PRODUCTION OF LACTULOSE BY β-GALACTOSIDASE FROM *Kluyveromyces lactis*: THE EFFECT OF THE SUBSTRATE CONCENTRATION AND THE TEMPERATURE

Tiago L. de Albuquerque[1], Sandy D. L. Gomes [1], Luciana R. B. Gonçalves[1], Maria Valderez P. Rocha[1]

[1] Universidade Federal do Ceará, Departamento de Engenharia Química

E-mail: tiagotla1@gmail.com/ valponterocha@yahoo.com.br/ lrg@ufc.br

ABSTRACT

The objective of this work is to evaluate the conditions of enzymatic production of lactulose, a disaccharide with wide application in the food and pharmaceutical industries. For this, it was investigated the influence of the lactose and fructose ratio and the total concentration these carbohydrates on the synthesis of lactulose by the enzyme β-galactosidase from Kluyveromyces lactis. The optimal temperature for this conversion was also evaluated. The results showed that the lactose and fructose ratio of 15% (w/v) and 30% (w/v), respectively, provided higher lactulose synthesis. Moreover, a total carbohydrates concentration of 200 g/L, in the proportion above mentioned, was required to achieve the highest production of lactulose (10.34 g/L). Regarding the temperatures evaluated, it was found that the temperature of 50 °C favored the synthesis of lactulose, reaching a concentration of 14.95 g/L, concluding that the use of this enzyme is promising in the process of lactulose production.

1. INTRODUCTION

The dairy industry is one of the most relevant waste-generating industries in the agro-business sector, responsible for large amounts of lactose-rich effluents (Gutiérrez et al., 2017). Whey is the liquid portion of milk and is rich in organic matter, for this reason, the main effluent of dairy industry, requires treatment before being disposed as wastewater. On the other hand, this residue can also be valued by being converted into high added-value products, such as a food additive or used in the manufacture of certain nutraceuticals (Wang et al., 2013).

The production of lactose derivatives such as lactulose is an alternative to the employment of whey in the formulation of nutritional compounds (Spalatelu, 2012), as lactulose, that has therapeutic and health-improving properties (Wang et al., 2013). Lactulose (4-O-β-d-galactopyranosyl-D-fructose) is a nondigestible synthetic disaccharide whose monomers, glucose and galactose, are bound by a β-glycosidic bond.

Methods commonly used for lactulose synthesis can be divided into chemical and biological routes. Chemically, lactulose is synthesized by an alkaline isomerization of lactose (Guerrero et al., 2011). However, problems such as the need for product purification, occurrence of lactulose degradation, undesired secondary reactions and waste generation arises (Kim et al., 2006). In order to overcome these bottlenecks, enzymatic lactulose synthesis have been studied (Kim et al., 2006). In this context, β-galactosidase has been used as a catalyst in lactose to lactulose conversion via transgalactosylation reaction (Song et al., 2013), and by using fructose as a galactosyl acceptor. Thus, the objective of this work was to evaluate the influence of substrate concentration (lactose and fructose) and temperature on the enzymatic production of lactulose by β-galactosidase from *Kluyveromyces lactis*.

2. MATERIAL AND METHODS

2.1. Materials

β-Galactosidase (EC 3.2.1.23) from *Kluyveromyces lactis* (Lactozyme® 2600L), lactose, fructose, and o-nitrophenyl-b-D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade.

2.2. Methods

2.2.1. Determination of the activity of β-galactosidase enzyme

The hydrolytic activity was determined using o-nitrophenyl-D-galactopyranoside as a substrate at pH 6.6 and 37 °C, according to Lima et al. 2013. A 50 µL enzyme sample was added to a test-tube containing a 2 mL solution of 1.25 mM of ONPG, mixed into a 50 mM potassium phosphate buffer solution with 0.1 mM $MnCl_2.4H_2O$ for 5 min. Finally, 0.5 mL of 1 M sodium carbonate was added to stopped the reaction and the samples were analyzed by spectrophotometry at 420 nm.

