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Permeabilização de células de *Saccharomyces fragilis* IZ 275 cultivadas em soro de queijo utilizando diferentes solventes orgânicos

Saccharomyces fragilis IZ 275 in cheese whey cell permeabilization using different organic solventes

Saccharomyces fragilis IZ 275 en permeabilización de células de suero de queso usando diferentes solventes orgánicos

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Resumo

O objetivo deste estudo foi verificar a eficácia de diferentes solventes orgânicos na permeabilização de células de *Saccharomyces fragilis* IZ 275, utilizando o Delineamento Composto Central Rotacional (DCCR) 2^3 e a Metodologia de Superfície de Resposta (MSR). Além disso, avaliar a eficácia do processo de permeabilização, monitorando a hidrólise da lactose e obtendo imagens de células não permeabilizadas e permeabilizadas por Microscopia Eletrônica de Varredura (MEV). A levedura *S. fragilis* IZ 275 foi cultivada em meio de fermentação composto por soro de queijo e nas células permeabilizadas foi determinada a atividade da β-galactosidase. A MSR foi utilizada por ser uma ferramenta eficiente para otimizar o processo de permeabilização, bem como identificar o solvente orgânico mais eficaz para esse processo. Nossos resultados mostraram que a concentração e o tipo de solvente orgânico, bem como a temperatura e o tempo de permeabilização, influenciaram o processo de permeabilização das células. Considerando os resultados experimentais, as melhores condições ao usar clorofórmio são 4 % de concentração a 25 ºC por 20 min com 81,03 % de hidrólise de lactose. Neste estudo, verificamos que o uso de etanol para permeabilização celular leva à obtenção da enzima β-galactosidase, um processo que pode ser usado em larga escala pela indústria de alimentos, sendo uma maneira mais barata e ambientalmente segura.

Palavras-chave: β-galactosidase; Rompimento celular; Microscopia electrônica de varredura; Processo biotecnológico; Solvente verde.

Abstract

The aim of this study was to verify the efficacy of different organic solvents, in the permeabilization of *Saccharomyces fragilis* IZ 275, by using a Central Composite Rotational Design (CCRD) 2^3 and Response Surface Methodology (RSM). Furthermore, we aimed to evaluate the effectiveness of the permeabilization process by monitoring lactose hydrolysis and obtaining images of non-permeabilized and permeabilized cells by Scanning Electron Microscopy (SEM). The yeast *S. fragilis* IZ 275 was grown in a fermentation medium composed of cheese whey, and the permeabilized cells was estimated by β-galactosidase activity. The response surface methodology was used as it is an efficient tool to optimize the permeabilization process as well as to identify the organic solvent, which was most effective for this process. Our results show that the concentration and type of organic solvent, as well as permeabilization temperature and time influence the cells permeabilization process.. Considering the experimental results, the best conditions when using chloroform are a concentration of 4 % at 25 ºC during 20 min with 81.03 % lactose hydrolysis. In this study,

we found that the use of ethanol for cellular permeabilization lead to obtaining β-galactosidase enzyme, a process which can be used in a large scale by the food industry, being a cheaper and more environmentally safe way of obtaining this enzyme.

Keywords: β-galactosidase; Disruption cell yeast; Scanning electron microscopy; Biotechnological process; Green solvent.

Resumen

El objetivo de este estudio fue verificar la eficacia de diferentes solventes orgánicos, en la permeabilización de *Saccharomyces fragilis* IZ 275, mediante el uso de un diseño giratorio compuesto central (CCRD) 2^3 y una Metodología de Superficie de Respuesta (RSM). Además, nuestro objetivo fue evaluar la efectividad del proceso de permeabilización mediante el monitoreo de la hidrólisis de lactosa y la obtención de imágenes de células no permeabilizadas y permeabilizadas mediante Microscopía Electrónica de Barrido (SEM). La levadura *S. fragilis* IZ 275 se cultivó en un medio de fermentación compuesto por suero de queso, y las células permeabilizadas se estimaron por la actividad de β-galactosidasa. Se utilizó la SEM ya que es una herramienta eficiente para optimizar el proceso de permeabilización, así como para identificar el solvente orgánico que fue más efectivo para este proceso. Nuestros resultados muestran que la concentración y el tipo de solvente orgánico, así como la temperatura y el tiempo de permeabilización influyen en el proceso de permeabilización de las células. Considerando los resultados experimentales, las mejores condiciones cuando se usa cloroformo son una concentración de 4 % a 25 ºC durante 20 min con 81.03 % de hidrólisis de lactosa. En este estudio, encontramos que el uso de etanol para la permeabilización celular conduce a la obtención de la enzima β-galactosidasa, un proceso que puede ser utilizado a gran escala por la industria alimentaria, siendo una forma más barata y más segura para el medio ambiente de obtener esta enzima.

