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Optimization of bioethanol production from cheese whey using *Kluyveromyces marxianus* URM 7404

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ABSTRACT

The objective of this study was to optimize the ethanol production from cheese whey, using the yeast *Kluyveromyces marxianus* URM 7404. The response surface methodology (RSM) with a central composite rotational design (CCRD) was used to evaluate the effects of pH (4–6), temperature (30–36 °C), and lactose concentration (50–70 $g.L^{-1}$). Cheese whey underwent 17 fermentations and further three fermentations were conducted for the validation of the equation obtained by RSM. After the validation of the optimization, the fermentation process was scaled-up to the volumes of 1 L, 6 L, 21 L, and 36 L, and ethanol production ($g.L^{-1}$), productivity (Q_P $g.L^{-1}.h^{-1}$), conversion ($Y_{P/S}$ $g.g^{-1}$), yield (η %), and chemical oxygen demand (mg COD. L^{-1}) were evaluated. Temperature was the most significant factor used for the optimization of the ethanol production, followed by pH and lactose concentration. The conditions for the ethanol production of higher than 90% were as follows: temperature range from 32.5 to 35.0 °C, pH from 4.8 to 5.3, and lactose concentration from 61.0 to 65.0 $g.L^{-1}$. The equation generated from the optimization process was validated and presented excellent values and precision for the use of this model in scale-up process, obtaining volume of 36 L, η 95.80%, Q_P 2.57 $g.L^{-1}.h^{-1}$, and $Y_{P/S}$ 0.50 $g.g^{-1}$. The COD of the cheese whey was reduced by 78.94%, proving it to be an effective process to reduce the environmental damages caused by this effluent.

1. Introduction

Among the various residues generated by the food industry, dairy is one of the most important, mainly due to the generation of their effluents that present a high organic load, with a COD (chemical oxygen demand) in the range of 50,000–80,000 mg COD. L^{-1} (Carvalho et al., 2013).

The organic matter content is mainly due to the presence of carbohydrates and milk proteins, such as lactose (4.5–5%) and casein, respectively. In addition, the content of lipids (0.4–0.5%), suspended solids (0.6–0.8%), and nutrients (N and P) contribute to the contamination. The rich nature of organic matter in dairy effluents makes its treatment necessary. Due to its composition (residual sugar, minerals, and nitrogen compounds), its potential use in the bioprocessing field has aroused interest to obtain bioproducts using submerged cultures, simultaneously carrying out an appropriate treatment and reducing environmental risks (Baldasso et al., 2011; Kushwaha et al., 2010; Rivas et al., 2011).

With the development of new techniques, whey has been studied and treated as an alternative substrate for the production of high commercial value co-products (ethanol, single cell protein, lactic acid, butanol, β–galactosidase, and other bioproducts) using microorganisms capable of directly metabolizing whey. Among the few microorganisms capable of fermenting lactose, the yeast belonging to the genus *Kluyveromyces* sp. stands out for its high metabolic diversity and its substantial degree of intra-specific polymorphisms and characteristics that are reflected by the various environments from which it is isolated (Geiger et al., 2016; Lane et al., 2011; Yadav et al., 2014).

In this context, an alternative to the use of whey would be the production of ethanol. In the last decades, many studies were carried out using the disaccharides (sugar cane molasses and beet) for the ethanol production. Due to the significant generation of coproducts, whey, whey permeate, and lignocellulosic residues have been gaining attention recently in order to boost the demand for cleaner renewable energy sources, with low environmental impact, and above all, as a way to solve global warming-related problems, associated with the use of fossil fuels and the exhaustion of their reserves (Castro and Roberto, 2014; Das et al., 2016; Rana et al., 2013).

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To obtain better ethanol production and yield from coproducts, one factor can be approached at a time in the process. However, this analysis disregards the synergistic effects between the variables, requires more time and experiments, and the optimal values achieved are far from ideal. However, with the use of response surface methodology (RSM), it is possible to combine all factors present in the process to find an optimal condition for *Kluyveromyces marxianus* growth and to obtain different products (ethanol, inulinase, β–galactosidase, ethyl acetate, and hydrogen) (Comelli et al., 2016; Löser et al., 2015; Mangayil et al., 2015). Therefore, the objective of this study was to produce ethanol from cheese whey using *K. marxianus* URM 7404, analyzing the factors that influence the bioproduction (temperature, pH, and lactose concentration) and the production profile in a fermentative scale-up.

