharides are catalyzed by β–fructofuranosidase enzymes, produced by many microorganisms. However, in order to have a rentable, costless and timeless process, researchers have been looking for alternatives. This work aims to select and identify yeasts able to produce fructooligosaccharides and evaluate the influence of pH and temperature at their synthesis. Yeasts suspensions, solutions of 500 g.L-1 sucrose and three pH values (4.5, 5.5, and 6.5) and temperatures (40 ºC, 50 ºC, and 60 ºC) were employed. Yeasts species were identified by molecular techniques. From 141 grape’s isolated yeasts, 65 were able to synthesize fructooligosaccharides. The maximum concentration of fructooligosaccharides was 4.8%(w/v), and *Saccharomyces cerevisiae* 222 produced 1-kestose and nystose.

***Keywords:*** Prebiotics, Biotransformation, Identification, PCR

***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 por alternativas. Este trabalho tem objetivo de selecionar e identificar leveduras capazes de produzir fruto-oligossacarídeos e avaliar a influência do pH e 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) temperaturas de (40 ºC, 50 ºC, e 60 ºC) 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), 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 consumers’ 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 (Nobre et al., 2014; Roberfroid et al., 2008). The U.S. market for prebiotics was estimated to achieve $225.1 million by 2015, and the European, $1.17 billion. Nowadays, world demand for prebiotics is estimated to be around 1,67.000 tons and fructooligosaccharides contributed to 10% of natural sweeteners (Singh et al., 2016).

Fructooligosaccharides are constituted by one molecule of sucrose and one or more fructose unities linked to it through 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 and Macfarlane, 2007; MacDonald et al., 2011; Quigley, 2011).

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

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

***Material and Methods***

 *Microorganisms*

The 141 yeast strains investigated were isolated from *Vitis labrusca* grapes from vineyards localized in Parana State, Brazil. The strains were stored at -20 °C until the moment of 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 media was sterilized at 121 ºC, 1 atm, using adequate time. After inoculation, the flasks were kept in orbital shaker for 24 hours at 150-rpm and 28 ºC.

To increase cell number, the yeasts cells were transferred to a 1000 mL Erlenmeyer flask containing 200 mL YPD medium. The flasks were kept in orbital shaker at the same conditions. Hence, the yeasts cells were separated from the media by centrifugation at 7000 *xg* for 5 minutes. The cells were suspended with a certain amount of sterilized distilled water, quantified by dried weight and maintained at 4 ºC 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 to 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 for a total of 12 hours at 50 ºC and 150 rpm agitation.

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

*Evaluation of pH and temperature parameters 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: 40 ºC, 50 ºC and 60 ºC.

*Analytical Methods*

*Thin Layer Chromatography*

Each sample of the screening experiment was tested by thin layer chromatography. Glass plaques measuring 20 x 20 cm with a Silica Gel 60 layer of 300 μm were used. A mobile phase of buthanol: 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-NH2 column (5 µm; 250 x 4.6 mm through an isocratic gradient employing 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 shown 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 Yeasts Identification*

*MALDI-TOF Mass Spectrometry*

For MALDI-TOF MS analysis, yeasts were grown in 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 et al., 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 of >1.7 indicated identification beyond to the genus level, and score values of >2.0 indicated identification to the species level. Scores of <1.7 was interpreted as no identification.

*PCR-RFLP technique*

DNA extractions were carried out using the freeze-thawing process described by other authors (Silva et al., 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 fragments size was estimated by comparisons with a 100-bp DNA ladder (Invitrogen, Brazil).

*Statistic Analysis*

Data were submitted to analysis of variance (ANOVA) and to *t* Student 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 producers was made trough 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 at the screening experiment. Among that, 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 in six hours. The samples from the yeasts that were positively identified as a possible prebiotic producer were analyzed by HPLC/RID. The trisaccharide identified as 1-kestose, and the tetrasaccharide as nystose.

*Evaluation of pH and temperature parameters at fructooligosaccharides production*

*Indigenous yeasts identification*

To evaluate the influence of pH and temperature on fructooligosaccharide synthesis, eight indigenous yeasts were randomly chosen and were numbered designated as 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(Fernández-Espinar et al., 2000; Guillamón et al., 1998; Pham et al., 2011).

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 employing ITS1 and ITS4 primers. (*b*) Restriction profile employing the endonuclease *Hae*III.



*Effect of pH and temperature at fructooligosaccharide production*

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

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 oC, 50 oC and 60 oC, and pH values of 4.5, 5.5 and 6.5.

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

Note: \* - there was no detection at the established conditions; numbers superscript with the same letters are considered statistically equals, p<0.05 for columns, or equivalent average and standard deviation for lines.

At the temperature of 40 ºC, as table 2 indicates, there was no detection of 1-kestose production by the 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 increases (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 at pH value 5.5 and 40ºC of temperature, achieving similar concentrations (14.78±1.02 g.L-1 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).

The indigenous yeast 67 produced its 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% e 71.35% obtained at the same temperature and pH values 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 higher monosaccharides concentration.

At the temperature 50 ºC, no statistical difference was observed 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 60 ºC, 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 40 ºC, had a similar behaviour.

At the temperatures 50 ºC and 60 ºC, pH value 6.5, yeast 222 produced its higher concentrations of 1-kestose, respectively, 10.69±5.59 and10.54±1.59 g.L-1. This strain was the most robust one, once 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 in the reactional condition using pH value 4.5. In this pH value, at temperature of 40 ºC, 50 ºC and 60 ºC 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 50 ºC 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 conduced 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 termostability of fructosyltransferase enzyme of *Rhodotorula sp.* LEB-V10 yeast and verified a loss of activity up to 61 ºC. They suggest the enzymes’ instability at higher temperatures led to the loss of transfructosylation capacity (Aguiar-Oliveira and Maugeri, 2011).

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

The fructooligosaccharide concentration are also influenced by pH values, due to alterations on hydrolytic and transfructosylation activities, and on 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 at the medium. This enzyme*,* at alkaline pH, suffers conformational modifications which benefits transfructosylation activity (Alvaro-Benito et al., 2007; Cruz et al., 1998; Ning et al., 2010).

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

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 value and temperature, are directly dependent on the strain employed. Further studies are necessary to determine the influence of other reaction parameters, such as agitation, oxygen concentration and medium composition (ions concentration) to determine the best productivity for these yeasts.

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