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MICROBIOLOGY

Porungo cheese whey: a new substrate to produce β-galactosidase

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Abstract: The bioconversion of porungo cheese whey to produce β-galactosidase in batch system was studied. The whey released after curd cutting and precipitation during porungo cheese production was collected in borosilicate flasks. Two strains of *Kluyveromyces marxianus*, CCT 4086 and CBS 6556, and whey supplementation with different nitrogen sources were evaluated. Different temperatures (30 °C and 37 °C) and pH values (5.0 to 7.0) were investigated to establish the best conditions for enzyme production. The highest enzymatic activity was obtained by *K. marxianus* CCT 4086 in porungo cheese whey supplemented with yeast extract (16.73 U mL^{-1}). *K. marxianus* CCT 4086 produced superior β-galactosidase activity when compared to CBS 6556 for all media tested (ranging from 11.69 to 14.40 U mL^{-1}). Highest β-galactosidase activity was reached under conditions of pH 7.0 and 30 °C using *K. marxianus* CCT 4086 in the better media composition. The lowest enzymatic activity was observed at 37 °C for all pH values tested (10.69 U mL^{-1} to 13.94 U mL^{-1}) and a highest β-galactosidase activity was reached in pH 7.0 for both two temperatures (11.42 to 15.93 U mL^{-1}). Porungo cheese whey shows potential for industrial β-galactosidase production by microbial fermentation.

Key words: Whey, β-galactosidase, *Kluyveromyces marxianus*, agroindustrial residues.

INTRODUCTION

Whey is the main by-product of the dairy industry, being a significant source of environmental pollution because of its high volumes of production and organic load. This by-product has a biological oxygen demand (BOD) ranging from 30 g L^{-1} to 50 g L^{-1}, which associated with improper disposal, can lead to serious environmental problems (Kosseva et al. 2009, Prazeres et al. 2012). Although about half of the total worldwide production of whey is disposed in wastewater treatment plants or reused in farms, whey presents an interesting composition of lactose (45–50 g L^{-1}), protein (6–8 g L^{-1}), lipids (4–5 g L^{-1}), and mineral salts (5–7 g L^{-1}), thus showing great potential for use in bioprocess to obtain compounds of interest, such as the enzyme β-galactosidase (Rech & Ayub 2007, Gupte & Nair 2010, Choonia & Lele 2013, Gabardo et al. 2014). Since the annual worldwide production of whey is estimated at around 160 million tons, the implementation of the technologies that contribute to obtain high added-value products using it in new processes characterizes an important industrial gain (Guimarães et al. 2010, Gabardo et al. 2014).

Porungo is an artisanal cheese traditionally manufactured by farmers using raw milk in the southwestern region of São Paulo State, Brazil, and has similar characteristics of mozzarella cheese. On its production process, fermented-whey (endogenous culture) is added in the milk to start a new production of cheese. Porungo cheese contains a large population of lactic

bacteria responsible for its aspect and flavor characteristics (Pezzo 2017). So far, there are no reports on the industrial use of porungo cheese whey, therefore, its biotransformation may be scientifically interesting.

The β-galactosidase enzyme, commercially known as lactase (EC 3.2.1.23), is classified as a hydrolase that catalyzes lactose hydrolysis, resulting in the equimolar mixture of glucose and galactose (Grosova et al. 2008, Fai & Pastore 2015). β-galactosidase is widely used in the food industry for low-lactose products, which offers benefits in health and food technology (Grosova et al. 2008, Husain 2010). This enzyme is found in vegetables (almonds, peaches and apples), animals (intestines and brains), and microorganisms (filamentous fungi, bacteria and yeasts) (Santiago et al. 2004, Grosova et al. 2008, Fai & Pastore 2015). However, microbial production is the industrial choice, since it allows for higher yields and controls, being yeasts and fungi the preferred microorganisms for commercial applications. In this context, the yeasts of the genus *Kluyveromyces (K. lactis*, *K. fragilis*, and *K. marxianus*), and the fungi *Aspergillus niger* and *A. oryzae* have been reported as the main sources of commercial enzymes (Santiago et al. 2004, Grosova et al. 2008, Pereira et al. 2012, Fai & Pastore 2015).

The production of β-galactosidase has been successfully studied by different research groups (Rech et al. 1999, Santiago et al. 2004, Manera et al. 2011) evaluating the influence of different lineages, media and process conditions. In this sense, several strategies were researched for the production of β-galactosidase, involving cultivation in fed batches (Rech & Ayub 2007, You et al. 2017), continuous culture (Ornelas et al. 2008) and genetic engineering (Oliveira et al. 2011, Zhou et al. 2013). Regarding batch cultivations, different yeast strains and supplementation of cheese whey, using different nitrogen sources have been investigated. Improvements in the growth kinetics of *K. marxianus* ATCC 46537 in the enzymatic synthesis were observed by Santiago et al. (2004), when cheese whey permeate was supplemented with different yeast extract concentrations. In this study, performed at 30 °C, 150 rpm and pH 5.5, the enzymatic activity was not influenced by the concentration of yeast extract (6 g L^{-1} or 12 g L^{-1}), however, the addition of this nitrogen source significantly increased the enzymatic activity when compared with media without supplementation. A similar result was obtained by Rech et al. (1999) when testing two different nitrogen source (yeast extract and urea) and two strains of *K. marxianus* (CBS 712 and CBS 6556) in cultivations using cheese whey at 30 °C, 200 rpm and pH 5.5. According to the authors, urea supplementation led to a limited growth of the strains due to the alkalinization of the culture medium. The highest cell growth and enzymatic activity occurred when media was inoculated with *K. marxianus* CBS 712 and supplemented with yeast extract. Manera et al. (2011) tested the influence of different concentration of two nutrient sources, corn steep liquor and Prodex-lac® yeast hydrolysate, and pH values, ranging from 5.0 to 7.0. They observed that the highest enzymatic activity of β-galactosidase from *K. marxianus* CCT 7082 was obtained at 30 °C and 180 rpm with the increased concentration of corn steep liquor and decrease of pH. β-galactosidase is an intracellular enzyme and requires cell disruption for its release, a fundamental step in the downstream processes. Different methods can be used to extract intracellular enzymes, depending of the microorganism type, its location within the cell and the desired use of the compound of interest. Mechanical, physical, chemical, enzymatic methods and the combination of these can be applied. However, because β-galactosidase has its main application in food, the disruption

