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Optimization of β-galactosidase production from *Penicillium* sp. for synthesis of galactooligosaccharides

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Abstract

Enzymes are of great importance and have been widely used for the synthesis of prebiotics. In this study, process conditions of hydrolysis of lactose (pH, temperature and substrate concentration) were optimized through a surface response methodology to maximize the production of β-galactosidase (β-gal). A quadratic mathematical model was obtained and the optimum conditions was predicted to be at pH 5.0 and 60°C which corresponded to 1.8 U mg^{-1} of lactase activity.The optimized broth was applied for synthesize galactooligosaccharides (GOS) in the presence of 25 or 35% (w/v) lactose source (whey).It was found that 25% whey gave higher lactose conversion.

Introduction

Among the fungal enzymes which have potential for industrial application, β-galactosidases (E.C. 3.2.1.23) have received attention because they are capable of recognizing and breaking the bond of the β-galactopyranosyl residual terminal of lactose (Gal-β-1→4-Glc) by hydrolysis, releasing glucose and galactose (Erich *et al*., 2015). Furthermore, this enzyme is able to perform reactions such as the hydrolysis of lactose and also the transgalactosylation of galactose, therefore can also be used to produce galactooligosaccharides (GOS) (Huber *et al*., 1976). This phenomenon occurs because the catalytic site of β-galactosidase can support lactose as well as galactose. In addition, in a high concentration of lactose, the β-galactosyl group is more likely to bind to lactose or an oligosaccharide than water, thereby enabling a greater production of oligosaccharides (Iwasaki *et al*., 1996). GOS are non-digestible oligosaccharides, which are considered to be prebiotic (Villaluenga *et al*., 2008; Gaggia *et al*., 2010). They contribute to the increase of the population of bifidobacteria in the colon, reducing the formation of toxic metabolites and consequently detoxifying the liver (Gullón *et al*., 2011). Besides, they have no anticancer effect, as well as increasing tolerance to lactose, the absorption of calcium, and reducing the risk of osteoporosis.

Thus, the production of GOS, catalyzed by β-galactosidase, may be carried out in excess of lactose by the hydrolysis of this disaccharide and the subsequent transgalactosylation of the galactose that is formed (Crittenden and Playne, 1996; Sako *et al*., 1999; Akiyama *et al*., 2001; Chen *et al*., 2002). In this case, the initial concentration of lactose is of great importance due to the increased availability of galactosyl saccharide (increase in synthesis rate) and the decrease in the availability of water (decreased degradation rate) (Zhou and Chen, 2001).

β-galactosidase is produced by a large number of yeasts, fungi and bacteria, and those that are most used for lactase production are *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Aspergillus oryzae*, *Aspergillus niger* and *Escherichia coli* (Gul-Guven *et al*., 2007; Guerrero *et al*., 2015). However, filamentous fungi (e.g. *Penicillium* sp.) are more thermostable, acid-stable and less demanding regarding the need for activators and stabilizers. They can be produced by both via solid-state fermentation (SSF) and via submerged fermentation (SF) with solid constituents suspended in media, which are released during fermentation (because they do not require induction for the enzymes to be released) (Kennedey, 1987; Archer and Wood,1995; Iwashita, 2002).

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Apart from the fact that the SSF process involves the growth of microorganisms in a moist substrate in the absence of free water (Mitchell *et al*., 2006; Singhania *et al*., 2009), this process can be performed by using different agro-industrial residues as substrates (Pandey *et al*., 2000; Rabelo *et al*., 2009) making it a potential alternative for industrial use aimed at producing chemicals with high added value (e.g. lactic acid, penicillin) (Barrios-Gonzalez *et al*.,1988 ; Kim *et al*., 2006) as well as enzymes (Nizamuddin *et al*., 2008 ; Paranthaman *et al*., 2009).

The *Penicillium* genus represents the largest number of lines of interest for enzyme production and it has long been used as a source of enzymes in various industries (Ruegger and Tauk-Tornisielo, 2004). This genus contains numerous species, and many of them are employed in the production of foodstuffs, particularly cheeses (Iwashita, 2002), because they have GRAS (generally recognized as safe) status. Due to this reason they have been used in the industrial preparation and production of traditional foods and drinks. Furthermore, the use of filamentous fungi from the Penicillium genus for the production of protein is desirable because fungi from this genus are defined as a nutritionally less demanding species; it is able to grow in more diverse environments and from a variety of carbon sources (Klich and Pitt, 1988).

