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Galacto-oligosaccharides Production Using Permeabilized Cells of *Kluyveromyces marxianus*

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Abstract

Galacto-oligosaccharides are non-digestible carbohydrates and are recognized as important prebiotics for than stimulation of the proliferation of lactic acid bacteria and bifidobacteria in the human intestine. GOS can be produced by a transgalactosylation reaction catalysed by β-galactosidase enzyme, and microorganisms can be used as a source of β-galactosidase. In this work, a process for producing GOS using permeabilized cells of *Kluyveromyces marxianus* CCT 7082 was proposed. The effects of the concentrations of lactose and enzyme, temperature and pH were studied using a fractional design followed by a central composite rotatable design. The optimum conditions for galacto-oligosaccharides production were found to be: lactose concentration 500 g/L, enzyme concentration 10 U/mL, 45°C and pH 7.0. Under optimized conditions, the GOS concentration, yield and productivity were 83 g/L, 16.5% and 27.6 g/L.h, respectively.

KEYWORDS: β–galactosidase, transgalactosylation, experimental design

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INTRODUCTION

Galacto-oligosaccharides (GOS) have become the focus of great attention as functional foods, owing to their known health benefits and potential to improve the quality of many foods. Because of these properties, they are currently used as low-calorie sweetners in fermented milk products, confectioneries breads, and beverages (Park and Oh, 2010). GOS can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the intestine, the predominance of this microorganisms in the colon has been suggested to cause beneficial effects for maintaining human health, such as reduction of the level of cholesterol in serum, decrease the population of pathogenic microorganisms, colon cancer prevention, enhancement of calcium adsorption (Rowland and Tanaka, 1993; Sako et al., 1999; Tomomatsu, 1994; Alander et al., 2001; Chonan et al., 2001; Sinclair et al., 2009).

The stability under acidic conditions of GOS during food processing makes them potentially applicable as ingredients for a wide variety of food products. Their excellent taste quality and relatively low sweetness make GOS interesting functional sweetners. They pass the small intestine without being digested and are therefore of low caloric value. In addition, GOS cannot be metabolized by microorganisms of the oral cavity and are thus not implicated in the formation of dental caries (Crittenden and Playne, 1996; Sako et al., 1999).

GOS are the products of transgalactosylation reaction catalyzed by the enzyme β-galactosidase, when using lactose or other structural related galactosides as the substrate. The hydrolysis of lactose to glucose and galactose, occurs predominantly at low lactose concentrations, while GOS production by the transgalactosylation reaction increases with increasing concentrations of lactose (Mahoney, 1998, Golsing et al., 2010).

β-galactosidase (EC 3.2.1.23) is one of the most promising enzymes, which has several applications in the food, fermentation and dairy industry (Kaur et al, 2008). Amongst various microorganisms, yeast has emerged as an important source of β-galactosidase, since the yeast enzyme has an optimum pH suitable for lactose hydrolysis in milk and sweet whey (Kondo et al., 2000). In this context, *Kluyveromyces marxianus* offers several great advantages, such as good growth yield, acceptability as a safe microorganism (GRAS) and higher β-galactosidase activity than other yeast (Kaur et al., 2008).

β-galactosidase produced by *K. marxianus* is an intracellular enzyme, therefore, the industrial applications of processes based on the enzymatic hydrolysis of lactose are limited (Panesar, 2008). The preparation of cell-free extracts is laborious and needs disruption of cells, which destroys the integrity of cells and may cause inactivation of enzymes. Therefore, cell permeabilization has been recommended as an alternative method for the study of intracellular

enzymatic reactions (Alamäe and Järviste, 1995). The permeabilizing agent may disrupt the membrane structures by decreasing the phospholipid content to allow the passive passage of low molecular weight solutes in and out of cells, including lactose and its products of hydrolysis (Siso et al., 1992; Panesar, 2008).