of *Kluyveromyces* cells should not be carried out by chemical methods, as it would require decontamination, increasing production costs. On the other hand, mechanical methods of cell disruption do not imply toxicity risks, as they do not include chemicals in the process (Medeiros et al. 2008). Among the mechanical methods, cell-breaking using glass beads as well as ultrasonic energy-break are considered highly efficient methods. Mechanical disruption using glass beads is considered simple, since it does not require a large operational apparatus, as it basically uses glass beads abrasion and shear, leading the cell wall rupture and release of the enzyme (Lemes et al. 2012).

From these considerations, we can devise the biotechnological potential of using porungo cheese whey as alternative carbon source to produce β-galactosidase enzyme, allowing the use of this agroindustrial by-product from the dairy farms to obtain added-value biomolecules to be used in the food industry, contributing to the regional development Therefore, the aims of this research were to investigate the synthesis of β-galactosidase enzyme using porungo cheese whey as substrate to *K. marxianus* culture as biocatalyst. Different strains and media supplement, pH, and temperature were evaluated in order to establish the optimal conditions of enzyme production.

MATERIALS AND METHODS

Microorganisms

Kluyveromyces marxianus CBS 6556 was obtained from Centraalbüreau vor Schimmel-Cultures (Amsterdam, The Nederlands) and *K. marxianus* CCT 4086 was provided by Tropical Culture Collection of André Tosello Foundation (Campinas, Brazil), both donated by Biotechnology & Biochemical Engineering Laboratory (BiotecLab), of the Food Science and Technology Institute (ICTA) of the Federal University of Rio Grande do Sul, Brazil. The strains were maintained on agar slants at 4 °C, as reported elsewhere (Furlan et al. 1995).

Porungo cheese whey characterization

Porungo cheese whey was provided by farmers located in the southwestern region of São Paulo State, in the Lagoa do Sino Territory, Brazil. The whey was obtained through the production process of the artisanal porungo cheese. After raw milk coagulation by the addition of rennet and fermented whey (which contains the endogenous microbiota), the curd was cut to obtain the separation of the solid fraction (proteins and fats) and the liquid (whey). In this stage, a small volume of approximately 2 L of whey were collected in plastic bottle and kept under room temperature for fermentation (used in the next day production) and the rest was collected in sterilized borosilicate reagent flasks and transported in isothermal boxes to the laboratory. This material was stored frozen at -20 °C until use.

The protein content of porungo cheese whey was determined by Micro-Kjeldahl method, using 6.38 as correction factor (Instituto Adolfo Lutz 2008). Ashes were determined by Adolfo Lutz Institute (2008) methodology by incinerating 20 mL of porungo cheese whey, followed oven burning at 550 °C in a muffle. The lipid content was evaluated by the butyrometric method using sulfuric acid and isoamyl alcohol as reagents (Lanagro, 2014). Total dry extract content was determined according to Adolfo Lutz Institute (2008) in an oven at 105 °C until constant mass. Lactose was evaluated by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959), from a lactose calibration curve.

β-galactosidase production in batch system

The enzyme production was studied in two steps. First, two strains of *K. marxianus* and three different media were tested in a rotary shaker to evaluate the conversion of porungo cheese whey into β-galactosidase. The media composition tested were porungo cheese whey without any supplementation (S); porungo cheese whey supplemented with 3 g L^{-1} of yeast extract (SE); and porungo cheese whey supplemented with 3 g L^{-1} of yeast extract and 5 g L^{-1} of bactopeptone (SEP). To avoid precipitation during autoclaving, whey proteins were hydrolyzed using a commercial protease (Alcalase 2.4 L, 2.4 UA-A g-1, Tovani Benzaquen Ingredients, São Paulo, Brazil) at pH 8.5, 55 °C for 3 h. Inocula were prepared by transferring isolated yeast colonies to a 250 mL conical flasks containing 50 mL of YEP-lactose medium (yeast extract, 10 g L^{-1}; bactopeptone, 20 g L^{-1}; lactose, 20 g L^{-1}), pH 7.0, and incubated in an orbital shaker at 200 rpm for 15 h at 30 °C. Cell concentration was adjusted for optical density at 600 nm (OD_{600nm}) of 1, which corresponded to 1.5 g L^{-1} for *K. marxianus* CBS 6556 and 1.4 g L^{-1} for *K. marxianus* CCT 4086. The fermentation experiments were carried out in conical flasks of 250 mL containing 45 mL of sterilized cultivation media and 5 mL of inoculum totalizing 50 mL of fermentation medium at 30°C and 200 rpm. Batch cultivations were carried out in duplicate. The samples were withdrawn periodically at each 5 h up to 25 h of cultivation.

