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Production and partial purification of galacto-oligosaccharides by sequential fermentation

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ABSTRACT

Galacto-oligosaccharides are compounds that are synthesized from lactose containing two to five galactose units and a terminal glucose residue. They are formed from lactose by the transgalactosylation activity of the enzyme β-galactosidase. During the galacto-oligosaccharides synthesis are formed significant amounts of glucose and galactose, products that are considered reaction inhibitors. This work aims to produce and partly purify galacto-oligosaccharides using sequential fermentation. Galacto-oligosaccharides were produced using *Aspergillus oryzae* ATCC 11488 by solid-state fermentation (first part); *Saccharomyces cerevisiae* was used for the selective removal of the monosaccharides from the GOS mixture, (second part). The substrate for the solid-state fermentation was moistened wheat bran with a solution of 2% (w/v) lactose whey permeate. For the production of the galacto-oligosaccharides, a solution of 25% (w/v) lactose whey permeate was used. Enzymatic activity of β-galactosidase was determined using *o*-nitrophenyl-galactopyranoside as substrate. β-galactosidase maximum activity was 0.43 U mL^{-1}, obtained in 96 hours of solid-state fermentation. Galacto-oligosaccharide yield was 13.7%, area percentage of glucose and galactose was 13.76%, and 7.93% respectively, after 24 hours of fermentation at 40 °C and pH 5.9. After sequential fermentation, 85% of glucose was removed in 48 hours of fermentation and a 63% increase in the area percentage of galacto-oligosaccharides after 60 hours of fermentation. It was shown that sequential fermentation can be used in the partial purification of mixtures of carbohydrates, which facilitates the subsequent separation stages.

Keywords: Solid-state fermentation; *Aspergillus oryzae*; β-galactosidase; sequential fermentation; *Saccharomyces cerevisiae*.

INTRODUCTION

Galacto-oligosaccharides (GOS) are indigestible oligosaccharides derived from lactose; they contain 2 to 5 units of galactose joined by β-1.4 and β-1.6 links and a residue of terminal glucose (SAKO *et al*., 1999; VERA *et al*., 2011). GOS are produced from lactose by the transgalactosylation activity of the β-galactosidase enzyme, this enzyme catalyses both the hydrolysis and synthesis reactions. In the hydrolysis reaction, the enzyme transfers the galactose unit to an acceptor containing a hydroxyl group, where water acts as an acceptor and then glucose and galactose are formed. The lactose present in the solution can also serve as an acceptor and then GOS are formed by the transgalactosylation reaction (LISBOA *et al*., 2012; MARTINS; BURKERT, 2009; OTIENO, 2010; TORRES *et al*., 2010). Due to the benefits provided by their consumption, GOS have become the focus of much attention as functional foods (PARK; OH, 2010; SANGWAN *et al*., 2011). GOS are one of the prebiotics that beneficially affect the host by selectively stimulating the growth and activity of a limited number of gastrointestinal microorganisms that confer health benefits (LU *et al*., 2010). Many GOS are not digested or absorbed by the intestine because the intestine does not have the enzymes needed to break the type of β bonds formed by monosaccharide units (MACFARLANE *et al*., 2007; MARTINS; BURKERT, 2009).

During the synthesis reactions, apart from GOS, a mixture of monosaccharides is formed. These monosaccharides do not have prebiotic properties (GIBSON; ROBERFROID, 1995) and they are considered as inhibitors of the transgalactosylation reaction, decreasing the formation of the product (FREITAS *et al*., 2011; GÄNZLE 2012; KLEIN *et al.*, 2013; NÉRI *et al*., 2009). The GOS yield can be increased by using substrates with a higher concentrations of lactose, reducing the water content, and using methods for the removal of glucose and galactose from the system (CHOCKCHAISAWASDEE *et al*., 2005; GOULAS *et al*., 2007, NÉRI *et al*., 2009; VERA *et al*., 2011). The removal of these monosaccharides facilitates the subsequent separation stages. The use of yeasts in the purification of mixtures of GOS has been shown to be effective compared with other methods of purification. It has the advantage of being performed directly on the synthesis of mixtures, and when added to the fermentation process these yeast cells prefer to consume monosaccharides such as glucose and galactose (LI *et al*., 2008; YOON *et al*., 2003; ZHOU *et al*., 2011).