The enzymatic synthesis of GOS from lactose and cheese whey using various crude, purified and immobilized β-galactosidases has been reported (Cho et al., 2003; Gaur et al., 2006; Hsu et al., 2007; Park et al., 2008; Martínez-Villaluenga et al., 2008) however, the use of yeast permeabilized cells as a source of β-galactosidase on the synthesis of GOS is an interesting alternative, which has been little explored (Park and Oh, 2010).

Several parameters such as the source and concentration of the enzyme, substrate concentration, pH and temperature can influence the equilibrium of the enzyme reaction catalysis in the synthesis of GOS. In this context, the aim of the present research was to optimize the production of GOS from permeabilized cells of *Kluyveromyces marxianus* CCT 7082. The optimization was carried out by experimental design and surface analysis methodology.

MATERIALS AND METHODS

Enzyme production

A strain of *Kluyveromyces marxianus* CCT 7082, previously selected as the best β-galactosidase producer by Manera *et al*. (2008), was used in this present study. The strain was maintained at -18ºC on YM broth (yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L, glucose 10.0 g/L) with glycerol 10% (v/v).

The fermentation media was composed by 17.0 g of yeast extract, 8.8 g of $(NH_4)_2SO_4$, 5.0 g of KH_2PO_4, 0.4 g of $MgSO_4.7H_2O$ and 28.2 g of lactose in 1 L of 0.2 M potassium phosphate buffer, pH 6.0. The cultures were incubated in an orbital shaking incubator for 96 h at 180 rpm and 30ºC (Manera et al. 2008). Cultivation was started with a 10% inoculum, incubated at 30ºC, 180 rpm for 24 h.

Permeabilization of cells

Cell permeabilization was carried out using isopropanol in a ratio biomass/isopropanol (w/w) of 0.03, in 10 mL of cold 0.1 M potassium phosphate buffer pH 7.0 and held at 25°C for 5 min. Cells were separated from solvent at 6000 × *g* for 5 min and washed twice with cold 0.1 M potassium phosphate buffer, pH 7.0 and lyophilized.

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Optimization of the galacto-oligosaccharides synthesis using permeabilized yeast cells

The reaction mixture, containing the enzyme β-galactosidase in permeabilized yeast cells and lactose prepared in 0.1 M potassium phosphate buffer, was incubated in a temperature-controlled water bath for 12 h. At regular intervals, an aliquot of the reaction mixture was removed and heated in boiling water for 5 min to inactivate the enzyme. The samples were stored at -18°C for subsequent analysis of sugars.

Two experimental designs were carried out; the first one, a fractional factorial design including 2^{4-1} trials with three central points (17 trials) (Rodrigues and Iemma, 2009) was carried out in order to evaluate the effects of lactose concentration, enzyme concentration, temperature and pH (independent variables) on the synthesis of GOS. The responses taken into account were GOS concentration, yield and productivity (dependent variables).

The preliminary fractional factorial design allowed for the selection of the statistically significant variables with respect to GOS concentration, yield and productivity. With these variables, a central composite rotatable design (CCRD) with three replicates at the central point and four axial points (11 trials) (Rodrigues and Iemma, 2009) was performed to obtain a second-order model for the prediction of GOS concentration, yield and productivity as a function of the variables studied. Statistical analyses were performed using the software Statistica 5.0 (Statsoft, 2001). Table 1 shows the range of the studied variables and the correspondent coded levels for both experimental designs.

Table 1: Values of coded levels and real values in fractional factorial design and CCRD.

Factorial design	Coded variable level	Lactose (g/L)	Enzyme (U/mL)	Temperature (ºC)	pH
Fractional	-1	400	5.0	40	6.0
	0	450	7.5	45	6.5
	+1	500	10.0	50	7.0
CCRD	-1.41	209	5.8	-	-
	-1	250	7.0	-	-
	0	350	10.0	-	-
	+1	450	13.0	-	-
	+1.41	491	14.2	-	-

Chromatographic determination of carbohydrates

The identification and quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD). A DIONEX (USA) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and PEAKNET software were used for the analyses. Sugars were eluted with 20 mM sodium hydroxide, at a flow rate of 1.0 $mL.min^{-1}$. Before injection, the samples were diluted with water and filtered through 0.22 μm filters.