In the second experimental step, the influence of the temperature (30 °C and 37 °C) and pH (5.0, 6.0, and 7.0) was evaluated. The fermentation was conducted using the best media obtained in the previous step, using porungo cheese whey supplemented with yeast extract and the *K. marxianus* CCT 4086 strain. This step was performed in 250 ml conical flask filled with 50 ml of the total fermentation volume, incubated under stirring of 200 rpm for a period of 25 h. The samples were withdrawn periodically at each 5 h. All experiments were performed in duplicate. Data were statistically evaluated by analysis of variance (ANOVA) using Statistica 7.0 software (StatSoft, USA).

Analytical Determinations

Mechanical Cell Disruption

Because β-galactosidase is an intracellular enzyme, a cell wall disruption step is required to release the enzyme from the cytosol of strains *K. marxianus* CCT 4086 and CBS 6556. Thus, cells collected at 3,000 × g for 15 min at room temperature, with the supernatant separated for lactose determinations and the cell pellet resuspended in 0.1 M phosphate buffer (pH 7.3) for subsequent enzymatic extraction. The cell wall was broken using 1.1 g of glass beads per ml of cell suspension (Medeiros et al. 2008) under vigorous agitation for 5 min using a vortex to allow cell wall abrasion and shear. The supernatant containing the enzyme was separated by the cell debris by centrifugation under the same conditions as described above and then used to determine enzyme activity. Tests were performed to determine which time, 5 or 10 min, lead to better cell disruption (data not shown). As time did not differ in the enzymatic activity, time of 5 min was chosen in this work.

Biomass And Lactose Concentration

Cell concentration was determined by optical density at 600 nm and correlated with dry cell weight (g L^{-1}). Lactose concentration was determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) using lactose calibration curve as standard.

β-galactosidase activity

The determination of β-galactosidase enzymatic activity was performed using ONPG

(o-nitrophenyl-β-D-galactopyranoside) as substrate, according to Klein et al. 2013. The reaction occurred by mixing and incubating 50 µL of enzyme and 0.5 mL of 0.1 M potassium phosphate buffer (pH 7.0) and 1.5 mM of magnesium chloride ($MgCl_2$) containing 10 mM of ONPG at 37 °C for 2 min. The reaction was stopped by adding 0.1 M sodium carbonate buffer (pH 10.0). The o-nitrophenol (ONP) released was determined using spectrophotometer at 415 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1 µmol of ONP per minute under analysis conditions.

Enzymatic Assay Of The Produced β-Galactosidase

To investigate the optimum temperature and pH of the β-galactosidase, the enzyme activity under different conditions were measured. The optimum temperature of β-galactosidase was evaluated ranging from 20 °C to 60 °C in activity buffer at pH 7.0. Likewise, the optimum pH of β-galactosidase was evaluated ranging from 5.5 to 8.0 at 37°C. The reaction occurred using 50 µL of β-galactosidase extract and 0.5 mL of activity buffer containing 10 mM ONPG for 2 min.

RESULTS AND DISCUSSION

Composition Of Porungo Cheese Whey

The centesimal composition of porungo cheese whey used in this work is presented in Table I. Cheese whey, in general, is described as containing water (94-95 %), lactose (4.5-5 %), proteins (0.8-1 %), lipids (0.4-0.5 %) and mineral salts (0.7-0.8 %) (Siso 1996, Christensen et al. 2011, Das et al. 2016). However, the composition depends on several factors, such as the type of milk used in cheese production, the technological processes used and the type of animal species and their diet (Vasconcelos et al. 2018). The values obtained in this study are

Table I. Centesimal composition of porungo cheese whey.

Component	%
Moisture	93.25 ± 0.118
Dry extract	6.76 ± 0.118
Lipids	0.30 ± 0.000
Protein	1.09 ± 0.025
Lactose	4.30 ± 0.026
Ash	0.53 ± 0.005

in agreement with those found by Carvalho 2007, in which the average lactose is around 5 % and the average protein amount is 0.95 %. Similar results were obtained by Boldrinia et al. 2011, who found percentages of approximately 4.7 % for lactose and 1.5 % for total proteins, and by Kosikowski 1979, who reported values of 4.9 % for lactose and 0.5 % for ashes. This composition, specially the lactose content, reinforces the idea that porungo cheese whey is rich in fermentable carbohydrate with high potential for β-galactosidase production.

β-galactosidase production by different strains and media

These experiments were carried out in order to determine the effect of media supplementation on the ability of the two strains of *K. marxianus*, to use the porungo cheese whey in the bioconversion to β-galactosidase. Once β-galactosidase enzyme remains intracellularly, cell disruption is an essential step in the process to obtain this enzyme (Medeiros et al. 2008, Dagbagli & Goksungur 2008). Although mechanical methods are not specific, they have high efficiency and wide application compared to other methods, such as enzymatic digestion of cell wall (Numanoglu & Sungur 2004, Medeiros et al. 2008). Although the chemical method has been described as more efficient in some studies (Becerra et al. 1998, Bansal at al. 2008) the chemical cell rupture would imply in the contaminant removal, with increased production costs (Medeiros et al. 2008).