Palabras clave: β-galactosidasa; Interrupción de levadura celular; Microscopía electrónica de barrido; Proceso biotecnológico; Solvente verde.

1. Introduction

Cheese whey can be considered as a potential resource for the production of various value-added products, partly due to its lactose, proteins, lipids and vitamins content (Nath et al., 2016). Alternatively, cheese whey can be used in the food industry for fermentation process which use lactose-consuming microorganisms, as it can be converted to glucose and

galactose (Panesar & Kennedy, 2012; Guimarães et al., 2010; Panesar et al., 2013). The process of lactose hydrolysis is catalyzed by β-galactosidase (Anisha, 2017).

β-D-galactosidase or lactase (EC 3.2.1.23; β-D-galactoside galactohydrolase) is an enzyme responsible for the hydrolyzation of disaccharide lactose into galactose and glucose monosaccharide (Panesar et al., 2006). The reduction of lactose levels, the sugar present in milk and dairy products, is important to people with lactose intolerance who cannot properly digest lactose, resulting in problems such as bloating, nausea, abdominal cramping, and diarrhea. Therefore, the lactase has interesting applications in the food dairy, and fermentation industries (Mlichova & Rosenberg, 2006; Hussain, 2010; Oak & Jha, 2018).

Yeasts can be used in the food industry to produce the β-galactosidase enzyme, due to its GRAS (generally regarded as safe) status (Panesar et al., 2013; Hussain, 2010). However, yeast β-galactosidase is an intracellular enzyme making the extraction process complex and expensive which presents difficulties in large scale applications (Panesar et al., 2007). Among the mechanical and enzymatic processes described in the literature, cellular permeabilization using organic solvents can be an alternative for yeast β-galactosidase production on an industrial scale (Prasad et al., 2013).

Cellular permeabilization using organic solvents (acetone, toluene, chloroform, ethanol, etc.) is rapid and not-destructive besides being a traditional method, which offers a low cost alternative and avoid the problem of enzyme extraction and purification (Panesar et al., 2007; Kaur et al., 2009). The biotechnological process involves the modification of the cell structure, causing disorganizations in its pores and therefore allowing molecules, such as substrates or products, freely cross the cellular membrane (Viana et al., 2018). The efficacy of permeabilization by organic solvents depends on incubation time, incubation temperature, and solvent type and concentration (Flores et al., 1994; Lee et al., 2004; Geciova et al., 2002). Kaur et al. (2009) using cetyltrimethyl ammonium bromide (CTAB) obtained a rate of lactose hydrolysis of 90.5 % with 200 mg DW after 90 min at 40 °C, while Panesar et al. (2007) concluded that optimal conditions for the permeabilization of yeast cells were a concentration of 50 % ethanol, at 25 °C and 15 min of treatment time. Flores et al. (1994) studied the permeabilization of *Kluyveromyces lactis* cells in relation to β-galactosidase activity using chloroform, toluene and ethanol. However, evaluation of the optimum conditions for cell permeabilization using different organic solvents, while assessing the rate of lactose hydrolysis by β-galactosidase and considering the concept of green extraction has not yet been reported (Chemat et al., 2012).

The aim of this study was to evaluate the efficacy of different organic solvents, using a Central Composite Rotational Design (CCRD) 2^3 and Response Surface Methodology (RSM), for the permeabilization of *Saccharomyces fragilis* IZ 275 cells, cultivated in supplemented cheese whey,. Moreover, we aimed to evaluate the effectiveness of the permeabilization process by monitoring lactose hydrolysis and obtaining images of non-permeabilized and permeabilized cells by Scanning Electron Microscopy (SEM).

2. Material and Methods

The chemical analysis were carried out in the Food Science laboratory and SEM analyzes were performed at the Electron Microscopy and Microanalysis laboratory, at the Universidade Estadual de Londrina, Londrina, Parana. All analysis are according describe in Pereira et al. (2018).

2.1 Microorganism identification

Saccharomyces fragilis IZ 275 cells were collected in the Colecao de Culturas Tropical (WDCM 885 number), maintained in tubes containing PDA (Potato Dextrose Agar, Acumedia) and stored at 4 °C. The yeast was reactivated on medium containing (w/v) malt extract (2 %) yeast extract (0.5 %) and incubated at 30 °C for 48 h in an orbital shaker (Tecnal®, TE-420). The inoculum was performed by using Neubauer chamber at a count of 1 x 10^7 cells mL^{-1} and was autoclaved at 121 °C for 15 min. An inoculum concentration of 10 % v/v in relation to the culture medium was used.

2.2 Production of yeast biomass in supplement cheese whey

The yeast *S. fragilis* IZ 275 was grown in a fermentation medium, namely supplemented cheese whey, containing sucrose (14 g L^{-1}), whey cheese (17.7 g L^{-1}), glucose (10 g L^{-1}), lactose (10 g L^{-1}), yeast extract (5.14 g L^{-1}), peptone (8.85 g L^{-1}), $MgSO_4$ (7 g L^{-1}) and K_2HPO_4 (5 g L^{-1}). The fermentation mediums were autoclaved at 121 °C for 15 min and the medium conditions were stabilized at 35 °C, pH 6 and 20 % concentration of inoculum.

