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Cell permeabilization of *Kluyveromyces* and *Saccharomyces* species to obtain potential biocatalysts for lactose hydrolysis

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ABSTRACT. Yeast's beta-galactosidase is an intracellular enzyme, through which it is possible to determine in vivo its activity as a biocatalyst in the lactose hydrolysis. Permeabilization process was used for transforming the microorganisms cells into biocatalysts with an enhanced enzyme activity. The potential application of this enzyme technology in industrial process depends mainly on the enzyme activity. Beta-galactosidase enzyme that hydrolyzes lactose, for instance, is largely dependent on the reaction time and its stability under different physical conditions, such as pH, temperature and enzyme concentration. The objective of this study was to optimize the cellular permeabilization process of *Kluyveromyces marxianus* CCT 3172 and *Saccharomyces fragilis* CCT 7586 cultured in cheese whey for lactose hydrolysis. Box-Behnken design was carried out for cell permeabilization with three independent variables, ethanol concentration, permeabilization time and temperature. The best permeability conditions for *K. marxianus* CCT 3172 were 27% (v v^{-1}) ethanol, 3 min at 20ºC, with specific enzymatic activity of 0.98 U mg^{-1}. For *S. fragilis* CCT 7586, a specific enzymatic activity of 1.31 U mg^{-1} was achieved using 45% (v v^{-1}) of ethanol, 17 min. of reaction under 17ºC. Thus, it was concluded that cellular permeabilization with ethanol is an efficient process to determine beta-galactosidase activity.

Keywords: beta-galactosidase; cheese whey; lactose-hydrolysis; biocatalyst.

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Introduction

The use of yeasts as biocatalysts is a promising technology in the food processing, textile, and pharmaceutical industries, as well as in medicine (Trawczyńska, 2019). *Saccharomyces fragilis* and *Kluyveromyces marxianus* can efficiently use lactose as carbon source for beta–galactosidase production (Morioka, Colognesi, & Suguimoto, 2016). Beta–galactosidase is generally utilized to hydrolyze lactose in milk and derivatives in the food industry (Fonseca, Heinzle, Wittmann, & Gombert, 2008). Cheese whey, for instance, can also be used as a source of lactose without the need of any additional supplement serving as an alternative culture medium (Marcel & Passos, 2001).

Yeasts like *S. fragilis* and *K. marxianus* are considered GRAS (Generally Regarded As Safe) by the FDA (Food and Drug Administration) of the United States, qualifying them as safe for human consumption, with huge potential application in biotechnology (Schaffrath & Breuinig, 2000; Fukuhara, 2006). Beta-galactosidase (lactase, EC 3.2.1.23) hydrolyzes lactose into glucose and galactose, and can be used in milk products intended for people that are lactose-intolerant or sensitive (Vidya, Palaniswamy, & Gopalakrshnan, 2014; Carevic et al., 2015).

Intracellular beta–galactosidase as whole cell biocatalyst is an interesting alternative for reducing lactose in milk and dairy products. However, a major drawback in the use of whole cells is the poor cell wall permeability to lactose. Therefore, cellular permeabilization modifies the cell wall structure allowing the contact of enzyme and lactose (Fontes, Passos, Passos, & Fontes, 2008). Cell permeabilization create pores in the yeast cell wall which allows access to the enzyme in its natural state and the enzymatic activity can be evaluated *in situ* (Kumari, Panesar, Bera, & Singh, 2011; Kumari. Panesar, Bera, & Panesar, 2013). Thus, permeabilization is frequently applied for transforming cells of microorganisms into biocatalysts, enhancing their cellular activity (Presecki & Vasić-Racki, 2005; Yu et al., 2007).

In this study a Box–Behnken Design to determine the optimal conditions for cell permeabilization of *S. fragilis* CCT 7586 and *K. marxianus* CCT 3172 cultivated in cheese whey was investigated.

Material and methods

Microorganisms and culture conditions

Kluyveromyces marxianus CCT 3172 and *S. fragilis* CCT 7586 were obtained from Andre Tosello Tropical Research and Technology Foundation, Brazil, and kept at 4ºC in tubes containing PDA (Potato Dextrose Agar). The inoculum was maintained in malt extract (15 g L^{-1}) and incubated at 35ºC, 100 rpm for 24 hours. Optical density (O.D. 570nm) was adjusted to 0.6 which corresponds to approximately 1 x 106 cells mL^{-1}.