However, Numanoglu & Sungur 2004 compared physical and chemical procedures, reporting that the activities were not significantly different. Medeiros et al. (2008), investigated the effect of the amount of glass beads (0.44 to 1.1 g mL^{-1} of cell suspension) and the time of cell rupture (10 min to 40 min), reporting best results for 1.1 g of beads per mL of cell suspension and that times over 10 min did not increased enzymatic activity. Dagbagli & Goksungur (2008), tested different chemical and mechanical methods for obtaining the intracellular β-galactosidase from *K. lactis*. The highest enzymatic activity (3,416.6 U g^{-1}) was obtained when the cells were permeabilized using isoamyl alcohol. Similar result was obtained by mechanical rupture of the cells using glass beads (3,038.9 U g^{-1}) and the lowest enzymatic activities were found when using Triton X-100 (1,888.8 U g^{-1}), liquid nitrogen (1,199.4 U g^{-1}), SDS (964.3 U g^{-1}), and sonication (152.6 U g^{-1}). Thus, the extraction process using glass beads under vortex agitation proved to be very efficient. Freitas (2013), studied two methods based on using 1.10 g of glass beads per mL: 1) rupture following 30 min vortex agitation with intervals of 2 min in ice-bath every 5 min and, 2) rupture by 40 min of ultrasound at 25 °C with an interval of 10 min preventing enzyme denaturing caused by water overheating. The first strategy showed a superior enzymatic activity (87.4 %) compared with second strategy. Based on these studies, in this work we used 1.1 g of glass beads per mL of cell suspension and tested two different times for cell rupture: 5 min and 10 min. Enzymatic activity did not differ for the two rupture cell times tested (data not shown). Thus, cells were disrupted for 5 min.

The enzymatic activity was influenced by both strain and culture media. The Tukey test showed statistical differences (p<0.05) between media S and the media with supplementation, SE and SEP, but not between these last two. The highest enzymatic activity was obtained for *K. marxianus* CCT 4086 strain in porungo cheese whey supplemented with yeast extract (SE) and the lowest values were obtained in porungo cheese whey without supplementation (S) for the two strains tested (Figure 1 and 2). In all experiments (three media and two strains) the highest enzymatic activity was achieved in 20 h of cultivation. Kinetics of lactose consumption, enzymatic activity and biomass formation for *K. marxianus* CBS 6556 in different media are shown in Figure 1. Lactose was practically consumed following 25 h of cultivation for all tested media, ranging from 92.4 % to 93.3 %, being the lowest consumption for the porungo cheese whey without any supplementation (S). Moreover, the lowest biomass concentration was observed in the whey without supplementation (5.35 g L^{-1}). The same behavior was observed to enzymatic activity, reaching of 11.69 U mL^{-1} for S media compared to a maximum of 14.45 U mL^{-1} in porungo cheese whey supplemented with yeast extract (SE). Lactose was totally consumed for *K. marxianus* CCT 4086 in 20 h of cultivation for the all media tested (Figure 2), however a slower lactose metabolization kinetics was observed for S media when compared to the other two cultivation media (SE and SEP). Biomass concentration was similar for all media evaluated, reaching of 4.55 g L^{-1}, 4.36 g L^{-1} and 4.24 g L^{-1} in S, SE and SEP, respectively, after 20 h of cultivation. Moreover, the enzyme activity was highest in 20 h of cultivation for all media tested, ranging from 14.41 U mL^{-1} to 16.73 U mL^{-1} in S and SE media, respectively. An enzymatic activity of 14.54 U mL^{-1} was obtained in SEP following 20 h of cultivation. The results are in agreement with Rech et al. (1999) and Santiago et al. (2004), who observed the highest cell growth and β-galactosidase synthesis using cheese whey or cheese whey permeate supplemented with yeast extract using different strains of *K. marxianus*. Rech et al. (1999) reported that specific β-galactosidase activity did not differ between *K. marxianus* CBS 6556 and