2.2.2. Effect of lactose and fructose concentration and of the temperature on lactulose production

Reactions were conducted in test tubes containing different ratios of the lactose-fructose substrate (w/v) [(1) 40%:5%; (2) 35%:10%; (3) 30%:15%, (4) 22.5%:22.5% and (5) 15%:30%] (450 g/L total carbohydrates) diluted in 50 mM potassium phosphate buffer at pH 6.6, at 40 °C and 100 rpm. From this initial experiment the most adequate lactose-fructose ratio for lactulose production was selected, and later experiments were carried out by varying the total sugar concentration, lactose and fructose (10, 25, 50, 100, 200, 300 e 450 g/L). To evaluated the temperature effect, the reactions were conducted in test tubes containing 5 mL of lactose and fructose solution, using the carbohydrates concentration according to the initial experiment. The temperatures tested were 37, 45, 50, 55 and 60 °C. Samples were analyzed using high performance liquid chromatography (HPLC).

2.2.3. Determination of carbohydrates

Lactose and lactulose concentrations were measured using high performance liquid chromatography (Waters, Milford, MA, USA), with a Waters refractive index detector (Model 2414) and a Supelco 610-H column at 65 °C. The eluent was 0.1% v/v H_3PO_4, and it was pumped through the column at a flow rate of 0.5 mL/min.

3. RESULTS AND DISCUSSION

3.1 Experiments to select the optimal ratio and concentration of lactose and fructose

The ratio between lactose and fructose in the reaction may influence the synthesis of lactulose (Table 1). There was no lactulose production using lowest concentrations of fructose, suggesting a positive effect when this monosaccharide is added to the medium in concentrations higher than lactose. The ratio of 15%:30% (w/v) (lactose and fructose) provided a lactulose concentration of 10.34 g/L.

Table 1. Production of lactulose using different ratios of lactose and fructose at 40 °C and 100 rpm

Ratio lactose : fructose (% w/v)	Lactose (g/L)	Fructose (g/L)	Lactulose (g/L)
	0h	0h	2h
1 (40:5)	390.92 ± 0.21	50.42 ± 0.50	0.00 ± 0.00
2 (35:10)	335.12 ± 0.40	94.28 ± 0.10	0.00 ± 0.00
3 (30:15)	297.73 ± 0.44	146,9 ± 0.30	2.30 ± 0.36
4 (22.4:22.5)	222.20 ± 0.35	230.50 ± 0.15	6.06 ± 0.55
5 (15:30)	148.80 ± 0.61	290.92 ± 0.21	10.34 ± 0.42

The most favorable concentration was 200 g/L (lactose and fructose) that reached 14.95 g/L (Table 2). The increase of the lactose and fructose concentration increased the production of lactulose, but from the 200 g/L of carbohydrate it was observed a reduction in lactulose production, probably due to inhibition by substrate. It was observed that when increasing the concentration up lactose 66.7 g/L, there is a reduction in the synthesis of lactulose, probably due to an inhibition of the active site of the enzyme by excessive substrate. This same behavior was observed in the experiments performed by Kim et al. (2006), that used β-galactosidase from *S. solfataricus* to produce approximately 50 g/L of lactulose in 6 h.