For culture conditions, the yeast strains were grown in 50 g L^{-1} cheese whey powder (Confepar®) solubilized in distilled water with 5% (v v^{-1}) inoculum. Cheese whey deproteinization was conducted by adding 85% of lactic acid at pH 4.6 and heated at 90ºC for 30 minutes. The precipitated protein was removed by filtration with Whatman nº 1 filter paper and the pH was adjusted to 5.0 with 0.5 M KOH solution. The pasteurization process was conducted at 65ºC for 30 minutes. Cultivation was performed at 100 rpm, 35ºC and 24 hours. The whole analysis was carried out in triplicate.

Experimental design for cell permeabilization

For cell permeabilization, a 5 mL aliquot of the culture was centrifuged at 3500 rpm (2640 x*g*) for 10 min and the biomass washed twice with distilled water. The biomass was resuspended in 0.1 M phosphate buffer (pH 6.8) and then ethanol was added as permeabilizing agent. The samples were incubated at different ethanol concentrations, time reaction and temperatures according to the experimental design as shown in Table 1 (*K. marxianus* CCT 3172) and Table 3 (*S. fragilis* CCT 7586). The enzymatic activity was determined via ONPG (o–nitrophenyl-β-D-galactopyranoside) colorimetric method (Inchaurrondo, Yauturno, & Voget, 1994).

For *K. marxianus* CCT 3172 the independent variables were X1: ethanol concentration; 27, 30 and 33 (% v v^{-1}), X2: time reaction; 3, 6 and 9 min.; and X3: temperature; 20, 25 and 30ºC.

For *S. fragilis* CCT 7586 the variables X1: ethanol concentration; 40, 45 and 50% v/v; X2: time reaction; 14, 17 and 20 min. and X3: temperature; 14, 17 and 20ºC. The dependent variable was the specific enzymatic activity of beta-galactosidase (U mg^{-1}). The statistical treatment was analyzed using response surface methodology by plotting the data in the program Statistic 6.0 (Statsoft Inc., Tulsa, USA). The level of statistical test was at 95% confidential interval ($p \leqslant 0.05$).

Beta-galactosidase activity

The specific enzymatic activity of the permeabilized yeast cells was determined using the o–nitrophenyl–β–D-galactopyranoside substrate (ONPG), following the methodology described by Inchaurrondo et al. (1994). In a test tube containing 2 mL of 1.25 mM ONPG, prepared in 50 mM KH_2PO_4 phosphate buffer with 0.1 mM $MnCl_2.4H_2O$ at pH 6.6, 50 µL of the enzyme solution were added. The solution was maintained at 37°C for 5 min. in a heating bath (Fisatom® 550, Fisatom *Equipamentos Cientificos Ltda*, São Paulo, Brazil), and then quenched by adding 0.5 ml of 1M Na_2CO_3. The samples absorbance was determined at 420 nm using a spectrophotometer (Femto® 600 Plus, Femto *Indústria e Comércio de Instrumentos*, São Paulo, Brazil). The enzymatic activity was calculated from a calibration curve of o–nitrophenol. In order to verify the effect of cell concentration on enzymatic activity, the unit of activity was expressed per milligram of cell mass, i.e., U mg^{-1}. Thus, the specific enzymatic activity (U mg^{-1}) was defined as 1 µmol o–nitrophenol produced per minute of reaction per milligram of dry biomass under the tested conditions. The whole analysis was done in triplicate.

Results

The optimization of the cell permeabilization conditions was carried out to find the optimal values for the independent variables, providing the maximum beta–galactosidase activity (U mg^{-1}). A preliminary study was applied to narrow the range of the independent variables tested. In Table 1, it is observed that the specific enzymatic activity ranged from 0.79 to 0.98 U mg^{-1}. Since yeast beta-galactosidase is intracellular and that permeabilized cells are the source of enzymes that remain 100% naturally immobilized, it was observed that the best experimental results were obtained in 27% v v^{-1} ethanol for 3 min. at 20ºC as well as in 30% v v^{-1} ethanol for 6 min. at 25ºC, that obtained a final concentration of 0.98 U mg^{-1}.

Table 1. Box–Benhken experimental design of the cellular permeabilization of *Kluyveromyces marxianus* CCT 3172 in the enzymatic activity (U mg^{-1}).