The *Penicillium* canescens genus has been reported as producing β-galactosidase, which immobilized in chitosan showed higher stability in the processing of whey with 4.8% lactose, resulting in over 90% lactose conversion in a time of 4 hours of hydrolysis at 55°C (Budriene *et al*., 2005). The optimal enzymatic activity against lactose was obtained in the pH range of 4 to 4.5 at 60°C. In this context, this study aimed to optimize the productions of β-galactosidase by *Penicillium* sp. using wheat bran enriched with whey in SSF and thus apply the whole enzymatic broth to produce GOS.

Materials and Methods

Materials

The strain used was kindly donated by Prof. Dr. Mareci Mendes de Almeida, from the Food Technology Centre laboratory at Ponta Grossa State University. The de-mineralized whey was kindly donated by the Sooro (Concentrado Indústria de Produtos Lácteos) company, while the wheat bran was obtained from the Ponta Grossa branch of Bunge Alimentos. All the other reagents that were used were of analytical grade.

Methods

The production of GOS was initiated with the growth of fungi in agar medium, and thus they were subsequently transferred to a medium rich in whey and wheat bran and they remained under incubation for 7 days at 28ºC. After the growth stage of the fungi, the enzyme activity subsequently optimized by GOS synthesis was determined.

Transfer of Penicillium *sp. strain*

The transfer of the fungal strain was performed in Sabouraud agar medium, which was sterilized at 121°C for 20 min. After being cooled it was acidified with 10% tartaric acid (w/v) in sterile conditions until reaching a pH of 4.0. Subsequently, the media (~15 mL) were conditioned in inclined tubes for solidification.

Preparation of the culture fermentation

The substrate was composed of 10 g of wheat bran with the addition of 2% lactose solution (w/v) and 50% humidity. Erlenmeyer flasks with a 250 mL capacity were used as fermenters. The medium was sterilized at 121°C for 20 minutes; 1 mL of sample with 10^8 conídios. mL^{-1} was inoculated and the flasks were incubated for 7 days at 26°C.

Obtaining extract of β-galactosidase

After the growth of *Penicillium* sp., the methodology cited by Almeida (2003) was followed (with modifications), in this case 50 mL of sterile solution of concentrated whey (25 or 35%) were added to the culture medium. The solution was allowed to stand for 60 minutes in an ice bath with mechanical agitation every 5 min. After this time the solution was homogenized and filtered to obtain the crude enzymatic extract, from which the enzymatic activity was evaluated.

Determination of β-galactosidase activity

The determination of enzymatic activity in the crude extract was carried out using ortho-nitrophenyl-galactopyranoside (ONPG) as a substrate (Wallenfels and Malhotra, 1961). In this case, the reaction medium was composed of 1.55 mL of 0.1M sodium acetate buffer, 0.15 mL of enzyme solution and 0.15 mL of 0.25% ortho-nitrophenyl-galactopyranoside. This mixture was incubated at the working temperature for 15 minutes. The reaction was stopped by adding 0.15 mL of 10% sodium carbonate and the reaction product was chromophore o-nitrophenol (ONP). The enzymatic activity was measured in a spectrophotometer at 420 nm (Park *et al*., 1979). To calculate the enzymatic activity the analytical curve

of ONP was performed, and one international unit of β-galactosidase activity (IU) was defined as the amount of enzyme that liberates 1 µmol of ONP per minute in 1 mL under the test conditions.

Optimization of β-galactosidase activity

The determination of the optimum temperature and pH of the β-galactosidase activity was performed through a central rotational design formula (CCRD) 2^2 in Response Surface Methodolgy (RSM). The experimental conditions of this design are shown in Table 1.

Using the data from the central composite design, multiple linear regression was performed to obtain a mathematical model capable of describing the experimental behavior of the temperature and pH variables. The model was then subjected to validation by analysis of variance (ANOVA) using Statistica® software v. 12.0.

Synthesis of galactooligosaccharides

To evaluate the effect of the initial lactose concentration in the conversion into galactooligosaccharides, concentrated milk whey containing 72% lactose in the presence of 1.8U. mL^{-1} of ONP activity was used as reaction media, which was optimized as previously described. To check the effect of the concentration of lactose on the GOS synthesis reactions, the whey concentrate was solubilized in de-ionized water to achieve concentrations of 25% and 35% lactose and it was sterilized at 121°C for 20 minutes. After sterilization, the whey was filtered because of the precipitation of proteins, which occurs due to exposure to high temperature.

The assays were performed for 12 hours (25%) and 40 hours (35%) in a Dubnoff bath at 45°C, with pendulum agitation at 80 rpm. An aliquot was withdrawn, and 0 time was considered to be the moment that the sample was placed at the optimal fermentation temperature. The samples were centrifuged at 9930 g for 4 minutes. The lactose conversion and the production of oligosaccharides were evaluated for each of the lactose concentrations (25% and 35%) using high-performance liquid chromatography.