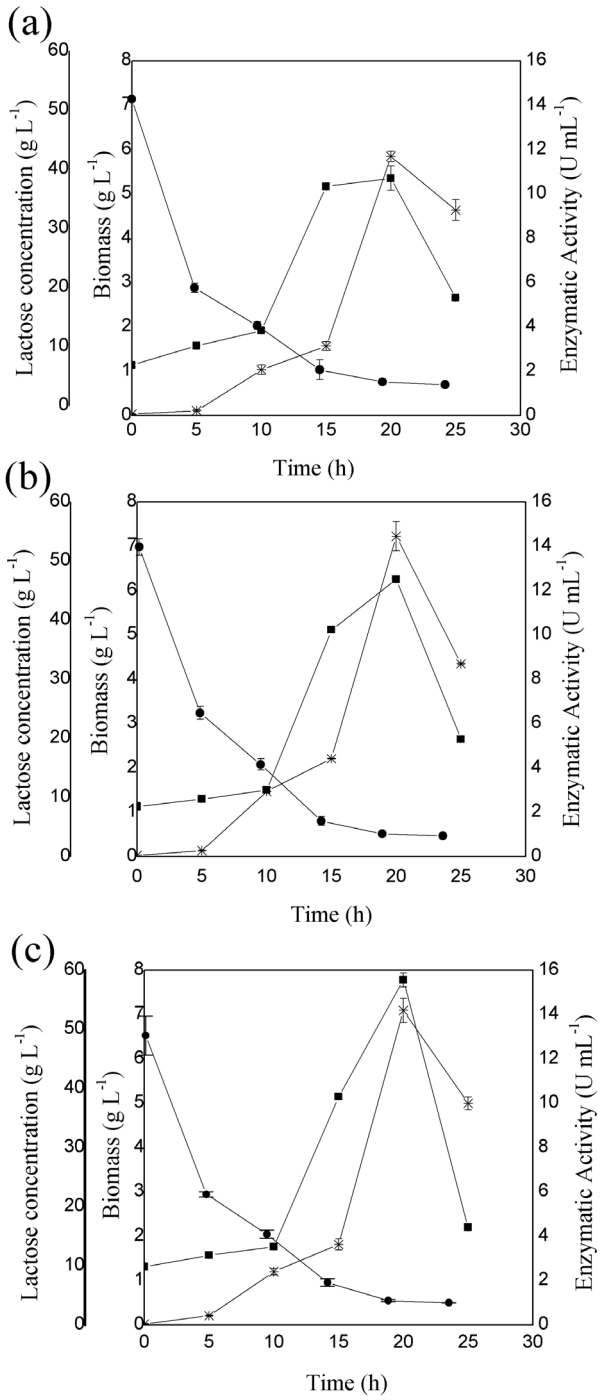

Figure 1. Kinetic of *Kluyveromyces marxianus* CBS 6556 in (a) porungo cheese whey, (b) porungo cheese whey suplemented with yeast extract and (c) porungo cheese whey supplemented with yeast extract and bactopeptone at 200 rpm and 30 °C. Lactose (-●-). Biomass (-■-). Enzymatic activity (-∗-).

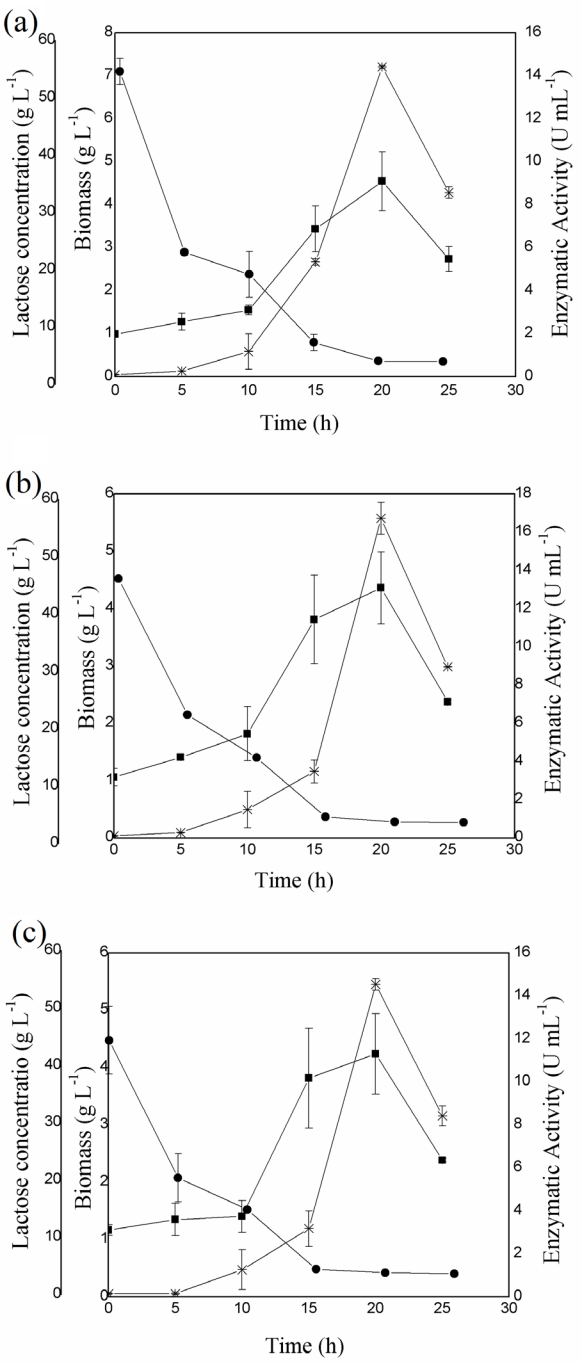

Figure 2. Kinetic of *Kluyveromyces marxianus* CCT 4086 in (a) porungo cheese whey, (b) porungo cheese whey supplemented with yeast extract and (c) porungo cheese whey supplemented with yeast extract and bactopeptone at 200 rpm and 30 °C. Lactose (-●-). Biomass (-■-). Enzymatic activity (-∗-).

CBS 712 strains, although the last strain showed a higher biomass concentration. Braga et al. (2012) investigated the optimal enzyme condition, using cheese whey and rice effluent in cultures of *K. marxianus* CCT 7082, and the supplementation of the media with yeast extract led to a highest enzymatic activity of 10.45 U mL^{-1}, lower than that obtained in this work. Contradictorily, Gupte & Nair (2010) observed that enzymatic activity was not influenced by supplementation of the cheese whey with different nitrogen sources using *K. marxianus* NCIM 3551. Although in this work the enzymatic activity did not differ between SE and SEP (p<0.05) for the two strains studied, subsequent tests were performed using SE because it would be a cheaper media. The strain *K. marxianus* CCT 4086 was chosen because it produced the highest enzymatic activity.