Run	Coded and (decoded) variables			Enzymatic activity (U mg^{-1})
	X1 Ethanol (% v v-1)	X2 Time reaction (min.)	X3 Temperature (°C)	
1	-1 (27)	-1 (3)	-1 (20)	0.98
2	-1 (27)	0 (6)	1 (30)	0.94
3	-1 (27)	1 (9)	0 (25)	0.84
4	0 (30)	-1 (3)	1 (30)	0.90
5	0 (30)	0 (6)	0 (25)	0.98
6	0 (30)	1 (9)	-1 (20)	0.79
7	1 (33)	-1 (3)	0 (25)	0.94
8	1 (33)	0 (6)	-1 (20)	0.86
9	1 (33)	1 (9)	1 (30)	0.79

The analysis of variance (ANOVA) demonstrates that the regression analysis is significant and shows good coefficient correlation (R2 = 0.91596) as verified in Table 2. This further demonstrates that 92% of validity was achieved, indicating the model exerted an adequate prediction on the enzyme activity.

Table 2. Analysis of variance (ANOVA) of the regression parameters for the Box–Behnken Design used to determine the ethanol, time and temperature, on the enzymatic activity of permeabilized cells of *Kluyveromyces marxianus* CCT 3172.

	SS	df	MS	F	p-value
Ethanol (%) L+Q	0.004822	2	0.002411	1.284024	0.437824
Time reaction (min.) L+Q	0.032356	2	0.016178	8.615385	0.104000
Temperature (ºC) L+Q	0.003756	2	0.001878	1.000000	0.500000
Error	0.003756	2	0.001878		
Total SS	0.044689	8			

Seq. SS, sequential sums of squares; df, the degrees of freedom; MS, adjusted mean square; F, F calculated; p, p-value at 0.05%.

Figure 1(A) shows the effect between time reaction and ethanol concentration, where the highest enzymatic activity was obtained between 3 and 6 min., with 27% ethanol. In Figure 1(B), the effect between ethanol concentration and temperature showed that the highest enzymatic activity occurred in the range from 20 to 30ºC, with 27% of ethanol. In Figure 1(C), the effect between time reaction and temperature, the highest enzymatic activity occurred at 4 min. and 25ºC. The yeast cells permeabilization has been conducted and the beta-galactosidase activity has been measured at the optimal conditions. The values close to below the central point of the response surface methodology indicated that maximum enzymatic activity (0.98 U mg^{-1}), occurred under the following conditions: 30% ethanol, 6 min. of reaction time at 25ºC.

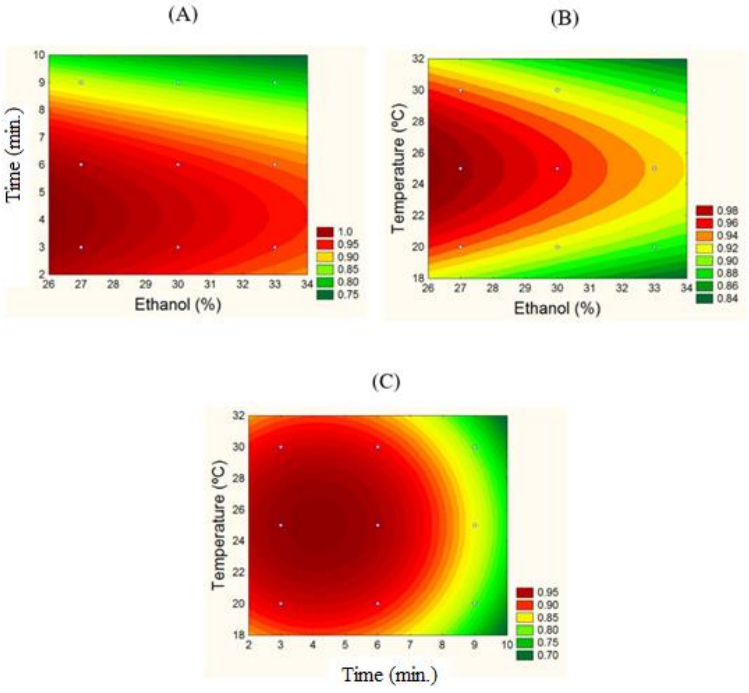

Figure 1. Enzymatic activity of beta–galactosidase obtained from permeabilized cells of *Kluyveromyces marxianus* CCT 3172.