β-galactosidase assay

β-galactosidase activity was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) following the method described by Inchaurrondo *et al*.(1994). A 50 μL sample of permeabilized cell suspension was mixed with 2 mL of 1.25 mM ONPG in buffer (50 mM of KH_2PO_4 and 0.1 mM $MnCl_2.4H_2O$, pH 6.6) and incubated for 5 min at 37°C. The reaction was stopped by adding 0.5 mL of 1 M sodium carbonate. Liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of β-galactosidase activity is defined as the amount of the enzyme necessary for the hydrolysis of 1 μmol of ONPG per minute under the conditions of the assay.

Cell concentration

Cell concentration was estimated by measuring the absorbance at 620 nm and converted to dry weight by using a standard curve. Samples were centrifugated at 6000 × *g* for 5 min, washed twice with distilled water and ressuspended in water, and absorbance read at 600 nm. For the standard curve cells were dried at 90°C to constant weight (Longhi et al., 2004).

RESULTS AND DISCUSSION

Table 2 shows the results of maximum GOS concentration, productivity and yield from the first experimental design. As it can be seen, GOS production changed from 28.3 g/L up to 95.1 g/L, the productivity from 7.1 to 23.8 g/L.h and yield from 5.7 to 19.0%. The maximal concentrations of GOS were obtained between 4 and 6 h of synthesis.

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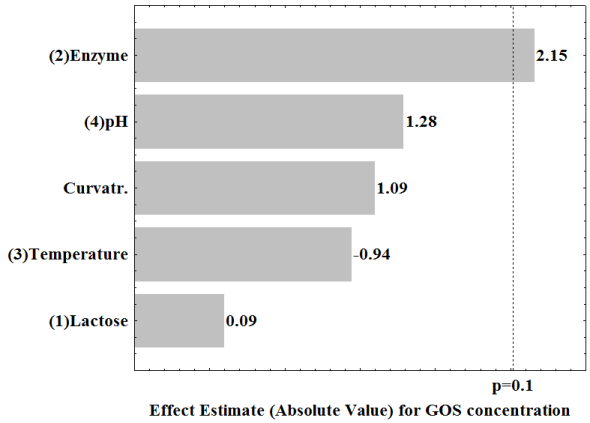
Table 2: The 2^{4-1} factorial design for GOS production.

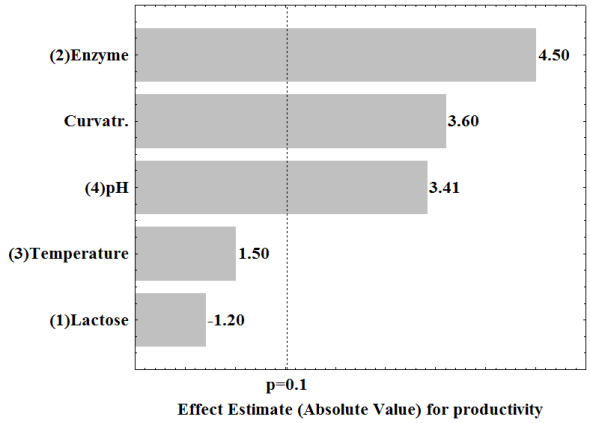
Essay	Lactose (g/L)	Enzyme (U/mL)	T (ºC)	pH	GOS (g/L)	t[a] (h)	P[b] (g/L.h)	R[c] (%)
1	-1 (400)	-1 (5)	-1 (40)	-1 (6.0)	66.8	6	11.1	16.7
2	1 (500)	-1 (5)	-1 (40)	1 (7.0)	75.3	6	12.6	15.1
3	-1 (400)	1 (10)	-1 (40)	1 (7.0)	75.2	4	18.8	18.8
4	1 (500)	1 (10)	-1 (40)	-1 (6.0)	92.1	6	15.4	18.4
5	-1 (400)	-1 (5)	1 (50)	1 (7.0)	71.4	4	17.9	17.9
6	1 (500)	-1 (5)	1 (50)	-1 (6.0)	28.3	4	7.1	5.7
7	-1 (400)	1 (10)	1 (50)	-1 (6.0)	73.6	4	18.4	18.4
8	1 (500)	1 (10)	1 (50)	1 (7.0)	95.1	4	23.8	19.0
9	0 (450)	0 (7.5)	0 (45)	0 (6.5)	81.6	4	20.4	18.1
10	0 (450)	0 (7.5)	0 (45)	0 (6.5)	83.7	4	20.9	18.6
11	0 (450)	0 (7.5)	0 (45)	0 (6.5)	85.7	4	21.4	19.0