This study aims to produce and partially purify a mixture of GOS through a sequential fermentation process using *Aspergillus oryzae* in the first stage of fermentation and *Saccharomyces cerevisiae* in the second stage for the selective removal of components resulting from the synthesis of GOS, which are considered to have an inhibiting effect on the reaction.

MATERIALS AND METHODS

In this study, two microorganisms were used. For the solid-state fermentation and production of GOS a strain of *Aspergillus oryzae* ATCC 11488 was used. For the selective removal of monosaccharides from the GOS mixture, a commercial strain of *Saccharomyces cerevisiae* r.f. *cerevisiae* was used, which was manufactured by the AEB Group.

The substrate for solid-state fermentation was wheat bran donated by Bunge Alimentos, Ponta Grossa, Brazil. For the production of the enzyme and GOS, was used whey permeate powder with lactose concentration of 88%, which was donated by Sooro (Concentrado Indústria de Produtos Lácteos), Marechal Cândido Rondon, Brazil. Enzymatic activity of β-galactosidase was determined using *o*-nitrophenyl-galactopyranoside as substrate, which was obtained from Sigma-Aldrich (St. Louis, MO, USA). Glucose, galactose, lactose, fructo-oligosaccharides (FOS) and melezitose were all of chromatographic grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA). The remaining reagents were of analytical grade. Figure 1 shows the process of sequential fermentation in a simplified form.

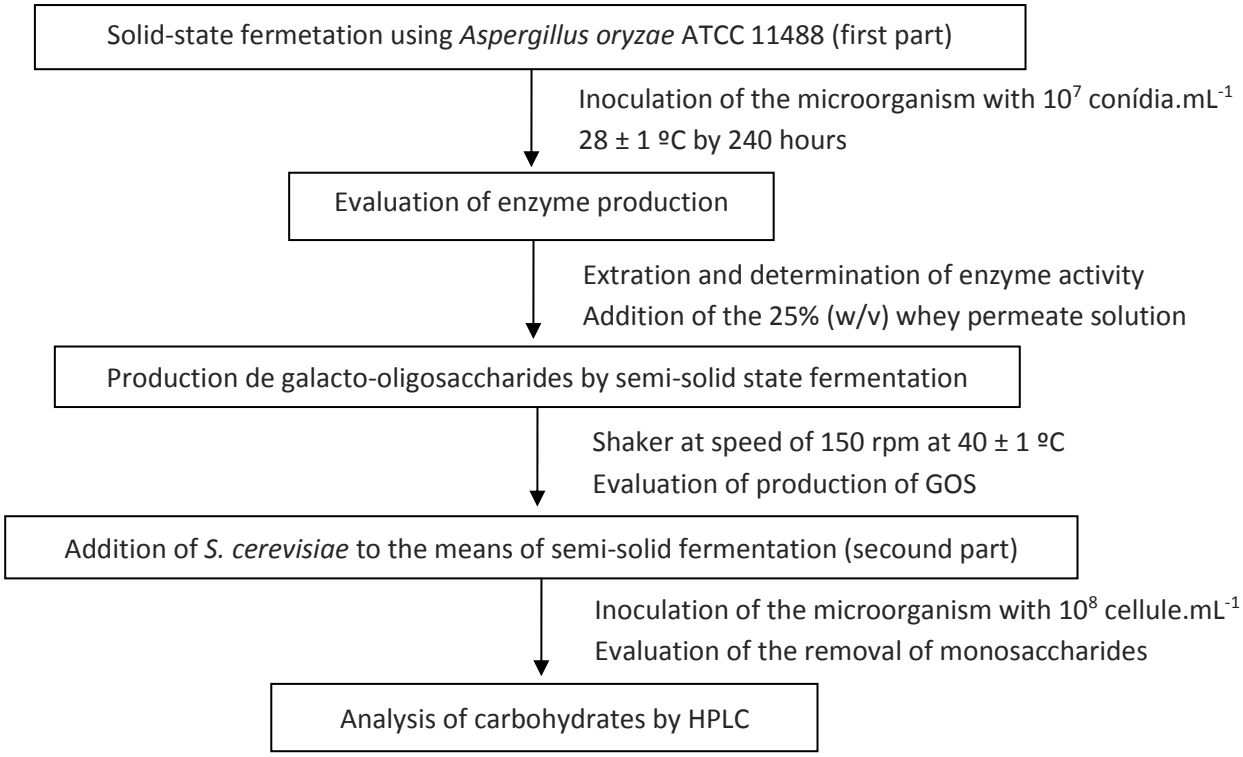

Figure 1. General diagram of the sequential fermentation process.