The results obtained from *S. fragilis* CCT 7586 for the different experimental sets are presented in Table 3. It can be observed that the enzymatic activity ranged from 0.60 to 1.31 U mg^{-1}. The permeabilization results for *S. fragilis* CCT 7586 show that maximum enzyme activity of 1.31 U mg^{-1} was obtained under optimum conditions of 45% ethanol for 17 min. and at 17ºC.

Table 3. Box–Benhken experimental design of the cellular permeabilization of *Saccharomyces fragilis* CCT 7586 in the enzymatic activity (U mg^{-1}).

Runs	Coded and decoded variables			Enzymatic activity (U mg^{-1})
	X1 Ethanol (% v v^{-1})	X2 Time reaction (min.)	X3 Temperature (°C)	
1	-1 (40)	-1 (14)	-1 (14)	1.03
2	-1 (40)	0 (17)	1 (20)	0.97
3	-1 (40)	1 (20)	0 (17)	1.12
4	0 (45)	-1 (14)	1 (20)	1.02
5	0 (45)	0 (17)	0 (17)	1.31
6	0 (45)	1 (20)	-1 (14)	0.96
7	1 (50)	-1 (14)	0 (17)	1.03
8	1 (50)	0 (17)	-1 (14)	1.26
9	1 (50)	1 (20)	1 (20)	0.60

The analysis of variance (ANOVA) demonstrates that the regression analysis is significant and presents good determination coefficient (R2 = 0.86458), Table 4. Approximately 86% of validity was achieved, indicating that the model exerted an adequate prediction on the enzyme activity. The maximum beta-galactosidase activity 1.31 U mg^{-1} was achieved in the following condition, ethanol 45% v v^{-1}, 17ºC and 17 min. (Table 1).

Table 4. Analysis of variance (ANOVA) of the regression parameters for the Box–Behnken Design used to determine the ethanol, time and temperature, on the enzymatic activity of permeabilized cells of *Saccharomyces fragilis* CCT 7586.

	SS	df	MS	F	p-value
Ethanol (%) L+Q	0.026867	2	0.013433	0.596154	0.626506
Time reaction (min.) L+Q	0.123467	2	0.061733	2.739645	0.267405
Temperature (ºC) L+Q	0.137400	2	0.068700	3.048817	0.246986
Error	0.045067	2	0.022533		
Total SS	0.332800	8			

Seq. SS, sequential sums of squares; df, the degrees of freedom; MS, adjusted mean square; F, F calculated; p, p-value at 0.05%.

The statistical model indicates that for maximum beta–galactosidase activity synthesized by *S. fragilis* CCT 7586, cell permeabilization should be performed using minimum values for ethanol concentration (40% v v^{-1}), time reaction (14 min.) and temperature (14°C).

Figure 2(A) shows the effect between time reaction and ethanol concentration, where the highest enzymatic activity was obtained between 15 and 18 min., with 45% of ethanol. In Figure 2(B) it is possible to visualize that the highest enzymatic activity occurred in the range from 14 to 17ºC, with 45% of ethanol. In Figure 2 (C), which shows the effect between temperature and time reaction, the highest enzymatic activity occurred in the range from 14 to 17ºC at 17 minutes. The values close to the central point of the response surface methodology indicated that maximum enzymatic activity (1.31 U mg^{-1}), occurred under the following conditions: 45% ethanol, 17 min. of reaction time at 17ºC.

The efficiency of *S. fragilis* CCT 7586 and *K. marxianus* CCT 3172 to produce beta-galactosidase was determined by permeabilizing the cells with organic solvent such as ethanol. Different parameters which influence the cell permeabilization such as, ethanol concentration, time reaction and temperature were determined for *S. fragilis* CCT 7586 and *K. marxianus* CCT 3172 and the optimum beta-galactosidase activity was found to be 1.31 U mg^{-1} and 0.98 U mg^{-1}, respectively. Strains of *Saccharomyces* and *Kluyveromyces* produced by low-cost biotechnology methods using cheese whey as substrates, have become important sources from which permeabilized yeast cells function as potencial biocatalysts for lactose hydrolysis. These sources offer the advantages of low cost and high volume of yeast biomass, which translates into high enzymatic potential. The differences in the enzymatic activities observed between the two species are probably due to the intrinsic characteristics of each one. There are some factors that influence the enzyme activity of yeast beta-galactosidase. Besides enzyme source, substrate concentration and the composition of the reaction medium are key factors that affect the enzyme activity (Gaur, Pant, Jain, & Khare, 2006). Lactose hydrolysis is a key industrial process, especially in the food industry, that generates galactose and glucose as reaction products (Basso & Serban, 2019; Andrade, Timmers, Renard, Volpato,