Analysis of carbohydrates

The carbohydrate analysis was performed by high-performance liquid chromatography using a Waters 2695 chromatograph with peristaltic pump, degasser and Waters 2414 refractive index detector, controlled by Waters Empower software. A Sugar Pack ion exchange column (6.5 x 300 mm) (Waters Model 085188) was used; the mobile phase was filtered and degassed ultrapure water (Milli-Q). The standards and samples were filtered through a Chromafil membrane (Xtra PA-45/25) 0.45µ pore with 25 mm diameter, before being injected.

Table 1. Factors and levels of the Central Composite Design (orthogonal and real values) as well as the eexperimental data obtained for the β-galactosidase production by *Peniccilium* sp

Run	Temperature (°C)	pH	Enzymatic Activity (IU.mL^{-1})
1	-1.00 (50)	-1.00 (4.0)	1.60
2	-1.00 (50)	1.00 (6.0)	1.30
3	1.00 (70)	-1.00 (4.0)	0.20
4	1.00 (70)	1.00 (6.0)	0.20
5	-1.41 (46)	0.00 (5.0)	1.50
6	1.41 (74)	0.00 (5.0)	0.20
7	0.00 (60)	-1.41 (4.6)	0.30
8	0.00 (60)	1.41 (6.4)	0.30
9 (C)	0.00 (60)	0.00 (5.0)	1.70
10 (C)	0.00 (60)	0.00 (5.0)	1.80
11 (C)	0.00 (60)	0.00 (5.0)	1.70

Table 2. Obtained values for the ANOVA between theoretical and experimental data

Source	SS	df	MS	F_{cal}	F_{tab}
Regression	5.0187	5	1.0037	25.3962	5.0503
Residue	0.1976	5	0.0395		
Lack of Fit	0.1910	3	0.0637	19.0951	19.1643
Pure Error	0.0067	2	0.0033		

Results and Discussion

Production of β-galactosidase

The data regarding to the optimization of production of enzymatic activity are shown in Table 1 (obtained according to the flowchart shown in Figure 2). Furthermore, such data were subjected to multiple linear regression in order to obtain a mathematical model that could describe the experimental data and to verify if the conditions were optimized (or not) through response surface analysis. The model that was found is shown in the following equation:

$$\text{Enzymatic activity} = 1.733 - 0.542T - 0.379T^2 - 0.038pH - 0.654pH^2 + 0.075TpH \quad (1)$$

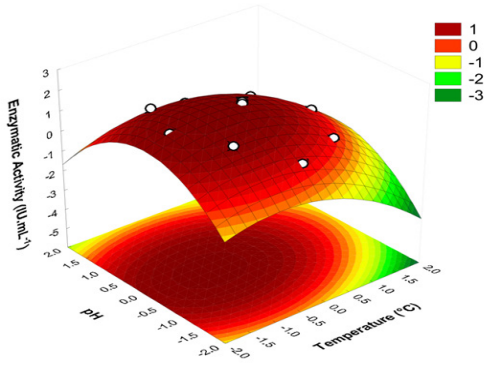

Figure 1. Response surfasse for the β-galactosidase production by *Penicillium* sp.

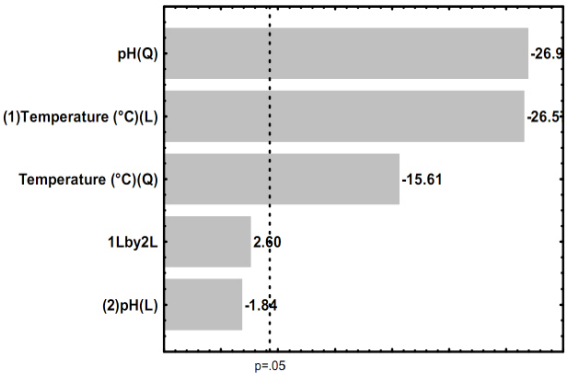

Figure 2. Pareto's chart for the effects found

Apart from the fact that temperature showed higher coefficient values than in the equation model, and therefore may have been more significant than pH in the production of enzymatic activity, it was difficult to measure which tendencies in these variables should be adopted so that even higher conversion values could be achieved. Consequently, in order to validate this model, analysis of variance (ANOVA) of the data was performed. The data obtained by ANOVA are shown in Table 2. The Lack of Fit Test table compare the residual error to the Pure Error from replicated design points.