a) t (h) = Time course for maximal GOS concentration
b) P (g/L.h) = GOS productivity = [g/L.h]
c) R (%) = GOS yield = [g GOS/g Initial lactose]*100

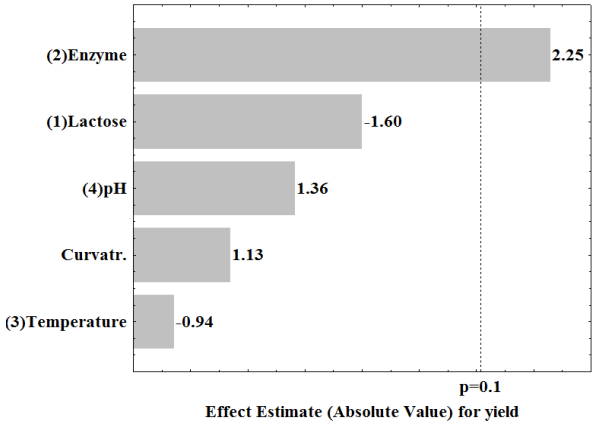
The variable effects were estimated according to the responses, for each variable, which changed from level -1 to +1. As shown in Figure 1, for the responses GOS concentration and yield, only the enzyme concentration was significant at 90% of confidence, in the range studied, and it was positive, what means that when the enzyme concentration changed from -1 to +1 levels, there was an increase in the GOS concentration. For the response productivity, both enzyme concentration and pH were significant at 90% of confidence, and both positives.



(a)



(b)



(c)

Figure 1: Pareto plot for the effect estimates: (a) GOS concentration; (b) productivity and (c) yield.

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According to the effect estimates concerning the first experimental design, a second one was planned, in which the temperature was fixed at 45°C (central point) and the pH at 7.0 (level +1). This pH value was chosen since it is the pH of maximum enzyme stability (data not shown). The new experimental design, a central composite rotatable design, was carried out, whose variables were enzyme and lactose concentrations. A higher range of enzyme activity was essayed, from 5.8 to 14.2 U/mL. For the lactose concentration, even if this variable was not significant in the factorial design, a lower concentration range was essayed (from 209 g/L to 491 g/L), in order to study the possibilities of using lower concentrations of this substrate, as an attempt for lowering GOS production costs.

The CCRD is shown in Table 3, together with the responses for each essay. It can be seen that the GOS concentration changed from 32.8 to 87.4 g/L, the productivity from 16.4 to 29.2 g/L.h and the yield from 15.7 to 19%. In all essays the maximum GOS concentration were lowers than those from the fractional design, but always achieved earlier, between 2 and 3 hours of time reaction, when compared to the same fractional design, which means, in other words, lower yields and higher productivities.