2. Material and methods

2.1. Microorganism and maintenance

The yeast *K. marxianus* URM 7404 was isolated in the Bioprocess Laboratory of São Paulo State University, São José do Rio Preto, São Paulo, Brazil, from the whey obtained after the production of Gorgonzola cheese. It was tested as a propelling fermentation agent and later identified and incorporated into the Collection of Cultures - Micoteca URM - of the Center of Biological Sciences at the Federal University of Pernambuco (Recife, Pernambuco, Brazil).

The microorganism was propagated on a Potato Dextrose Agar (PDA) slant at 30 °C for 24 h. Subsequently, it was stored in a refrigerator at 4 °C (Arora et al., 2017).

2.2. Preparation of the inoculum

Five milliliters of sterile peptone water was added to the activated microorganism (previous step) and the surface of the medium was scraped so as to obtain a cell suspension. The formed suspension was transferred to 250 mL Erlenmeyer flasks containing 100 mL of medium (5.0 $g.L^{-1}$ lactose, 1.0 $g.L^{-1}$ yeast extract, 0.1 $g.L^{-1}$ $(NH_4)_2SO_4$, 0.1 $g.L^{-1}$ KH_2PO_4, 0.1 $g.L^{-1}$ $MgSO_4$, and 0.2 $g.L^{-1}$ $CaCl_2$) previously sterilized at 121 °C for 15 min. The suspension was then incubated at 30 °C and 150 rpm for 24 h or the time required for cell concentration to reach an optical density of 0.8 at 600 nm (Comelli et al., 2016).

After the incubation period, the cultivation medium was centrifuged at 3000 × *g* for 20 min. The pellet was washed in 0.1% peptone water and the procedure was repeated again. The pellet was resuspended in distilled water and denominated standard inoculum.

2.3. Culture medium

2.3.1. Supplementation and fermentation of the culture medium

The first stage of the study consisted of cheese whey supplementation with different nutrients (Table 1), as described by Comelli et al. (2016).

The substrate used was the cheese whey (El primo - Buenos Aires - Argentina) powder (atomized) derived as a coproduct during production of ricotta cheese. The cheese whey powder was reconstituted in distilled water (50 $g.L^{-1}$ lactose concentration) containing various supplements (yeast extract, mineral salts, or the combination of both).

Hundred milliliters of supplemented medium was kept in 250 mL Erlenmeyer flask and autoclaved under constant steam for 15 min at 100 °C. Once the initial inoculum was standardized, 10% of this inoculum, previously centrifuged (3000 × *g* for 20 min) was added in the medium, which was incubated at 30 °C, without stirring, for 20 h. Samples were taken after every 2 h to monitor the ethanol production, yield, and productivity.

Table 1
Composition of culture medium based on cheese whey for fermentation.

Experiment	Medium Composition
Medium 1	Cheese whey *in natura*
Medium 2	Cheese whey *in natura* + Yeast extract
Medium 3	Cheese whey *in natura* + Nutrients (salts: $(NH_4)_2SO_4$; K_3PO_4, $MgSO_4$; $ZnSO_4$)
Medium 4	Cheese whey *in natura* + Yeast extract + Nutrients (salts $(NH_4)_2SO_4$; K_3PO_4, $MgSO_4$; $ZnSO_4$)

Nutrient solution according to Comelli et al. (2016).

2.3.2. Culture medium for experimental design

The medium was supplemented with abovementioned materials, which resulted in higher ethanol production, productivity, and yield. The substrate was reconstituted in distilled water and sterilized in an autoclave under constant steam for 15 min at 100 °C, and the fermentation of the cheese whey was conducted in 250 mL Erlenmeyer flask containing 100 mL of culture medium. The inoculum was added to the medium (10%) and placed in a BOD incubator for 12 h. The cheese whey concentration (lactose), pH, and the temperature values were regulated by CCRD.

2.4. Experimental design

The process (ethanol production, yield, and productivity) was optimized according to a central composite rotational design (2^3), with three replicates at the central point.

Supplemented culture medium that presented more efficient ethanol production results was fixed. The independent variables (factors) studied were temperature (30–36 °C), initial pH (4–6), and lactose concentration (50–70 $g.L^{-1}$). These factors were selected due to their known effects on ethanol production by *K. marxianus* yeast. The studied responses were ethanol concentration, yield, and productivity.