Based on the data of the quadratic sums, the quadratic means were calculated, and using these means ANOVA was performed between the quadratic averages obtained from the regression and the residue of the modeling. In this case, ANOVA showed that the model was not within the error estimate for the necessary degree of freedom because the calculated F value (F_{Rcal}25.40) was 25.40, and was therefore greater than the tabulated value (F_{tab},0.05;5,5=5.05). Furthermore, the ANOVA of the residue, which was related to a lack of model fit with the pure error, demonstrated that the value of (F_{rcal}=19.10) was slightly lower than the tabulated value (F_{tab},0.05;3,2=19.16). This indicated that the

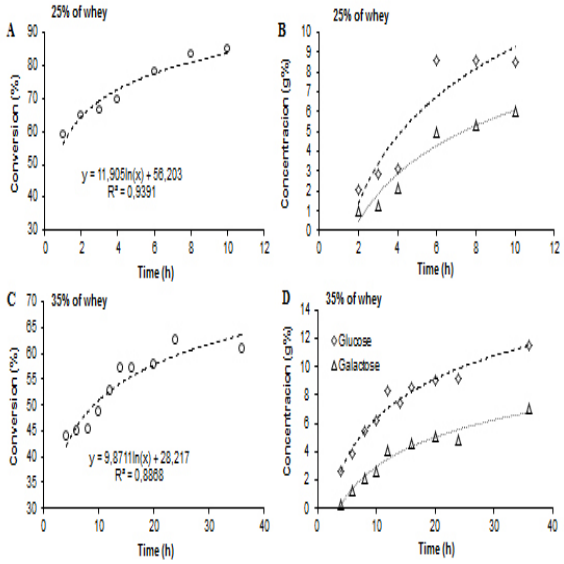

Figure 3. Kinetic behavior of galactose conversion with (A) and (C) as well as concentration of glucose and galactose (B and D)

residuals of the model were within the expected error for such a degree of freedom. Therefore, once the model had been validated by ANOVA the graphics were generated in order to develop the discussion about the optimization of process conditions for producing β-galactosidase. Figure 1 shows the response surface generated with the proposed model.

Based on the graphic behavior of the data, it could be seen that both pH and temperature showed parabolic behavior for the production of β-galactosidase. Furthermore, it could be seen that there was synergistic behavior, indicating that conditions of moderate pH and temperature resulted in increased production of β-galactosidase. However, the surface analysis chart did not show the statistical significance of the tendencies that were observed in the process conditions that were used. Consequently, the statistical effects are presented below in Figure 2, in the form of a Pareto chart.

Taking into consideration the effects shown in Figure 2, it is evident that there was synergy between pH and temperature and the effect of this interaction (temperature x pH) was no significant. In addition, the importance of the parabolic behaviour found in the response surface for temperature was confirmed by the presence of a statistically significant quadratic effect. However, the curvature present in the pH axis in the response surface was not significant, because according to the analysis of the Pareto chart the respective linear effect was not statistically significant.

With reference to Figures 1 and 2, it can be concluded that the more extreme pH and temperature

conditions were unfavorable for β-galactosidase activity, with optimum activity at 50°C and pH 5.0. In this context, the condition of the best result was considered to be optimized because the peripheries of the sample space showed low conversion values and they also coincided with the center point of the CCD. Nevertheless, the theoretical data were also compared to the experimental data through a graphic showing the predicted and observed values, in which it was observed that the mathematical model was suitable for predicting the data.

Production of GOS

Once the enzymatic activity was optimized, the pools that were obtained were packed in a Dubnoff bath at 45°C in pendular agitation at 80 rpm for the GOS synthesis. Whey was used in order to apply a natural source of disaccharide for the GOS synthesis. In this case, two concentrations of whey were used (25 and 35%). In both cases GOS were produced but they showed different behaviours (Figure 3).

From the behaviour shown in Figure 3, it is evident that when using a 25% concentration of whey the rate of consumption of galactose was greater compared to 35% concentration. However, even with a lower rate, the production of GOS with a high substrate load was preferred due to the higher levels of the product that was formed. Moreover, it was noted that using a 25% substrate load and a time period of only 10 hours the production of glucose and galactose reached a plateau because the lactose conversion reached 85%. On the other hand, employing a 35% substrate load the plateau was reached only after 24 hours of reaction and the conversion of 63% of lactose.

Conclusion

The use of whey in the production of enzymes and GOS synthesis is a promising alternative to be used as a renewable source from the dairy industry in the production of value-added products in a renewable process. Furthermore, *Penicillium* sp. was robust in the production of β-galactosidase and the lactose hydrolysis was optimized. The quadratic model can be used to explian the effect of pH and temperature on the β-galactosidase production process".

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