Equations (1) and (2) were obtained from data in Table 3, for GOS concentration and yield, respectively, with 95% of confidence. For the response productivity only the linear correlation of lactose was statistically significant, so that no statistical model was possible.

$$GOS\ (g/L) = 64.80 + 19.17Lactose - 2.74Lactose^2 - 3.02Enzyme^2 \quad (1)$$

$$R\ (\%) = 18.51 + 1.68Lactose - 1.89Lactose^2 - 1.62Enzyme^2 \quad (2)$$

Table 3: The CCRD for GOS production.

Essay	Lactose (g/L)	Enzyme (U/mL)	GOS (g/L)	t[a] (h)	P[b] (g/L.h)	R[c] (%)
1	-1 (250)	-1 (7)	39.1	2	19.6	15.7
2	1 (450)	-1 (7)	74.8	3	24.9	16.6
3	-1 (250)	1 (13)	39.4	2	19.7	15.8
4	1 (450)	1 (13)	79.6	3	26.6	15.9
5	-1.41 (209)	0 (10)	32.8	2	16.4	15.7
6	1.41 (491)	0 (10)	87.4	3	29.1	17.8
7	0 (350)	-1.41 (5.8)	60.6	3	20.2	17.3
8	0 (350)	1.41 (14.2)	58.4	2	29.2	16.7
9	0 (350)	0 (10)	62.9	3	20.9	18.0
10	0 (350)	0 (10)	65.0	3	21.7	18.6
11	0 (350)	0 (10)	66.5	3	22.2	19.0

a) t (h) = Time course for maximal GOS concentration
b) P (g/L.h) = GOS productivity = [g/L.h]
c) R (%) = GOS yield = [g GOS/g Initial lactose]*100

The ANOVA of these equations are shown in Tables 4 and 5, for GOS concentration and yield, respectively. According to the F test, both models are predictive, since the calculated F is higher than listed one, 61.6 times for GOS concentration and 6.88 times for yield. The regression coefficients were 0.99 and 0.92, for GOS concentration and yield, respectively. Therefore, the models can be used for the surface generations.

Table 4: The ANOVA for GOS concentration.

Source of variation	Sum of squares	Degrees of freedom	Means squares	F_{calc}	F_{tab}	F_{calc}/F_{tab}
Regression	3006.7	3	1002.2	265.2	4.3	61.6
Residual	26.4	7	3.7			
Total	3033.1	10				

Table 5: The ANOVA for yield.

Source of variation	Sum of squares	Degrees of freedom	Means squares	F_{calc}	F_{tab}	F_{calc}/F_{tab}
Regression	12.4	3	4.1	29.6	4.3	6.88
Residual	0.97	7	0.13			
Total	13.4	10				

Responses surfaces for GOS concentration and GOS yield are shown in Figure 2. According to the surfaces, GOS concentration can achieve more than 80 g/L if lactose concentration is higher than 444 g/L, no matter the enzyme activity, for the range beginning at 5.8 up to 14.2 U/mL (Figure 2a). Yields higher than 17% can be achieved with lactose concentration between 303 and 491 g/L and enzyme activity between 7.2 and 12.8 U/mL (Figure 2b). Also, it can be seen that the lower lactose concentration the lower GOS production. Considering the essays with low lactose concentration and higher glucose and galactose concentrations, lower GOS concentration are found. The transgalactosilation is a reaction where the enzyme β-galactosidase hydrolyses lactose producing glucose and galactose, transferring a galactose molecule to a hydrosylated compound, which can be galactose, lactose or galacto-oligosaccharides, instead of transferring it to a hydroxyl group of the water molecule. However, at low lactose concentrations, the transgalactosilation is lower than hydrolysis, since the quantity of hydroxyl groups from carbohydrates is low, resulting in a higher glucose and galactose concentrations in the reaction solution (Prenosil et al., 1987). It has also been reported that the lactose initial concentration is a major factor in the GOS synthesis and that higher concentrations lead to higher GOS production (Hsu et al., 2007: Martínez-Villaluenga et al., 2008; Park et al. 2008).