Table 2. Production of lactulose under different concentrations of substrate at 40 °C and 100 rpm

Concentration of lactose + fructose (g/L)	Final concentration of lactulose (g/L)	Concentration of lactose + fructose (g/L)	Final concentration of lactulose (g/L)
10	0.00 ± 0.00	**150**	10.35 ± 0.30
25	0.00 ± 0.00	**200**	14.95 ± 0.24
50	1.23 ± 0.40	**300**	12.13 ± 0.40
100	7.62 ± 0.51	**450**	10.87 ± 0.21

3.2 Effect of the temperature on lactulose synthesis by β-galactosidase

Lactulose synthesis from lactose and fructose is a kinetically controlled reaction where the enzyme first acts on lactose forming the galactosyl–enzyme complex with liberation of one molecule of glucose per molecule of lactose reacted. The galactosyl–enzyme complex is then reacted with a molecule of fructose (Kim et al., 2006). At 45 °C was obtained the highest conversion of lactose, reaching almost 100% of hydrolyses into glucose and galactose. However, from 50 ° C the hydrolysis of lactose was reduced to values of up to 50%. On the other hand, (Figures 1A and 1B), at 50 °C the highest conversion of lactose to lactulose was observed, reaching a concentration of 14.95 g/L. The second most favorable temperature was 55 °C, but from that temperature, probably, the enzyme begins to be denatured, losing the potential to be used as a biocatalyst. At 37 °C and 45 °C the conversion courses of lactulose are similar, yielding approximately 10.6 g/L and 11.3 g/L, respectively. At 60 °C there was no conversion, the enzyme was rapidly inactivated (Figure 1A).

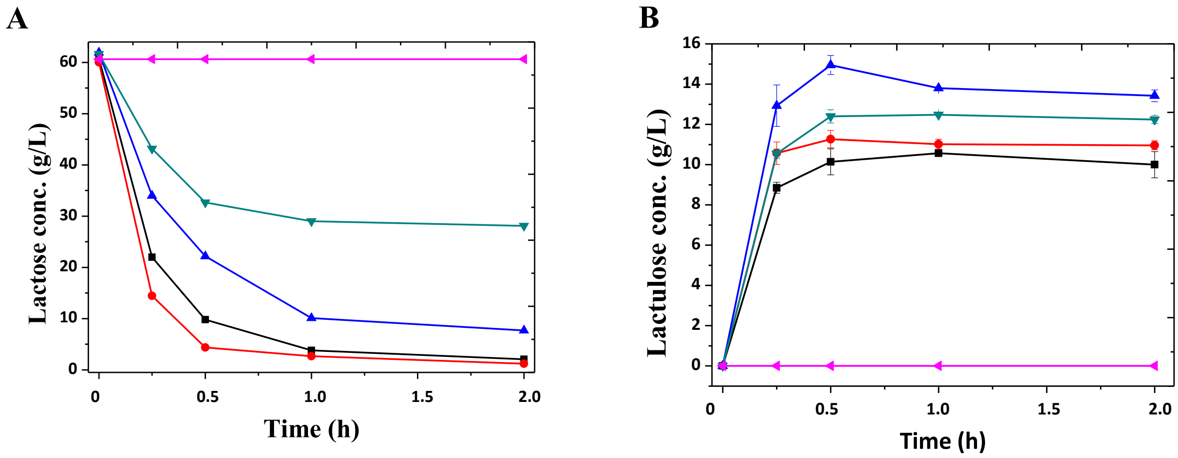

Figure 1. Conversion of lactose to lactulose at different temperatures: (■) 37 °C; (●) 45 °C; (▲) 50 °C; (▼) 55 °C; (◄) 60

Song et al. (2013) evaluated the lactulose synthesis from whey lactose by immobilized β-galactosidase and glucose isomerase. The lactulose concentration increased as temperature increased, and maximum values were observed at 53.5 °C (4.81 g/L, approximately 3 times less than the concentration achieved in our study). Above this temperature the lactulose synthesis decreased, likely, due to inactivation of β-galactosidase at elevated temperatures.

4. CONCLUSIONS

It is possible to produce lactulose through the enzymatic route by the enzyme β-galactosidase from *Kluyveromyces lactis* using lactose and fructose as substrates. The optimum conditions of lactose and fructose concentration were 15% (w/v) lactose and 30% fructose (w/v), and the optimum temperature observed was 50 °C in which the lactulose production was 14.95 g/L.

5. REFERENCES

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