FERMENTATION USING *ASPERGILLUS ORYZAE* (FIRST PART)

A. oryzae ATCC 11488 was used in the solid-state fermentation, which was stored in test tubes at 8 ± 1 °C in modified *Sabouraud* agar containing the following: 10 g L^{-1} of bacteriological peptone (HiMedia, Mumbai, India), 40 g L^{-1} of lactose (Sigma-Aldrich, St. Louis, USA) and 20 g L^{-1} agar (HiMedia, Mumbai, India), acidified with 10% tartaric acid (w/v) sterile until pH 4.0. Microorganism was activated in modified *Sabouraud* agar and incubated for 7 days at 28 ± 1 ºC. The inoculum was prepared by adding spore suspension to 10 mL of sterile distilled water in a tube and this was standardised by counting in a Neubauer chamber. Solid-state fermentation (SSF) was in 250 mL capacity Erlenmeyer flasks. Ten grams of wheat bran was used as substrate; with the addition of the solution of 2% (w/v) whey permeate powder at a ratio of 1:1 (w/v), with final moisture content of about 54%.

The medium was sterilised at 121 °C for 15 minutes and then 1 mL of inoculum of *A. oryzae* was added, with a concentration of 10^7conidia mL^{-1}, and maintained at a temperature of 28 ± 1 °C for 240 hours. During this period, the assessment of the production of β-galactosidase was performed in order to determine the time at which the selected fungus produced the best yield in the production of the enzyme.

OBTAINING THE CRUDE EXTRACTS AND DETERMINATION OF ENZYME ACTIVITY OF β-GALACTOSIDASE

Crude extract was obtained by adding 50 mL of 25 % (w/v) whey permeate to the fermentation medium in a solid state. The medium was homogenised for enzyme release and remained in an ice bath for one hour, with mechanical stirring every 5 minutes, and it was then filtered on Whatman No.1 paper. In the crude extract, the enzymatic activity of β-galactosidase was measured using *o*-nitrophenyl-galactopyranoside as substrate (WALLENFELS; MALHOTRA, 1961). Reaction medium for the determination of enzymatic activity was prepared with 1.55 mL of sodium acetate buffer at pH 5.0, 0.1 $mol.L^{-1}$; 0.15 mL of enzyme solution; and 0.15 mL of 0.25% (w/v) *o*-nitrophenyl-galactopyranoside, incubated at 60 °C for 15 minutes, and the reaction was stopped with 0.15 mL of 10% (w/v) sodium carbonate. Enzymatic activity was measured in a spectrophotometer at 420 nm (PARK *et al*., 1979) and calculated using a standard curve of *o*-nitrofenol. One unit of β-galactosidase activity was defined as the amount of enzyme required to liberate 1 µmol of *o*-nitrofenol per minute of reaction.

PRODUCTION OF GALACTO-OLIGOSACCHARIDES BY SEMI-SOLID STATE FERMENTATION

After fermentation in solid-state, 50 mL of a solution of whey permeate at 25% (w/v) prepared indistilled water previously sterilized at 121 °C for 15 minutes was added to the medium. Medium was homogenised with a glass rod for enzyme release and remained in the ice bath for one hour, with mechanical stirring every 5 minutes. Then the samples were placed in a cooled incubator chamber with orbital agitation (shaker), Marconi brand, with a speed of 150 rpm at 40 ± 1 ºC to define the time of greatest production of GOS. The zero time was considered the time at which the sample was placed in the temperature of production GOS. Samples were taken at zero time and after 2, 6, 8, 12, 24, 32, 36, 48, 52 and 56 hours of reaction. To halt the enzymatic reaction, the samples were placed in a boiling bath for 10 minutes, filtered through Whatman No. 1 paper and frozen for later analysis of lactose, glucose, galactose and GOS by high performance liquid chromatography (HPLC). Results were expressed as area percentage for plotting in conjuction with the production of GOS.