2.3 Effect of different organic solvents on the permeabilization the *Saccharomyces fragilis* IZ 275 cells

The permeabilization of the *Saccharomyces fragilis* IZ 275 cells was carried out using three different organic solvents. To evaluate the effect of each organic solvents on the permeabilization cells process was employed a Rotational Central Composite Design (RCCD) with three variables (organic solvent concentration, permeabilization temperature and permeabilization time) and replicates at the central point (Table 1). The coded independent variables (x_1, x_2, x_3) and uncoded variables (X_1 = % of organic solvent, X_2 = permeabilization temperature, °C and X_3 = permeabilization time, min) are shown in Table 1 with their variation levels. The RCCD was conducted in 2 blocks (Table 2 and Table 3), and exploratory modeling block 1 was conducted with 11 random assays (8 factorial points and 3 central points). Modeling was conducted in block 2 with 9 random assays (6 axial points and 3 central points) for optimization, with a total of 20 assays in the two blocks.

Table 1. Independent variables and levels of variation in RCCD.

	Levels				
Independent variables	-1.80	-1.00	0	+1.00	+1.80
	Ethanol or Acetone				
Organic solvent (%) X_1	11.90	20	30	40	48.10
Permeabilization Temperature (ºC) X_2	20.95	25	30	35	39.05
Permeabilization time (min) X_3	3.82	20	40	60	76.18
	Chloroform				
Organic solvent (%) X_1	1.19	2.00	3.00	4.00	4.81
Permeabilization Temperature (ºC) X_2	20.95	25	30	35	39.05
Permeabilization time (min) X_3	3.82	20	40	60	76.18

Source: Software Statistic 7.0.

2.4 Permeabilization of Biomass

Cell permeabilization was carried out following the method Panesar et al. (2007) with modifications. Different concentrations of organic solvents (Table 1) were added to the yeast biomass (cells non permeabilized). The contents were homogenized on a vortex and the cells were permeabilized in each specific solvent, temperature and time (Table 1). After treatment with different organic solvents (Tables 2 and 3), and to stop the permeabilization process, the

cells were taken in to the ice bath and separated by centrifugation at 6000 g, 10 min to 5 °C and then, were washed twice with phosphate buffer to remove excess solvent.

The permeabilized cells (biomass) were used to measure the β-galactosidase activity in relation the percentage of lactose hydrolysis. The permeabilized cells were washed with phosphate buffer to remove the solvent excess and were used to measure the enzyme activity.

The permeabilized cells or biomass were used to evaluate the followings response functions: Y_1, Y_2 and Y_3 = percentage (%) of lactose hydrolysis using ethanol, acetone and chloroform, respectively. The model equation was as follows:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{23} x_2 x_3 + e$$

Where Y = Y_1, Y_2 and Y_3 (response function), x_1, x_2 and x_3 (coded variables), β (estimated coefficients for each term of the response surface model) and e = pure error. The response functions (Y_1, Y_2 and Y_3) were used to perform regression analysis and analysis of variance (ANOVA) for the regression. The equation model was fitted to the experimental data to create the proposed model. Response surface graphs were generated for each response function (Y_1, Y_2 and Y_3). All the executed analysis, and response surfaces were performed with STATISTICA 7.0 software (StatSoft Inc. 2007).

2.5 Hydrolyze of lactose using permeabilized *Saccharomyces fragilis* IZ 275 cells

Five milliliters of permeabilized biomass was added in 50 mL of whole milk and placed in a water bath at 37 °C for 60 min. The samples were then incubated in a boiling water bath for 5 min to stop the hydrolysis reaction.

Glucose concentration was determined by the glucose oxidase method using Glucose-Laborclin Bioliquid kit. The absorbance was measured in a spectrophotometer at 505 nm. The percentage (%) of lactose hydrolysis was determined as the difference between initial lactose concentration and the released glucose concentration (final).

2.6 Scanning Electron Microscopy (SEM)

The images of non-permeabilized and permeabilized cells were acquired using Scanning Electron Microscopy (FEI Quanta 200). All chemicals used in SEM were obtained from ESM — Electron Microscopy Science. Samples of each type of cells were fixed in a solution of 3 % glutaraldehyde and 2 % of paraformaldehyde (in 0.1M sodium cacodylate buffer, pH 7.2) for 12 h at 25 ºC.

Afterwards, the samples were washed 3 times with the same buffer, post-fixed in a 1 % osmium tetroxide solution for 12 h at room temperature, and washed again three times. Samples were then dehydrated in a series of solutions with increasing alcohol concentrations (from 70 % to 100 %), and dried in a CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). The samples were assembled on aluminum stubs with carbon tape and covered with gold (Sputter coater, BAL-TEC SCD 050).