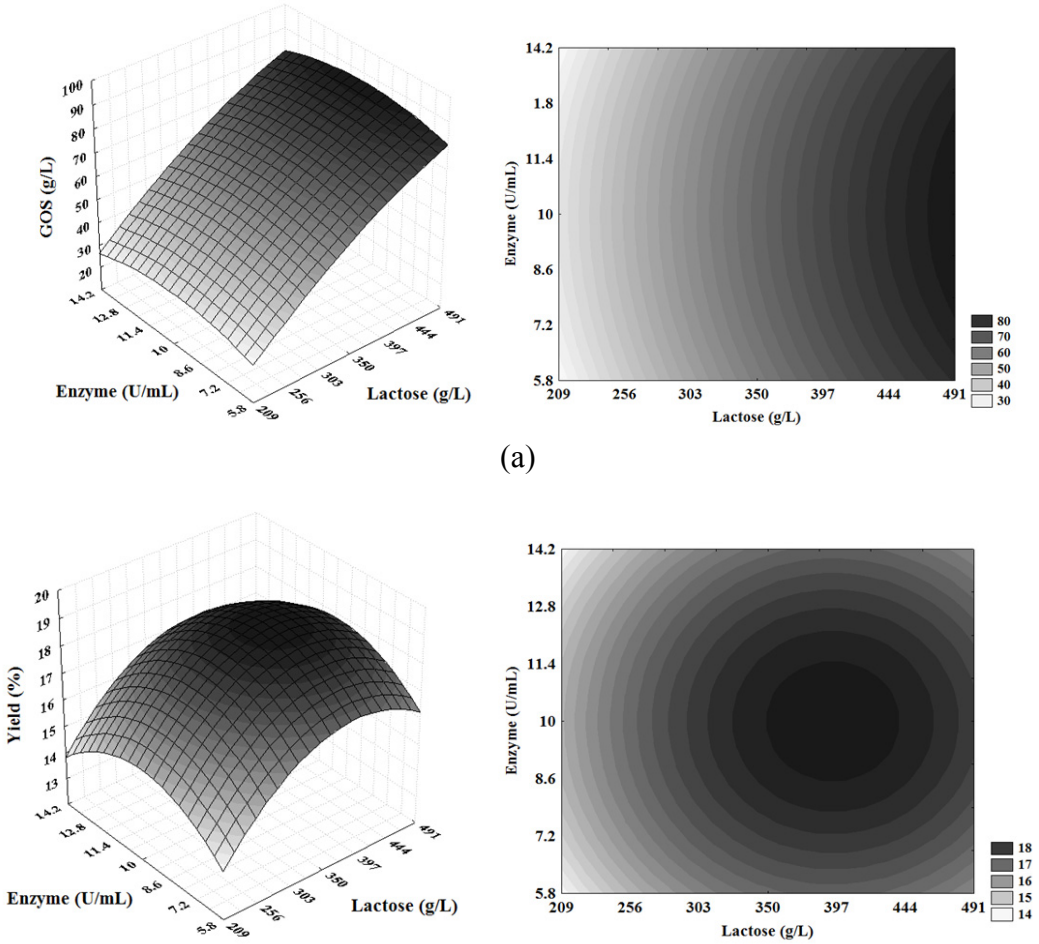

Figure 2: Surface responses for (a) GOS concentration and (b) yield, as a function of enzyme activity and lactose concentration.

A set of three experiments were carried out, following the indications of the previous experimental designs, in which the enzyme activity was kept at 10 U/mL, lactose concentration at 500 g/L, 45ºC and ph 7.0. The GOS production was monitored and is shown in Figure 3, where the experimental data are the average of the three essays. The use of these synthesis conditions led to a maximum GOS production of 83 g/L, after three hours of reaction, with a productivity of 27.6 g/L.h and a yield of 16.5%. The total lactose conversion to glucose, galactose and GOS was around 90%. After three hours of reaction there was a gradual decrease of galacto-oligosaccharides as a consequence of the continuous GOS hydrolysis. Similar results about GOS production have been

reported using β-galactosidases from different microorganisms (Albayrak & Yang, 2002; Chockchaisawasdee et al., 2005; Hsu et al., 2007). These results show that the transgalactosilation happens predominantly at the beginning of the reaction, resulting in higher GOS concentration, while the β-galactosidase hydrolytic activity increases during the reaction time.