FERMENTATION USING *SACCHAROMYCES CEREVISIAE* (SECOND PART)

Yeast activation was performed with 0.25 g of lyophilised yeast in synthetic medium, containing 80 g L^{-1} glucose obtained from Synth (Diadema, Brazil); 5 g L^{-1} of KH_2PO_4 from Dinâmica (Diadema, Brazil); 15 g L^{-1} of NH_4Cl from CAQ (Diadema, Brazil); 1 g L^{-1} of $MgSO_4.7H_2O$ from Reagen (Rio de Janeiro, Brazil) and 20 g L^{-1} yeast extract from Acumedia (Michigan, USA). The inoculum remained under agitation for 24 hours at a temperature of 28 ± 1 °C. The cell patterning was performed by counting the cells using a Neubauer chamber. After the production of GOS by semi-solid-state fermentation, *S. cerevisiae* was added to the medium for the removal of monosaccharides. Then, 10^8 mL^{-1} yeast cells were inoculated in fermentation medium for the best time defined by the results obtained in the production of GOS. Fermentation was conducted at 35 ± 1 ºC under orbital shaking at 150 rpm. Samples were collected at zero time and after 24, 36, 48, 60, 72, 84, 96 hours, filtered through Whatman No. 1 paper, and frozen for subsequent analysis of lactose, glucose, galactose and GOS (by HPLC).

FERMENTATION WITHOUT THE ADDITION OF YEAST

This fermentation aimed to compare the carbohydrate profile after adding the yeast was performed simultaneously with the sequential fermentation, with the same conditions of temperature and agitation. Samples were collected at time zero and after 24, 36, 48, 60, 72, 84 and 96 hours, filtered through Whatman No. 1 paper, and frozen for subsequent analysis of lactose, glucose, galactose and GOS by HPLC.

ANALYSIS OF CARBOHYDRATES BY HPLC

Analysis of the carbohydrates obtained from the SSF and sequential fermentation was performed by HPLC using a Waters 2695 chromatograph with peristaltic pump, degasser and Waters 2414 refractive index detector, controlled by Empower Waters software. Column used was a Sugar Pack ion exchange (6.5 x 300 mm) Waters, model 085188; mobile phase was degassed ultra pure water for 15 minutes. Flow of the mobile phase was 0.6 mL min^{-1}, detector temperature was 50 °C and column temperature was 80 °C. The standards and samples were filtered through a 0.45 μm membrane of 25 mm diameter before being injected. Concentrations of lactose, galactose and glucose were determined using calibration curves. Production of GOS was determined by the percentage of the area obtained in the chromatograms due to lack of standards for these compounds. In order to prove the elution of the peaks of the oligosaccharides, fructooligosaccharide (FOS) and melezitose standards were used.

STATISTICAL ANALYSIS

Results were submitted to an analysis of variance and Fisher's test at 5% probability using the Statistica (Statsoft) version 5.0 statistical programme. The results were expressed as means ± standard deviation.

RESULTS AND DISCUSSION

EVALUATION OF THE PRODUCTION OF B-GALACTOSIDASE BY *ASPERGILLUS ORYZAE* (FIRST PART)

Medium was prepared in the same way as in Section **materials and methods** and every 24 hours the samples were removed and to determine enzyme activity. Production of β-galactosidase by *A. oryzae* is shows in Figure 2.

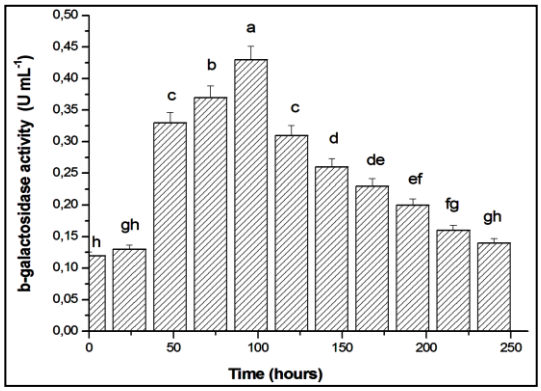

Figure 2. β-galactosidase activity during the 240 hours of solid-state fermentation with *A. oryzae* in wheat bran medium. The same lowercase letters represent no statistically significant difference according to Fisher's test (5% significance).

The zero time showed activity of 0.12 U mL^{-1} because of the extraction stage, where the enzyme remained in contact with the substrate for one hour in an ice bath. Maximum activity was obtained after 96 hours of fermentation with a value of 0.43 U mL^{-1}. Enzyme activity decreased by 30% compared to a time of 96 hours. Decrease in β-galactosidase activity may have been due to the synthesis of proteases, which use enzymes as a substrate (PESSOA; KILIKIAN, 2005), and the decrease in pH during the fermentation process (RODRIGUEZ-NOGALES; DELGADILLO, 2005).