3. Results and Discussion

3.1 Effect of different organic solvents on the permeabilization the *Saccharomyces fragilis* IZ 275 cells and lactose hydrolysis

From the exploratory model of the first RCCD block (Tables 2 and 3, assays 1-11), the ANOVA and the regression analysis showed that, the effects of the variables, X_1 (organic solvent concentration, %), X_2 (permeabilization temperature, °C), and X_3 (permeabilization time, min) and all their interactions (X_1X_2, X_1X_3 and X_2X_3) were significant**.** Therefore, to improve the investigation of the variables as well as the process optimization, assays were performed in block 2, which contained 6 axial points and 3 central points (Tables 2 and 3, assays 12-20). By using a regression analysis of the response function $Y_{1,2,\text{and } 3}$, independent variables X_1, X_2 and X_3 showed significant linear and quadratic effects. Moreover, excepting the interaction between organic solvent concentration and permeabilization time for acetone and chloroform, all others were significant. Considering the organic solvents ethanol and acetone, it was observed (Table 2) that lactose hydrolysis (%) was higher in assay 8 (Y_1 = 77 %) and assay 15 (Y_2 = 81.48%), respectively.

Table 2. RDCC for *Saccharomyces fragilis* IZ 275 cells permeabilization and response function Y_1 and Y_2 using ethanol and acetone organic solvents.

Assays	Independent variables coded and uncoded			Function Response	
	$X_1(x_1)$	$X_2(x_2)$	$X_3(x_3)$	Y_1	Y_2
1	(-1) 20	(-1) 25	(-1) 20	41.96	72.55
2	(1) 40	(-1) 25	(-1) 20	64.27	76.92
3	(-1) 20	(1) 35	(-1) 20	69.32	75.52
4	(1) 40	(1) 35	(-1) 20	70.26	73.97
5	(-1) 20	(-1) 25	(1) 60	49.40	67.32
6	(1) 40	(-1) 25	(1) 60	76.15	70.07
7	(-1) 20	(1) 35	(1) 60	74.36	72.11
8	(1) 40	(1) 35	(1) 60	77.00	75.32
9	(0) 30	(0) 30	(0) 40	73.93	73.34
10	(0) 30	(0) 30	(0) 40	73.42	72.89
11	(0) 30	(0) 30	(0) 40	74.20	72.76
12	(-1.80) 11.9	(0) 30	(0) 40	48.87	54.44
13	(1.80) 48.10	(0) 30	(0) 40	64.33	63.27
14	(0) 30	(-1.80) 20.95	(0) 40	67.17	66.15
15	(0) 30	(1.80) 39.05	(0) 40	63.54	81.48
16	(0) 30	(0) 30	(-1.80) 3.82	67.70	66.76
17	(0) 30	(0) 30	(1.80) 76.18	60.97	71.54
18	(0) 30	(0) 30	(0) 40	74.07	71.96
19	(0) 30	(0) 30	(0) 40	73.36	72.71
20	(0) 30	(0) 30	(0) 40	73.79	72.88

X_1(concentration of solvent, %); X_2 (temperature of permeabilization, °C); X_3 (time of permeabilization, min) and Y_1 (% of lactose hydrolysis by β-galactosidase in presence of ethanol); Y_2 (% of lactose hydrolysis by β-galactosidase in the presence of acetone).
Source: Software Statistic 7.0.

The model proposed for ethanol and acetone can be described as follows:

Ethanol

$$Y_1 = 73.83 + 11.08x_1 - 9.78x_1^2 + 7.23x_2 - 4.43x_2^2 + 2.60x_3 - 5.06x_3^2 - 11.37x_1x_2 + 1.54\,x_1x_3 - 1.88x_2x_3$$

Acetone

$$Y_2 = 72.83 + 2.65x_1 - 6.05x_1^2 + 5.20x_2 + 2.17x_2^2 - 0.76x_3 - 0.68x_3^2 - 1.37x_1x_2 + 2.51x_2x_3$$

The model showed a significant lack of fit (at 95 %) and approximately 80 % (R^2) of the experimental data was properly adjusted to the model. Afterward, residual evaluation (observed vs. predicted), a distinct behavior of the normal distribution and heteroscedastic characteristics was not observed. In addition, the quadratic mean values of the pure error for the models (MSpe) had very low values (ethanol = 0.1423 and acetone = 0.1775). As such, the F value for the fit of the model to the responses can be overestimated with the underestimation of the MSpe value, resulting in the observed lack of fit (Ballus et al., 2011).

According to Bruns et al. (2010) and Silveira et al. (2016) when there is a good agreement between the predicted and observed values, it is possible to decide to maintain the proposed model.