Table 2 shows the treatments that were performed. The three variables (temperature, pH, and lactose concentration) were studied at three levels: low (−1), medium (0), and high (+1). The results were evaluated by linear regression, exploring the complete quadratic model with interaction. The non-significant parameters were eliminated from the model by Student's t-test at a 5% level of significance.

The quality of the developed models was evaluated by three types of values: coefficient of determination–R^2 (ability to explain the variance), *p*–value, and the F–test of the model (considering $F_{calculated}$ of the model is higher than $F_{tabulated}$). The adjusted polynomial empirical equations were expressed in the form of contour plots to illustrate the relationship between the responses as a function of the combination of two factors to be optimized, setting the other variables in central positions. Contour surfaces were used to facilitate the interpretation of any two response variables. Thus, the optimized numerical method was used to obtain optimal regions (optimal solutions).

2.5. Validation of the experimental model

To confirm the theoretical results obtained from the adjusted polynomial equations, the fermentation, under the conditions described as optimal, was carried out in triplicate to confirm the legitimacy of the models generated by the Statistica® software version 10.0. The ethanol production, yield, and productivity were compared to the values

Table 2
Variables used in the fermentation of cheese whey.

Variables	Levels				
	−1.68	−1	0	+1	+1.68
Temperature (°C)	30	31.3	33	34.7	36
pH	4.0	4.4	5.0	5,6	6.0
Lactose (g L^{-1})	50	54	60	66	70

predicted by the mathematical model.

2.6. Bioreactor cultivations

After the process optimization in Erlenmeyer flasks, the fermentation process was scaled up (with the best established conditions). The volumes of 1 L, 6 L, 21 L, and 36 L were examined in property sterilized polyethylene bioreactor, with natural agitation by the formation of CO_2, and with coupled peristaltic pump for withdrawal of samples during the fermentation until 18 h.

Initially, an inoculum standardization was performed, with the medium, in the 500 mL Erlenmeyer flasks, receiving 10% pre-inoculum. After 12 h, the medium, 10% inoculum was added in the 1 L fermenter, with optical density of 0.8 at 600 nm. The same procedure was repeated for 6 L, 21 L, and 36 L fermenters.

2.7. Analytical procedure

The ethanol content was determined by gas chromatography, after separation of the cells by centrifugation, using Chromatograph - HP-5890 Series II - detector FID (Flame Ionization Detector), HP-FFAP column (25 m × 0.2 mm × 0.3 μm); oven temperature of 70 °C (maintaining this temperature throughout the run); run time of 3.2 min; temperature of the injector at 250 °C; detector temperature of 250 °C; and injection of 40 μL of sample steam. The samples were left in "dry block" at 40 °C until they reached equilibrium (Santos and Cruz, 2017).

The reducing sugar (lactose) content was determined using the 3.5-dinitrosalicylic acid method described by Miller (1959). The standard curve was prepared from serial dilutions of a pure lactose solution.

To perform the COD analysis, the closed reflux colorimetric method was used, using a digester and a spectrophotometer at 620 nm (Hach Co.), according to APHA (1995).

2.8. Kinetic parameters of fermentation

The parameters used to evaluate the performance of the ethanol fermentation process included normalization against product yield coefficient, volumetric productivity of ethanol, and theoretical conversion of lactose to ethanol (Equations (1)–(3), respectively).

$$Y_{P/S}\ (g.\ g^{-1}) = (E_f - E_0)\ /(S_t - S_f) \quad \text{(Eq. 1)}$$

$$Q_P\ (g.\ L^{-1}.\ h^{-1}) = (E_f - E_0)/t_f \quad \text{(Eq. 2)}$$

$$\eta\ (\%) = \frac{(E_f - E_0) \times 100}{(S_t - S_f) \times 0.5368} \quad \text{(Eq. 3)}$$

Where: E_f = final product concentration ($g.L^{-1}$); E_0 = initial product concentration ($g.L^{-1}$); S_t = total substrate concentration ($g.L^{-1}$); S_f = final substrate concentration ($g.L^{-1}$); 0.5368 = theoretical value of the coefficient of conversion of lactose to ethanol.