A. oryzae fungus grow in a medium composed of wheat bran and 2% whey permeate solution milk at a ratio of 1:1 (w/v), which synthesised the β-galactosidase. Wheat bran is a by-product of the food industry, which has with low added value, and it is often used as a substrate for the production of different enzymes (BUDRIENE *et al*., 2004; KO, 2003; MENONCIN *et al*., 2009; RUEGGER; TAUK-TORNISIELO, 2004; SEYES; AKSOZ, 2004; TERRASAN, 2007). These results showed that this enzyme may be inductive, or synthesised as a response to the presence of lactose in the culture medium (MURRAY *et al*. 2002). According to literature data, most microorganisms show a better production of β-galactosidase when induced by lactose (NAKAO *et al*., 1994; ONISHI; TANAKA, 1997; SANTIAGO *et al*., 2004).

In a study by Schuber *et al*. (2012), the production of the β-galactosidase enzyme by *Penicillium* sp in solid-state fermentation was performed using wheat bran with lactose solution at 1% (w/v). The microorganism showed highest enzyme activity after 168 hours of fermentation. When the fungus grown in a medium of

wheat bran without lactose, there was no significant production of the enzyme, showing that the fungal strain synthesised the enzyme when lactose was present.

The time required to obtain the maximum enzyme activity depends on the type of microorganism and other factors such as moisture content and composition of the medium, supplementation of the growth medium, water activity, inoculum density, temperature, pH, agitation and aeration (NIGAM; PANDEY, 2009).

PRODUCTION OF GALACTO-OLIGOSACCHARIDES BY SEMI-SOLID STATE FERMENTATION

Figure 3 shows production of galacto-oligosaccharides with an initial lactose concentration of 25% (w/v) in relation to time.

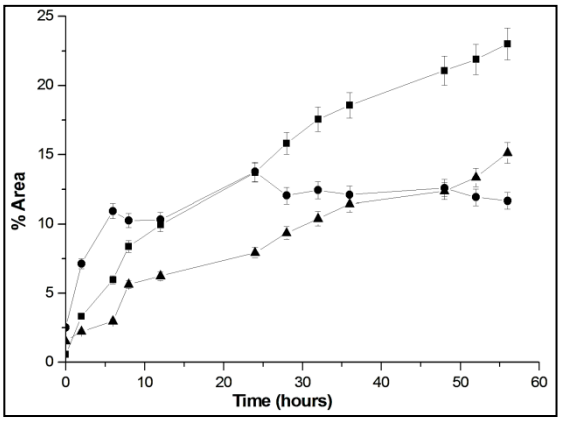

Figure 3. Production of galacto-oligosaccharides with an initial 25% (w/v) lactose concentration in relation to time. The symbols represent ■ Glucose, ▲ Galactose, ● GOS.

Conversion of the lactose at zero time was 4.64% in relation to the initial concentration of 25% (w/v); the GOS had an area percentage of 2.52%. Area percentage of glucose was 0.58% (0.70 g%) and for galactose it was 1.54% (0.54 g%).

Formation of GOS at zero time occurred during the extraction stage because of contact between the enzyme and the substrate when the mixture remained in the ice bath for one hour. Small percentage of the areas of glucose and galactose indicates that the hydrolysis reaction and transgalactosylation were occurring simultaneously. The conversion of the lactose after 12 hours fermentation was 26.5%, with an area percentage of glucose of 9.95% (4.28 g%), 6.24% (2.81 g%) galactose, and 10.32% of GOS. Synthesis of GOS was maximal after 24 hours of fermentation, with 36% conversion of lactose, and area percentage of 13.76%; an increase of about 500% compared to zero time. Glucose and galactose at 24 hours of fermentation showed area percentages of 13.71% (5.46 g%) and 7.93% (3.44 g%), respectively. The level of galactose was lower than that of glucose from the 24th hour because the molecules of galactose were mainly used in the synthesis

of GOS. These results show that transgalactosylation occurs predominately early in the reaction, resulting in a higher concentration of GOS, whereas the hydrolytic activity of β-galactosidase increases during the reaction time (ALBAYARK; YANG, 2002; BOON *et al*., 2000; GUVEN *et al*., 2007; GOULAS *et al*., 2007; HSU *et al*., 2007; MARTÍNEZ-VILLALUENGA *et al*., 2008).