Table 3. RDCC for *Saccharomyces fragilis* IZ 275 cells permeabilization and response function Y_3 using chloroform organic solvent.

Assays	Independent variables coded and uncoded		Function Response	
	$X_1(x_1)$	$X_2(x_2)$	$X_3(x_3)$	Y_1
1	(-1) 2.0	(-1) 25	(-1) 20	77.18
2	(1) 4.0	(-1) 25	(-1) 20	81.03
3	(-1) 2.0	(1)35	(-1) 20	76.41
4	(1) 4.0	(1) 35	(1) 60	74.53
5	(-1) 2.0	(-1) 25	(1) 60	64.02
6	(1) 4.0	(-1) 25	(1) 60	70.77
7	(-1) 2.0	(1) 35	(1) 60	78.03
8	(1) 4.0	(1) 35	(1) 60	75.47
9	(0) 3.0	(0) 30	(0) 40	73.08
10	(0) 3.0	(0) 30	(0) 40	73.38
11	(0) 3.0	(0) 30	(0) 40	72.01
12	(-1.80) 1.19	(0) 30	(0) 40	45.32
13	(1.80) 4.81	(0) 30	(0) 40	63.45
14	(0) 3.0	(-1.80) 20.95	(0) 40	49.77
15	(0) 3.0	(1.80) 39.05	(0) 40	47.79
16	(0) 3.0	(0) 30	(-1.80) 3.82	68.14
17	(0) 3.0	(0) 30	(1.80) 76.18	64.42
18	(0) 3.0	(0) 30	(0) 40	71.76
19	(0) 3.0	(0) 30	(0) 40	71.86
20	(0) 3.0	(0) 30	(0) 40	70.88

X_1(concentration of solvent, %); X_2 (temperature of permeabilization, °C); X_3 (time of permeabilization, min) and Y_3 (% of lactose hydrolysis by β-galactosidase using chloroform).
Source: Software Statistic 7.0.

When using the organic solvent chloroform (experimental data), it was observed (Table 3) that lactose hydrolysis (%) was the highest in assay 2 (Y_3 = 81.03 %) in others words,4 % of solvent at 25 °C of temperature during 20 min. The model proposed to chloroform can be described as follows:

$$Y_2 = 72.41 + 5.36x_1 - 5.48x_1^2 + 1.08x_2 - 8.90x_2^2 - 3.79x_3 + 1.79x_3^2 - 3.76x_1x_2 + 6.50x_2x_3$$

Similarly, as for ethanol and acetone, the model showed a significant lack of fit (at 95 %) and approximately 80 % (R^2) of the experimental data was properly adjusted to the model. The proposed model led to very high residues (observed vs predicted); therefore, it was not considered.

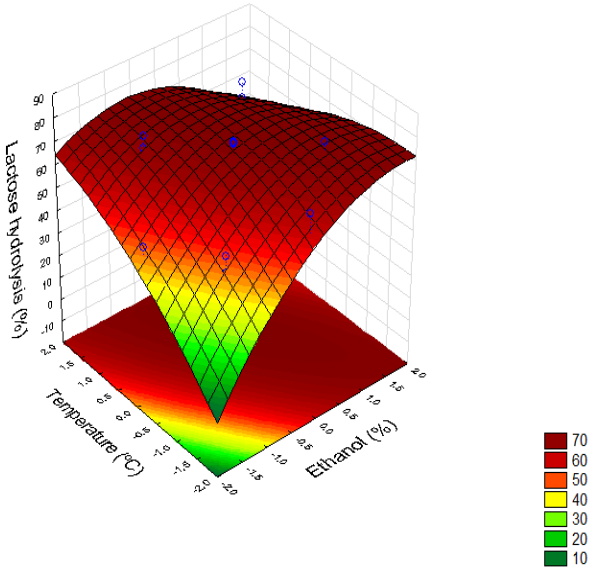
It was also observed that there was an extensive difference between the blocks, probably due to the extreme conditions provided by the axial points that prevented the permeabilization and consequently, the increase in the hydrolysis percentage, either by low solvent concentration (assay 12), insufficiently short time for the solvent action (assay 14) or excessively long time (assay 15), which may have led to cell disruption and extravasation of the enzyme of interest. The highest response observed for assay 2 (81.03% lactose hydrolysis)

was, for acetone, 8.5 units higher than the mean of all other assays (72.41%).