β-galactosidase production on different ph and temperature

Enzyme activity of *K. marxianus* CCT 4086 growing in SE was influenced by both pH and temperature (p <0.05), reaching the highest amount of 15.93 U mL^{-1} at pH 7.0 and 30 °C (Table II). Lactose was completely consumed following 25 h of cultivation for all tested pH (5.0, 6.0 and 7.0), with a slower lactose metabolization at pH 5.0 for both temperatures tested (Figure 3 and 4). The pH also influenced the biomass formation. While the lower pH (5.0) produced less biomass in both temperatures (4.49 g L^{-1} and 5.27 g L^{-1}), the highest biomass concentration of 7.68 g L^{-1} and 6.95 g L^{-1} at 30 °C and 37 °C, respectively, was achieved at pH 7.0. The enzymatic activities of all cultures peaked at 20 h of fermentation. Again, highest values of enzymatic activity were influenced by pH, the lowest at pH 5.0 and the highest at pH 7.0. Regarding temperature, at 37 °C the lowest values were found, and the highest, at 30 °C (Table II). Similar results were observed by Furlan et al. (2001), who reported maximum β-galactosidase enzymatic activity at 30 °C and minimum at 37 ° C in cultures of *Kluyveromyces marxianus* CDB 002 in sugar-cane molasses medium (100 g L^{-1}).

Table II. Enzymatic activity for different pH (5.0, 6.0 and 7.0) and temperatures (30 °C and 37 °C) in 20 h of cultivation of *K. marxianus* CCT 4086 cultured in porungo cheese whey.

pH	Temperature (°C)	
	30	37
	Enzymatic Activity (U mL^{-1})	
5	11.42 ± 0.003^{Ba}	10.69 ± 0.103^{Aa}
6	14.35 ± 0.009^{Bb}	11.29 ± 0.344^{Ab}
7	15.93 ± 0.010^{Bc}	13.94 ± 0.011^{Ac}

Note: **Tukey test (p<0.05) on lines (uppercase letter) and in column (lowercase letter).**

The maximum specific growth rate (μ_{max}), the yields of biomass formation ($Y_{X/S}$), specific product ($Y_{P/X}$), and yields of product per substrate ($Y_{P/S}$), calculated at 20 h of cultivation are shown in Table III. The lowest temperature (30 °C) led to the highest values of all kinetics parameters for all pH tested. The kinetic parameters increased with increased of pH values, except for $Y_{P/X}$, which was decreasing with pH. Rech et al. (1999) found highest values of μ_{max} (0.49-0.61 h^{-1}) and $Y_{X/S}$ (0.29-0.71 g g^{-1}) for the two *K. marxianus* CBS 712 and CBS 6556, in experiments conducted in bioreactors. Although Machado et al. (2015) found the enzymatic activity of 41.7 U mL^{-1} in cultures of *K. marxianus* CCT 7082 in cheese whey and glycerol, pH 5, the specific product concentration was 1.85 U g^{-1} of dry cell, a value very smaller than that obtained in this work (2.55 U mg^{-1}). Alves et al. (2010) evaluated the kinetic parameters using synthetic lactose media and different aeration conditions in bioreactor and found the range of enzymatic activity ranging from 4.7 U mL^{-1} to 14.6 U mL^{-1}, the $Y_{X/S}$ range of 0.07 g g^{-1} to 0.35 g g^{-1} and $Y_{P/X}$ range of 0.26 g g^{-1} to 0.59 U g^{-1}. Our results indicate the potential of using porungo cheese whey in this bioprocess.

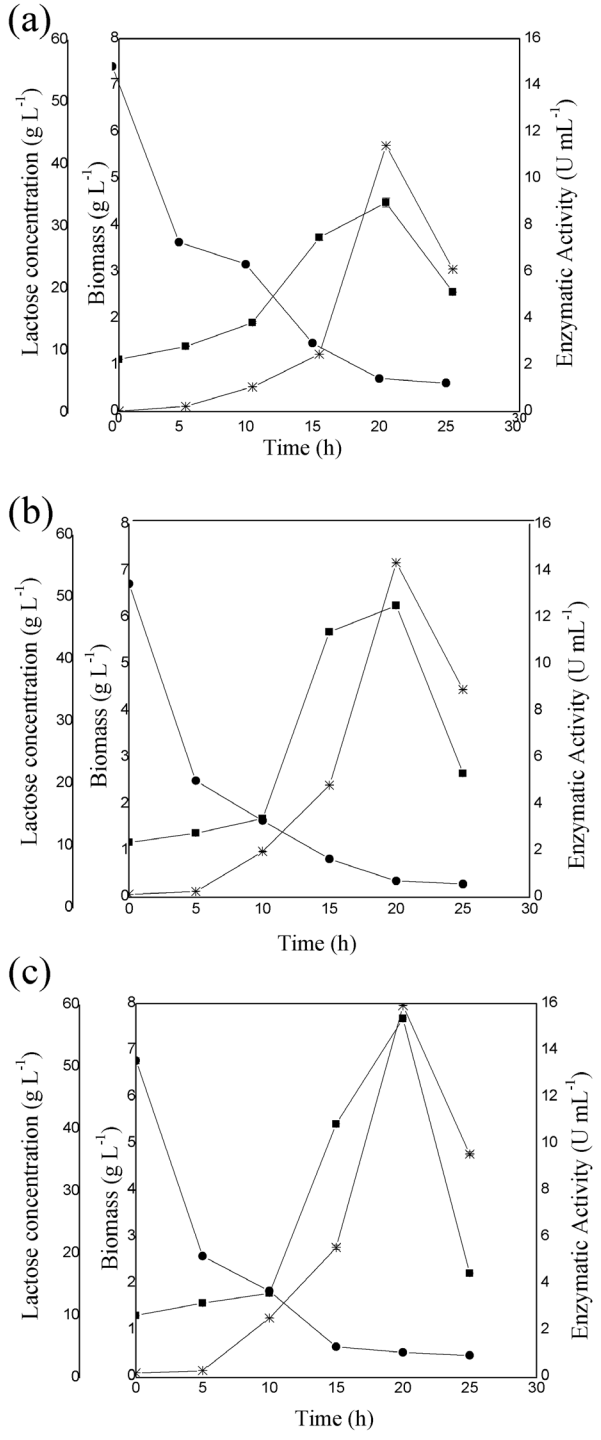

Figure 3. Kinetic of *Kluyveromyces marxianus* CCT 4086 in porungo cheese whey supplemented with yeast extract at 200 rpm and 30 °C in (a) pH 5.0, (b) pH 6.0 and (c) pH 7.0. Lactose (-●-). Biomass (-■-). Enzymatic activity (-∗-).