Accumulation of glucose during GOS synthesis can lead to inhibition of the formation of the product; this occurs because glucose competes with lactose and trisaccharides as an alternative to the galactosyl group, favouring the formation of disaccharides instead of trisaccharides and tetrasaccharides (CHOCKCHAISAWASDEE *et al*., 2005; GAUR *et al*., 2006; GÄNZLE, 2012, KLEIN *et al*., 2013).

Area percentage of galactose increased after 48 hours of fermentation from 7.93% (3.44 g%) at 24 hours to 12.37% (5.36 g%), surpassing the area percentage of GOS (11.92%). This increase in the area percentage of galactose also contributed to a decrease in the synthesis of GOS. According to literature data, galactose can also be an inhibitor of β-galactosidase, competing with the active site of the enzyme and decreasing the synthesis of GOS. (ALBAYARK; YANG, 2002; FREITAS *et al*., 2011; GUIDINI *et al*., 2011; NÉRI *et al*., 2009; PORTACCIO *et al*., 1998).

In this work, GOS yield was 13.7% after 24 hours of fermentation, area percentage of glucose was 13.76% and of galactose 7.93%, from an initial lactose concentration of 25% (w/v), temperature 40 °C and pH 5.9 ± 0.11. After 56 hours of fermentation, the conversion of lactose was 49.8%, area percentage of GOS was 11.67%, glucose was 23.01% (8.47 g%) and galactose was 15.63% (5.90 g%). Reduction in the area percentage of the GOS may have been due to the formation of some disaccharides, such as allolactose (gal-β-1,6-glu) and galactobiose (gal-β-1,6-gal). In addition, the formation of molecules of galactobiose may have occurred due to the hydrolysis of trisaccharides. However, it was not possible to identify the peaks of these disaccharides because the amount of these compounds in the medium was very small and was neglected (ALBAYARK; YANG, 2002). All GOS, including transgalactosylated disaccharides, are considered to be non-digestible oligosaccharides and they have similar physiological characteristics (SAKO *et al*., 1999).

Néri *et al*. (2009) investigated the synthesis of GOS with β-galactosidase from *A. oryzae*. The initial lactose concentration was 50%, pH was 4.5 and temperature was 40 °C. They achieved a conversion of 55% of the lactose that was present in the medium. The concentration of GOS obtained was 26% (w/w) of total sugars. pH or temperature not affected a formation of GOS, but the concentrations of glucose and galactose significantly inhibited the reaction rate, reducing the yield.

Goulas *et al*. (2007) used *Bifidobacterium bifidum* NCIMB 41171 cells to synthesise oligosaccharides; they compared lactose and whey permeate as substrates. These authors found that the production of oligosaccharides initially increased, reached a maximum, and subsequently decreased. Significant quantities of glucose (109-140 mg mL^{-1}) and galactose (57-90 mg mL^{-1}) were formed from lactose hydrolysis. The yield of GOS using whey permeate was 36-38% and using pure lactose it was 39-43%.

Chockchaisawasdee *et al*. (2005) produced GOS with β-galactosidase from *Kluyveromyces lactis* (Maxilact® L2000). Synthesis was performed using different initial concentrations of 220 and 400 mg mL^{-1} lactose, and of enzyme between 3

and 9 U mL^{-1}, at a temperature of 40 °C and pH 7.0. The rate of consumption of lactose decreased when glucose and galactose were added to the mixture; the inhibition by galactose was higher.

The yield can be increased with the use of substrates with higher concentrations of lactose, reducing the water content of the system, and by using methods for the removal of glucose and galactose from the system (GOULAS *et al*., 2007, NÉRI *et al*., 2009; VERA *et al*., 2011).

FERMENTATION USING *SACCHAROMYCES CEREVISIAE* (SECOND PART)

Sequential fermentation can use more than one microorganism to achieve the desired effect in the final product. After the solid-state fermentation to produce the enzyme, GOS production was performed and in the 24th hour of the production of GOS the yeast was added. Figure 4 shows the profile of carbohydrates in the sequential fermentation.