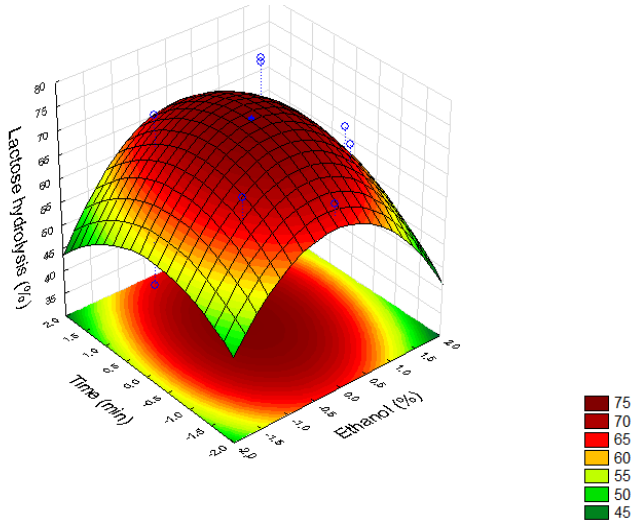
Analyzing the mathematical model for the response function Y_1 and the response surface Figure 1 (a,b and c), it was observed that there was a region in which the percentage of lactose hydrolysis was greater (> 70 %); when x_1 was between 0 and 1 or X_1 was between 30 and 40 % ethanol, x_2 was between 0 and 1 or X_2 was at a permeabilization temperature between 30 and 35 °C and x_3 was between 0 and 1 or X_3 was between 40 and 60 min for the permeabilization process of *Saccharomyces fragilis* IZ 275 cells.

For the response function Y_2 and the response surface Figure 1 (d, e and f), it was observed that there was a region in which the percentage of lactose hydrolysis was approximately 80 % when x_1 was between 0 and 1 or X_1 was between 30 and 40 % of acetone, x_2 was between 1 and 1.80 or X_2 was at a permeabilization temperature between 35 and 39.05 °C and x_3 was between 0 and 1 or X_3 was between 40 and 60 min for the permeabilization process of *Saccharomyces fragilis* IZ 275 cells.

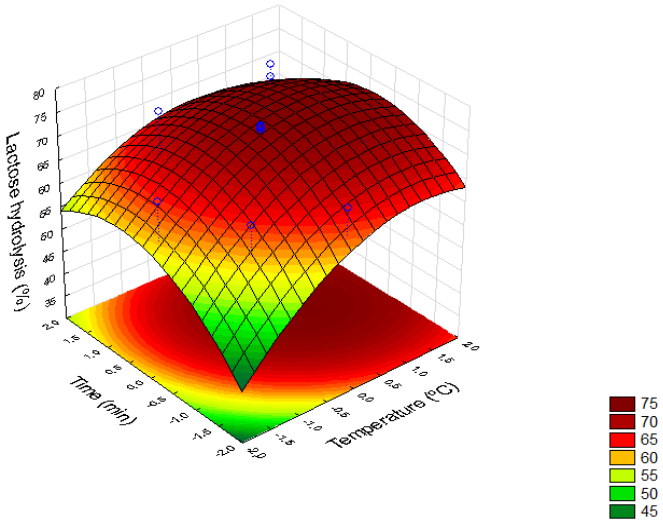
Figure 1. Response surface: (a) percentage of lactose hydrolysis as function of temperature (°C) and ethanol (%); (b) as function of time (min) and ethanol (°C) and (c) as function of temperature (°C) and ethanol (%); (d) percentage of lactose hydrolysis as function of temperature (°C) and acetone (%); (e) as function of time (°C) and acetone (%) and (f) as function of time (min) and temperature (°C).



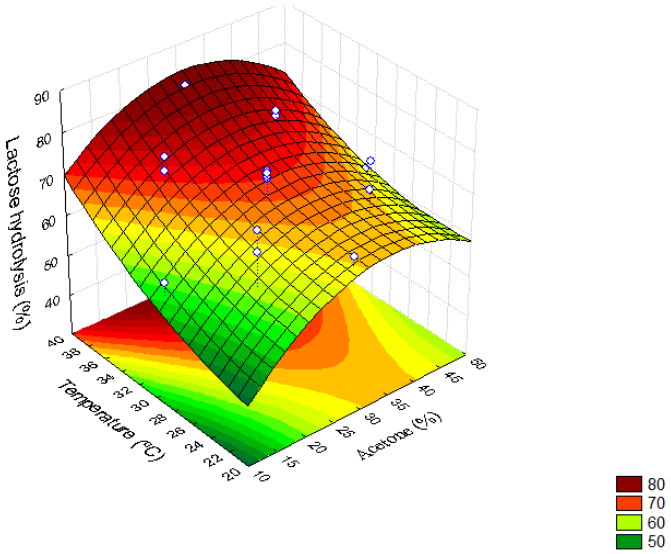
(a)