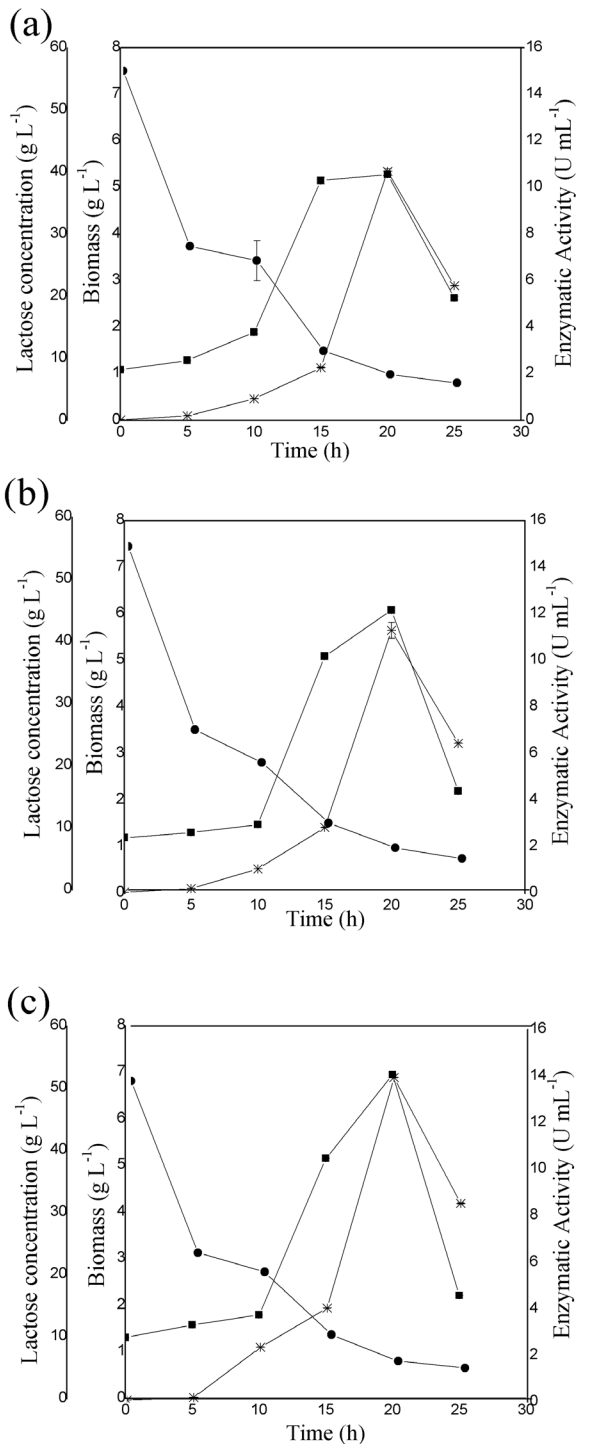

Figure 4. Kinetic of *Kluyveromyces marxianus* CCT 4086 in porungo cheese whey supplemented with yeast extract at 200 rpm and 37 °C in (a) pH 5.0, (b) pH 6.0 and (c) pH 7.0. Lactose (-●-). Biomass (-■-). Enzymatic activity (-∗-).

Table III. Kinetics parameters of *K. marxianus* CCT 4086 cultured in porungo cheese whey supplemented with yeast extract at 200 rpm in shaker under different pH (5.0, 6.0 and 7.0) and temperature (30 °C and 37 °C).

pH	Temperature (°C)							
	30				37			
	μ_{max} (h^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/X}$ (U g^{-1})	$Y_{P/S}$ (U g^{-1})	μ_{max} (h^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/X}$ (U g^{-1})	$Y_{P/S}$ (U g^{-1})
5	0.23	0.09	2.55	0.23	0.20	0.11	2.03	0.22
6	0.30	0.13	2.29	0.30	0.25	0.12	1.86	0.23
7	0.34	0.16	2.07	0.34	0.21	0.14	2.01	0.31