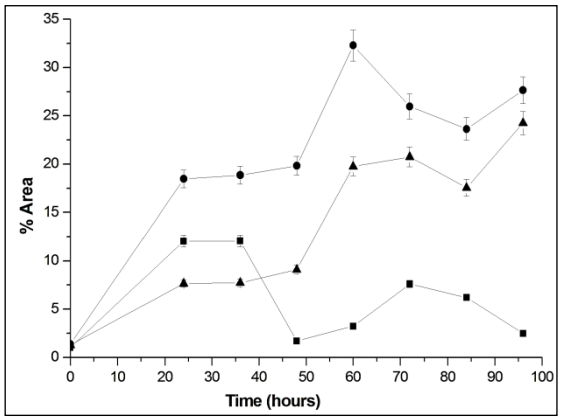

Figure 4. Profile of carbohydrates in sequential fermentation. The symbols represent ■ Glucose, ▲ Galactose, ● GOS

At 24 and 48 hours of fermentation the area percentage of glucose was 12.05% and 1.71%, respectively. Glucose showed a reduction of 14.2% in its area percentage, with a removal of 85% in 48 hours of fermentation. The removal of this monosaccharide resulted in a 63% increase in the area percentage of GOS, from 19.82% after 48 hours fermentation, to 32.29% after 60 hours. As well as increasing the production of GOS, the consumption of glucose in the medium by *S. cerevisiae* resulted in the formation of larger amounts of trisaccharides and tetrasaccharides.

Total area percentage of GOS was 18.47% and 32.29%, after 24 and 60 hours of fermentation, respectively. The trisaccharides showed an increase in area percentage of 30.8%, from 15.87% after 48 hours of fermentation, to 22.95% after 60 hours. Area percentage of tetrasaccharides at 48 hours was 3.95% and it increased to 9.34%, an increase of 57.7%. After 96 hours of fermentation, the area percentage of GOS was 27.65%, glucose was 2.47% and galactose was 24.67%.

Galactose presented an area percentage of 24.67% after 96 hours fermentation, increasing its concentration about 18 times, compared to zero time

(1.28%). A decrease in the concentration of galactose was expected because the glucose concentration after 48 hours of fermentation was 0.25%; when glucose is below 0.5% the activation of galactose uptake by *Saccharomyces cerevisiae* occurs. (YOUNG, 1996). For galactose to be metabolised by the yeast, it must first be transported into the cell. The membrane of yeast cells have hexose transporters (proteins) which function as channels that facilitate the diffusion by a concentration gradient, rather than pumps that use energy to transport molecules across the membrane. However, not all hexose transporters are expressed under the same conditions; some are expressed under conditions of high concentrations of glucose, while others are expressed at lower concentrations of sugar. For the mechanism of uptake of galactose to be activated, in addition to low levels of glucose, the induction of galactose transporter proteins (Gal2p), which transport the galactose in the medium into the cell, must also occur. Inside the cell, the conversion of galactose to glucose-6-phosphate occurs, which is catalysed by galactokinase, and which can be directed to fermentative and respiratory metabolism or for cell growth (OSTERGAARD *et al*., 2000; TOMSON, 2007; HOLDEN *et al*., 2003).

Since there was increased production of galactose, it can be inferred that the higher levels of ethanol production did not permit the assimilation (GOULAS *et al*., 2007). Ethanol alters the organization and the permeability of the plasma membrane by inhibiting the transport of nutrients. This change can be harmful to the cell because it causes the leakage of intracellular components and entry of toxic extracellular components, causing disruption to the transport systems of the cell, such as the inhibition of hexose transporters (ANSANAY-GALEOTE *et al*., 2001; LEÃO; VAN UDEN, 1982; QUINTAS *et al*., 2000).

The use of yeast in the removal of monosaccharides results in the purification of GOS. Compared with other purification methods, it has the advantage of being conducted directly in the synthesis mixture, without the need for dilution. For purification by nanofiltration it is necessary to perform dilution to obtain 100-150 mg mL^{-1} of total sugars (GOULAS *et al*. 2002). However, the removal of yeast and ethanol cells is required to obtain a mixture of GOS with a higher degree of purification (GOULAS *et al*., 2007).

Yeast cells are separated from ethanol by centrifugation, and the ethanol can be removed by distillation processes, such as vacuum distillation and membrane distillation (FURTADO; SCANDIFFIO, 2006; LIMA *et al*., 1975). Vacuum distillation allows the separation of ethanol at low temperatures, reducing the loss of thermo-sensitive compounds. In membrane distillation, such as reverse osmosis, the selective diffusion of compounds through the membrane is based on the molecular weight or pore size of the membrane. This process has been used in industries for the de-alcoholisation of wines and beers (MASSOT *et al*, 2008; PICKERING, 2000).