Lactose hydrolysis (%)
Time (min)
Ethanol (%)
75
70
65
60
55
50
45

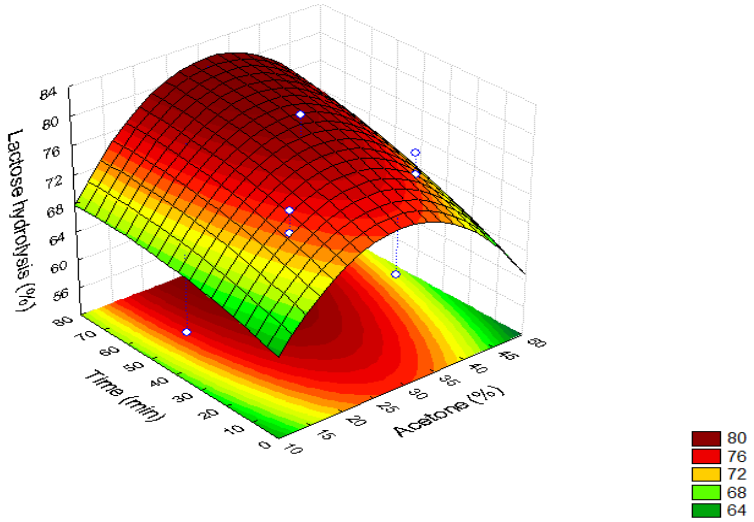
(b)


Lactose hydrolysis (%)
Time (min)
Temperature (°C)
75
70
65
60
55
50
45

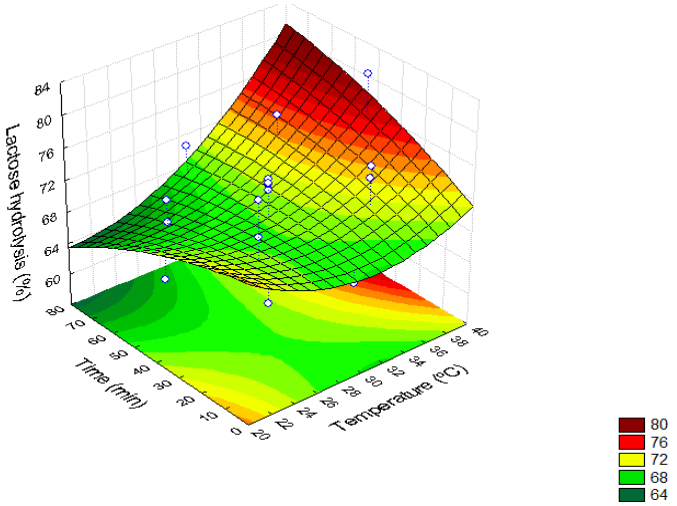
(c)


Lactose hydrolysis (%)
Temperature (°C)
Acetone (%)
80
70
60
50

(d)



(e)



(f)

Source: Software Statistic 7.0.

In this study, it was observed that the optimum conditions for the permeabilization of *Saccharomyces fragilis* IZ 275 cells evaluated by assessing the maximum level of lactose hydrolysis were the following: 40 % ethanol at 35 °C for 60 min; 30 % acetone at 39.05 °C for 40 min, and 4 % chloroform at 25 °C for 20 min.

Ethanol showed the lowest percentage of lactose hydrolysis (77 %) when compared with acetone (81 %) and chloroform (81%). Chloroform was used in a concentration which was 10-fold lower and, for approximately a quarter of the total, time to produce similar results to acetone, i.e. approximately 81 % of lactose hydrolysis.

The use of organic solvent ensures accessibility to the intracellular enzymes, and it is a rapid and no-destructive method which offers a low-cost alternative and avoids the problem

of enzyme extraction and purification (Panesar et al., 2007; Kaur et al., 2009).

However, many chemicals such as chloroform and acetone, are toxic and non-biodegradable, which hinders their application in the food processing industry. In this context, ethanol is considered the most common bio-solvent and is considered safe for use in this purpose.

Moreover, it has a low cost and environmental impact, it is water soluble and easy to obtain. Ethanol is also considered to be a green solvent (Chemat et al., 2012) which will reduce energy consumption and ensure a safe and high quality product. Considering this, we decided to use ethanol for the permeabilization of *Saccharomyces fragilis* IZ 275 cells.

Few studies have evaluated the permeabilization of yeast cells using organic solvents monitoring lactose hydrolysis Kaur et al. (2009) using cetyltrimethyl ammonium bromide (CTAB) permeabilized *Kluyveromyces marxianus* cells, thereby avoiding the problem of enzyme extraction and optimization of different process parameters.

The authors concluded that a maximum level of hydrolysis (90.5 %) was observed after 90 min of incubation at 40 °C. Panesar (2008) used the response surface methodology to investigate the effect of different parameters (concentration, temperature and treatment time) on the permeabilization of *Kluyveromyces marxianus* cells and concluded that the optimum operating conditions for the permeabilization process were 49.6% (v/v) ethanol concentration, 23 °C temperature and a process duration of 18 min.

These permeabilized yeast cells hydrolyzed 89.7% of the lactose in skimmed milk after 150 min of incubation time. By comparing these results, we were able to determine that the type and concentration of organic solvent, temperature permeabilization and time permeabilization are related and that the optimum conditions for cell permeabilization depend on these variables.