& Souza, 2020). However, some authors have highlighted that this reaction can be hindered by product inhibition, such as glucose and galactose, which reduces the reaction rates of lactose hydrolysis. In some cases, this phenomenon was even reported to completely halt the reaction, preventing it from being fully carried out (Mateo et al., 2004; Zhang et al., 2018). Temperature and pH are the main influencing factors in the activity of lactose hydrolysis. Yeasts of the species *K. marxianus*, *K. lactis* and *S. fragilis* are the most used to produce beta-galactosidase in cheese whey, due to the growth pH being in the neutrality range (6.0 - 7.0) and milder temperatures (37ºC) (Diniz, Rodrigues, Fietto, Passos, & Silveira, 2014).

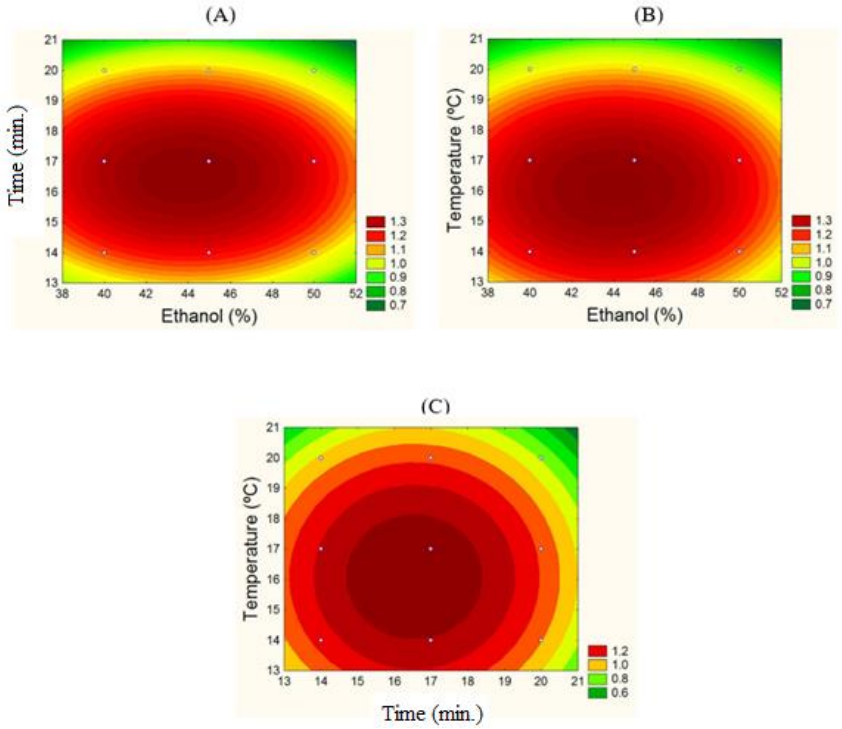

Figure 2. Activity of beta–galactosidase obtained after the permeabilization of *Saccharomyces fragilis* CCT 7586.