Optimal Conditions For Enzyme Activity

The effect of the temperature and pH on the relative activity of the β-galactosidase produced by *K. marxianus* 4086 using porungo cheese whey as substrate is presented in Figure 5. The enzyme showed optimal activity at 37 °C and pH 6.5, in agreement with other studies using β-galactosidase from *Kluyveromyces* sp. (Siso 1996, Jurado et al. 2002, Mlichová & Rosenberg 2006, Klein et al. 2013). The enzymatic activity is dependent on the temperature (Figure 5a), in which an ascendant activity was observed up to reach the maximum activity at 37 °C. Above this temperature, the activity sharply declined due to enzyme denaturation. This occurs because the activation energy (E_a) values for enzyme deactivation is typically higher than the E_a values for activation effect, once protein denaturation involves unfolding of large segments of the polypeptide chain (global process), requiring greater free energy change than that required for stabilization of the transition state at the active site (localized process) (Damodaran et al. 2007). The pH effect on the relative activity of β-galactosidase was evaluated in the range of 5.5 to 8.0 (Fig. 5b). The enzymatic activity was upward until pH 6.5, when it reached the maximum value and from which activity started to decline. Ionizable groups in enzymes can undergo transitions dependent of the pH based on the p*Ka* values of the amino acid residues (Damodaran et al. 2007). The low enzymatic activity at pH 5.5 occurs because the isoelectric point (p*I*) of the β-galactosidases is 5.42 (Zhou & Chen 2001). The enzyme showed optimal activity at pH 6.5 probably due to that the β-galactosidase has two active-site carboxyl groups in active site, one protonated (Glu^{482}) and one ionized (Glu^{551}), being both able to coexist in the same time at neutral pH (Zhou & Chen 2001).

The results obtained in this work are similar to Jurado et al. (2002) that studied the effect of temperature and pH on the activity of β-galactosidase produced by *Kluyveromyces fragilis* and found the highest enzymatic activity at temperature 37 °C and pH 6.6. When investigating the influence of temperature (17-42 °C) and pH (5.0 to 9.0) on the activity of β-galactosidase from *Kluyveromyces lactis* in the free form, Song et al. (2010) also found the optimum temperature of 37 °C and pH value of 7.0. Although in the study of Klein et al. (2013) the authors observed the maximum β-galactosidase activity at 45 °C using β-galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000) in free form, the optimum pH of 6.5 was in accordance with results in this work. Since the optimum pH of the β-galactosidase is near neutral, the enzyme is quite suitable for saccharifying milk and sweet whey, setting up a higher demand to be used in these products compared to β-galactosidases from fungi species (Siso 1996, Mlichová & Rosenberg 2006). The knowledge of

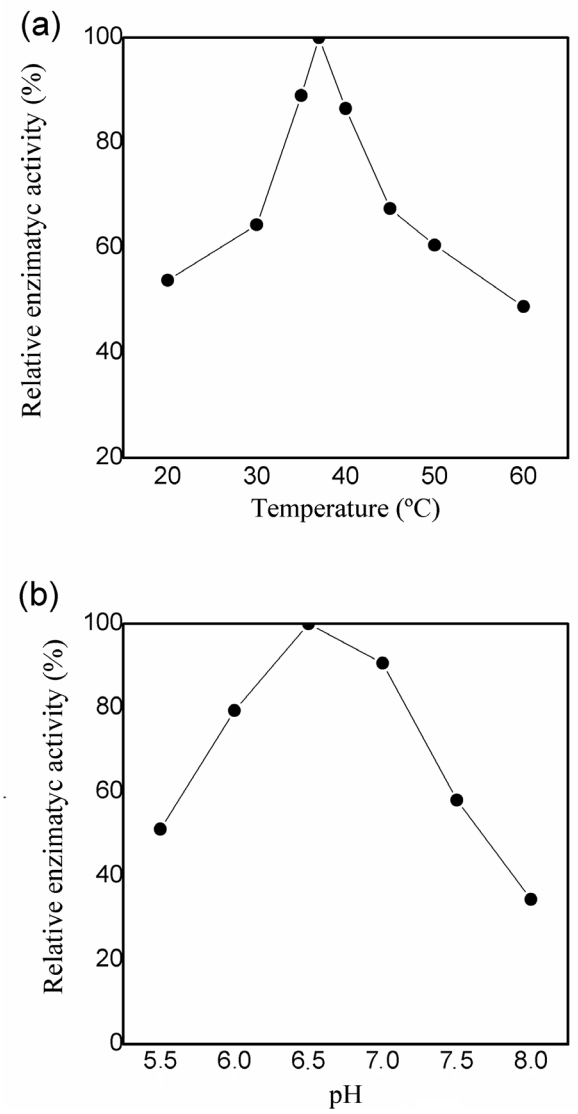

Figure 5. Effect of temperature (a) and pH (b) on the β-galactosidase activity from *Kluyveromyces marxianus* CCT 4086 produced in the better conditions.

the optimum enzymatic conditions consists as an important tool for its industrial application.

Porungo cheese whey proved to be an alternative and inexpensive carbon source for β-galactosidase production, even when non-supplemented. Moreover, the use of this by-product allows to associate both the reduction of environmental impacts caused by its inadequate disposal, with the production of a biomolecule that can be applied in the development of new products of the food industry. This study is a pioneer in the investigation of the biotechnological potential of the whey obtained from the porungo cheese, which may contribute to advances in scientific studies in the area of bioprocesses and food engineering.

CONCLUSIONS

Results showed the promising use of porungo cheese whey to produce β-galactosidase enzyme, using minimally media supplementation. The two yeast strains were able to produce biomass and the target enzyme. *K. marxianus* CCT 4086 was more efficient in the production of β-galactosidase using porungo cheese whey as substrate when supplemented with yeast extract at pH of 7.0 and temperature of 30 °C. Porungo cheese whey has not been used in bioprocess before and this is one of the first research on its potential use in biotechnology.

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R.J.C carried out the most of the experiments (almost fullness), the validation and writed the original draft. S.G A designed, coordinated and supervised the study, carried out the project administration, the funding, review and editing the manuscript, figures and tables. L.B.S contributed quietly with the first experimental stage. A.V.C.M contributed with the last experimental stage and writing these results. M.A.Z.A and N.J.P.F contribute with the resources and carried out the review and editing of the manuscript.