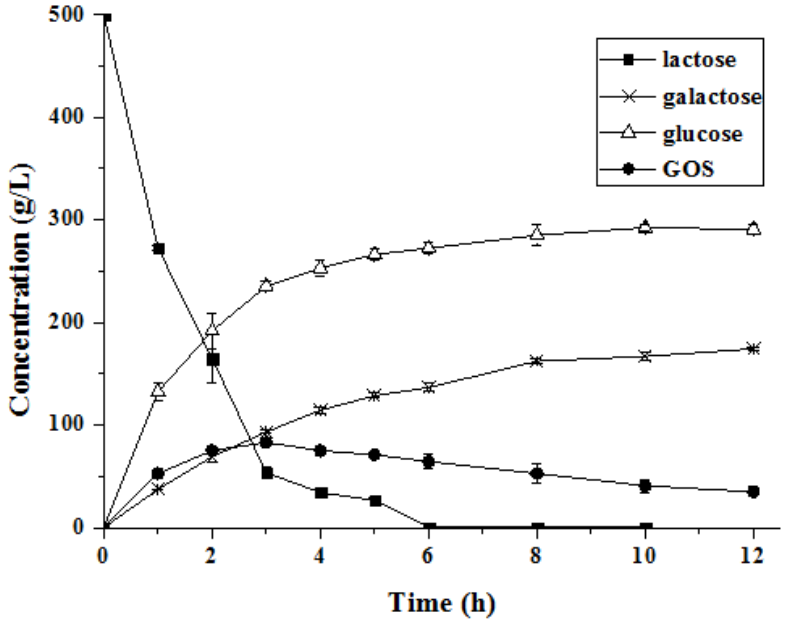

Figure 3: Time course of GOS synthesis at the following reaction conditions: 500 g/L lactose, 10 U/mL enzyme activity, 45°C, pH 7.0. The data are from triplicate essays.

Onishi and collaborators (1995) obtained GOS from permeabilized cells of the yeast *Sterigmatomyces elviae.* In their work, 135 g/L of GOS were produced after 20 hours of synthesis, with a yield of 37.5% and a productivity of 6.75 g/L.h. In a similar work, Onishi *et al.* (1996) studied the synthesis of GOS with permeabilized cells of *Sirobasidium magnum*, in which 136 g/L of GOS were produced after 42 hours of synthesis, with a yield of 37.7% and a productivity of 3.2 g/L.h. Onishi and Yokozeki (1996) working with *Rhodotorula minuta* cells, which had been treated with toluene, obtained a maximum GOS production of 76 g/L after 24 hours of reaction, with a yield of 38% and a productivity of 3.2 g/L.h. When these results of GOS production by permeabilized yeast cells are compared to the one obtained in this work, it can be asserted that in this work the GOS yields were lower and productivity 7 times higher, so that the lower yields can be compensate by higher productivities

CONCLUSIONS

The use of permeabilized cells for GOS synthesis can be an advantage in industrial processes since the enzyme can easily be recuperated and reused. In this work, the use of permeabilized cells of *Kluyveromyces marxianus* CCT 7082, containing β-galactosidase, to produce galacto-oligosaccharides was focused. The methodology of experimental factorial design and surface analysis led to the more suitable reaction conditions, which were: 500 g/L lactose concentration, 10 U/mL enzyme activity, 45ºC and pH 7.0. These reaction conditions led to a GOS production of 83 g/L, with a yield of 16.5% and productivity of 27.6 g/L.h. The reaction time for the highest production was 3 hours.

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