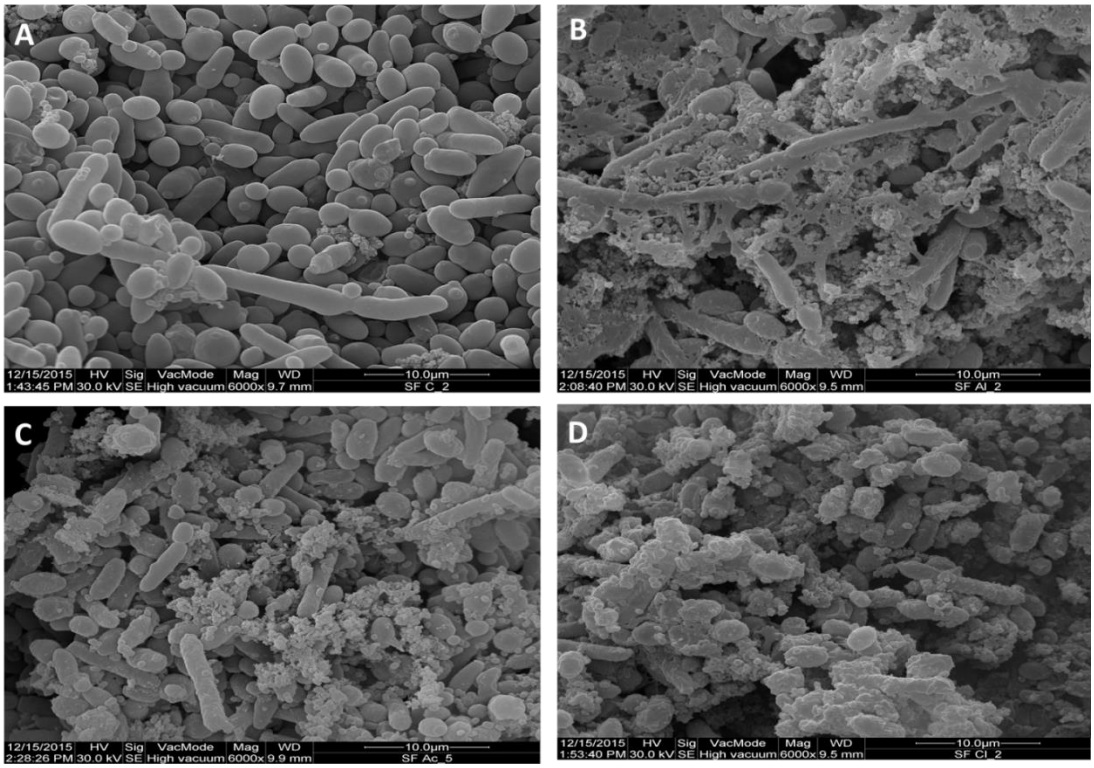
3.2 Scanning electron microscopy (SEM)

Cells permeabilization using organic solvents is a simple and fast method which promotes lactose hydrolysis, does not leave cellular fragments and modifies the cell wall structures causing disorganization in their pores (Panesar et al., 2007). This disorganization can be observed in Figure 2 which shows images of non-permeabilized (A) cells, permeabilized with ethanol (B), acetone (C) and chloroform (D).

The non-permeabilized cells have an intact and smooth surface, whereas the permeabilized cells present deformations and roughness in the cell wall caused by the

solvents. The deformity of the cell surface is due to the reduction of membrane phospholipids, which allows the passage of low molecular weight solutes into and out of the cell, including sugars such as lactose and its hydrolysis products (Becker et al., 1996).

Figure 2. Scanning electron microscopy (SEM) of *S. fragilis* IZ 275 cells non-permeabilized (A) and permeabilized with ethanol (B), acetone (C) and chloroform (D).



Source: SEM FEI Quanta 200.

4. Final Considerations

The response surface methodology employed proved to be an efficient tool to optimize the cell permeabilization process by monitoring lactose hydrolysis. The concentration and type of organic solvent, and the temperature and time of permeabilization influence the process of cells. The optimum conditions for the permeabilization of *S. fragilis* IZ 275 cells using ethanol are a concentration of 30 % at 35ºC for 40 min with 74.44 % lactose hydrolysis.

The optimum conditions for the permeabilization of *S. fragilis* IZ 275 cells using acetone are a concentration of 40 % at 39.05ºC for 76.18 min with 79.82 % lactose hydrolysis while for chloroform they are a concentration of 4 % at 25ºC for 20 min with 81.03 % lactose hydrolysis. As such, in this study, we showed that the use of ethanol for cell permeabilization

can improve β-galactosidase enzyme production, with applicability on a large scale in the food industry, being a cheaper and environmentally safe way to produce this enzyme.

Future works could apply the conditions established in the present work to obtain the enzyme in a large scale.

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