Discussion

The choice of the appropriate microorganism to produce the enzyme must take into account some factors such as cultivation in a simple and economical substrate; nutritional requirements; ease of enzyme recovery from the medium, concentration and purification; approval by health authorities, for food applications and high multiplication rates. The rapid substrate hydrolysis is what provides the yeasts *K. marxianus* and *S. fragilis* rapid growth rate and formation of biomass, ethanol and enzyme. During permeabilization, the cell retains its intracellular compounds within it, but allows small molecules and solutes, including lactose, to pass through the cell membrane. Hence, the enzyme remains inside the cell (Kaur, Panesar, Bera, & Kumar, 2009). Cell permeabilization is simple, fast and allows access to the enzyme in its natural state, making them potential biocatalysts (Kumari, et al., 2011). Yeast cell permeabilization with ethanol was used to obtain biocatalysts with an enhanced enzyme activity. In this study, the response surface methodology (RSM) was used to evaluate the influence of ethanol concentration, time reaction and temperature in the cell permeabilization with high enzyme activity. A preliminary study was applied to narrow the range of the independent variables tested. Since yeast beta–galactosidase is intracellular and that permeabilized cells are the source of enzymes that remain 100 % naturally immobilized, 0.98 U mg^{-1} were obtained in 27% (v v^{-1}) ethanol for 3 min. at 20ºC. According to Faria et al. (2013) cell permeabilization was improved by an increase in ethanol concentration and simultaneous decreases in the incubation temperature and retention time. Arellano, Quicaña, Flores, and Obeso (2012) focused on making the membrane permeabilization of *Kluyveromyces* sp. to determine the activity of the beta-galactosidase in wicth 30% ethanol for 5 min. and at 20ºC showed the highest enzymatic activity, with a value of 2.9642 µmol of glucose $minutes^{-1}$.

The maximum beta-galactosidase activity for *S. fragilis* CCT 7586 was 1.31 U mg^{-1}. Similar conditions were observed by Kumari et al. (2011), which the optimum operating conditions for permeabilization process to achieve maximum beta-galactosidase activity (1.68 U mg^{-1} DW) obtained by RSM were 1:1 ratio of toluene

(25%, v v^{-1}) and ethanol (50%, v v^{-1}), 23°C temperature and retention time of 12 minutes. In another study, Kumari et al. (2011) investigated the enzyme activity of *Kluyveromyces* sp. by RSM and obtained the following conditions: 1:1 ratio of toluene (25%, v v^{-1}) and ethanol (50%, v v^{-1}), 25°C temperature and treatment time of 12 min. which displayed an enzyme activity of 1.71 U mg^{-1}.

The maximum beta–galactosidase activity of 1.220 U g^{-1} dry weight was obtained using permeabilized cells under the following optimized conditions, anionic detergent N–lauroyl sarcosine (N–LS) concentration of 1.5% w v^{-1}, solvent (water) volume of 1 mL (or 1.0 g wet weight mL^{-1} of N–LS solution), temperature of 25°C and incubation time of 20 min, (Yadav et al., 2015). Confirming that growing conditions can influence the microorganism metabolic characteristics, Mahadevaiah, Basavaiah, Parida, and Batra (2020) showed that the efficiency of *Lactobacillus fermentum* to produce beta-galactosidase of 4.254 U mL^{-1} was determined by permeabilizing the cells with solvents such as sodium dodecyl sulfate (SDS) and chloroform. Different parameters contributing beta-galactosidase production including reaction time, temperature, pH, carbohydrates, and substrate concentration on *L. fermentum* were studied and optimum enzyme activity was found to be 6.223,13 U mL^{-1}. It was observed that different experimental parameters for pH (7.0), temperature (35°C), and carbohydrates (galactose) were statistically significant ($p < 0.05$).

Therefore, using whole cells as lactase biocatalysts can be seen as a natural enzyme immobilization method. Compared to isolated enzymes, the use of whole cells may reduce costs and resources otherwise spent on enzyme purification or transport. Since the cells will potentially be present in the final product, only organisms safe for human consumption will be useful for this approach, even though they might be unavailable upon consumption. Another essential consideration for whole-cell biocatalyst production is that the cells should only be treated with food-grade agents, such as ethanol, which might be required in the various stages of the process of obtaining permeabilized cells.

Conclusion

In this study, the use of response surface methodology proved to be a useful tool to optimize the best conditions for cells permeabilization, obtaining live yeasts as biocatalysts in lactose hydrolysis. The results showed that cell permeability was efficient for the two yeasts, and the optimal condition for *K. marxianus* CCT 3172 was 27% ethanol, 3 min. at 20ºC and enzymatic activity of 0.98 U mg^{-1}, while for *S. fragilis* CCT 7586, the optimized conditions were 45% v v^{-1} ethanol, for 17 min. of reaction at 17ºC with maximum enzyme activity of 1.31 U mg^{-1}.

Permeabilization is one of the effective tools, used to increase the accessibility of intracellular enzymes, so transforming cells of microorganisms into biocatalysts with an enhanced activity. Therefore, such biocatalysts can find applications in the lactose hydrolysis from dairy products.

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