FERMENTATION WITHOUT THE ADDITION OF YEAST

Fermentation without the addition of yeast was performed simultaneously with sequential fermentation using the same inoculum (*A. oryzae*) and the same

temperature and agitation conditions. This fermentation showed a different behaviour to that of the sequential fermentation, as can be seen in Figure 5.

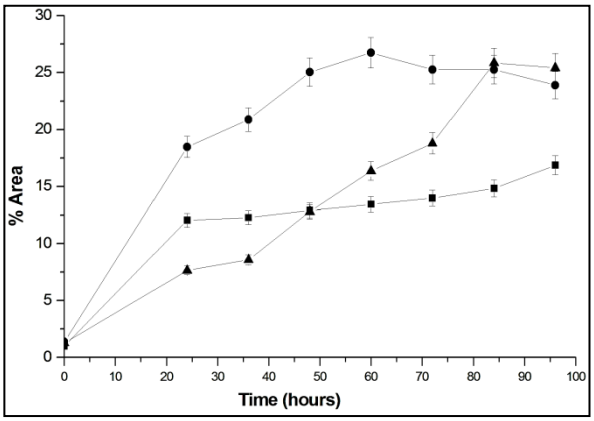

Figure 5. Profile of carbohydrates in fermentation without the addition of yeast
The symbols represent ■ Glucose, ▲ Galactose, ● GOS

Area percentage of glucose after 24 hours was 12.05% (2.23 g%), a value similar to that found for sequential fermentation. After 48 hours the figure was 12.91% (2.32 g%), while in the fermentation with the addition of yeast the figure was 1.71%. An increase of 7.1% in the area percentage of glucose in the SSF was observed and in the sequential fermentation, there was a reduction of 14.2%.

Galactose showed an area percentage after 24 hours of 7.66% (1.39 g%), a value similar to that found for sequential fermentation. After 48 hours fermentation the figure was 12.81%. After 84 hours of fermentation, the area percentage of galactose was 25.86% (1.48 g%); an increase of 100% in relation to the 48th hour. At this same time, the area percentage of GOS was 25.26%, leading to a decrease in its production, probably due to an inhibition mechanism. Galactose is considered to be a competitive inhibitor of the synthesis of GOS because it competes with the active sites of the enzyme (GUIDINI *et al*., 2011; HATZINIKOLAOU *et al*., 2005; HU *et al.*, 2010; JURADO *et al*., 2002; KLEIN *et al*., 2013; ÖZDURA *et al*., 2003; VERA *et al*., 2011). In the lactose molecule, it is galactose which binds to the active site of the enzyme. When galactose and lactose are present at the same time they both compete for the same binding sites on the surface of the enzyme. The galactose can then combine with the enzyme to form the enzyme-inhibitor complex rather than the enzyme-substrate complex (MURRAY *et al.,* 2002).

The carbohydrate profile after 96 hours of fermentation was 2.5 g% glucose and 4.10 g% galactose, with a 55% conversion of lactose and area percentage of GOS of 23.90%.

In terms of the SSF, comparing the graphs of the carbohydrate profile shown in Figures 4 and 5, after 60 hours there was a 7% increase in the area percentage of GOS, and in the sequential fermentation, there was a 63% increase in relation to the 48th hour. GOS results obtained using sequential fermentation and SSF were submitted to Fisher's test (5% significance), and there were significant differences between the samples. With the sequential fermentation it was observed that after

the removal of most of the glucose (85%) present in the medium there was an increase in the production of GOS

CONCLUSION

A. oryzae fungus grew in medium composed of wheat bran and 2% whey permeate solution; it synthesised the β-galactosidase enzyme and the maximum activity was 0.43 U mL^{-1}, which was obtained after 96 hours of fermentation. The highest yield of GOS was after 24 hours fermentation, with a yield of 13.7%. Using sequential fermentation for 48 hours, there was 85% removal of glucose present in the medium, and the highest production of GOS was obtained after 60 hours of fermentation (32.29%). Using solid-state fermentation, with *A. oryzae* and without the addition of yeast, the production of GOS was 26.74% in 60 hours. However, the sequential fermentation was more effective because the fungus synthesised the enzyme and the GOS, and the yeast removed most of the glucose, which was considered to inhibit the reaction, resulting in partial purification of the GOS and increasing their yield.

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