The reduction in chemical oxygen demand was calculated by:

$$\%reduction = \frac{(COD_{initial} - COD_{final}) \times 100}{COD_{initial}} \quad \text{(Eq. 4)}$$

3. Results and discussion

Initially, the cheese whey was supplemented with different nutrients to verify the change in behavior of the yeast *K. marxianus*. As shown in Table 3, the best condition for higher ethanol production, yield, and productivity involved whey based medium supplemented only with yeast extract (M_2), with the maximum production after fermentation for 8 h.

The minerals used as supplements for *K. marxianus* are described as stimulant and inhibitor based on the performance of the culture as described by Arora et al. (2017). In our studies, the ethanol productivity

Table 3
Ethanol production, yield, and productivity obtained from the fermentation culture medium based on cheese whey containing different supplements.

Experiment	Ethanol ($g.L^{-1}$)	η (%)	Q_P ($g.L^{-1}.h^{-1}$)
M_1 [1]	16.34 ± 0.51[c]	94.80 ± 0.10[b]	1.63 ± 0.02[b]
M_2 [2]	20.60 ± 0.44[a]	95.70 ± 0.13[a]	2.58 ± 0.01[a]
M_3 [3]	15.56 ± 0.10[c]	72.10 ± 0.09[d]	0.78 ± 0.01[d]
M_4 [4]	18.54 ± 0.18[b]	79.90 ± 0.11[c]	0.93 ± 0.03[c]

The values denote the mean of triplicate (± SD). Different letters in the same column are significantly different ($p < 0.05$). $\mathbf{Q_P}$: productivity in ethanol ($g.L^{-1}.h^{-1}$), **η**: ethanolic yield (%). [1] cheese whey *in natura,* [2] cheese whey *in natura* + Yeast extract, [3] cheese whey *in natura* + Nutrients (salts: $(NH_4)_2SO_4$; K_3PO_4, $MgSO_4$; $ZnSO_4$), [4] cheese whey *in natura* + Yeast extract + Nutrients (salts $(NH_4)_2SO_4$; K_3PO_4; $MgSO_4$; $ZnSO_4$).
*$\mathbf{M_1}$ (10 h); $\mathbf{M_2}$ (8 h); $\mathbf{M_3}$ (20 h); $\mathbf{M_4}$ (20 h).

($g.L^{-1}.\ h^{-1}$) and yield (%) were not satisfactory (M_3 and M_4), when the *in natura* whey medium was compared with the media supplemented with yeast extract (M_1 and M_2).

It is already known that cheese whey is rich in vitamin B complex and minerals, including NaCl, KCl, and calcium salts (mainly phosphate) (Dragone et al., 2009). Thus, the additional use of ionic salts inhibited ethanol production. Casey et al. (2013) observed a significant reduction (50%) in the ethanol productivity after adding ionic salts during xylose and glucose co-fermentation by *Saccharomyces cerevisiae*. According to Sayed et al. (2018) the use of salts (NaCl and Na_2SO_4) had negative influence on cell growth, decreasing the conversion rate in ethanol from 77% to 74% with *S. cerevisiae* in synthetic medium. The presence of salts in the culture medium lead to change in yeast behavior due to osmotic pressure, since the micro-organisms need to adapt to a higher pressure; in addition, other neutral solutes are produced as organic acids that interfere with ethanol production (Blomberg, 2000).

The yeast extract is described as an efficient source of nitrogen, which is present in B complex vitamins (niacin, thiamine, pyridoxine, pantothenic acid, folic acid, and biotin) and amino acids (methionine, cysteine, glutamic acid, aspartic acid, glycine, and alanine) (Hälvin et al., 2013; Vieira et al., 2016). The addition of yeast extract in the medium increased the ethanol production. This promoted the biomass growth, and consequently, increased the ethanol production, as observed in similar studies on *Kluyveromyces* (Gethins et al., 2015; Parrondo et al., 2009).

The composition of medium M_2, formulated with cheese whey and yeast extract, was selected for use in the study. A central composite rotational design (CCRD) was used to obtain an empirical model to optimize the culture conditions (temperature, initial pH, and lactose concentration) for cheese whey, evaluating the various combinations of the factors.

Table 4 presents the coded and actual values of CCRD and their responses in terms of ethanol production, yield, and productivity.

In CCRD (Table 4), ethanol production varied from 12.64 $g.L^{-1}$ (assay 8) to 29.78 $g.L^{-1}$ (assay 15), the ethanol yield varied from 55% (assay 7) to 98% (assay 15 and 17), and the ethanol productivity from 1.05 $g.L^{-1}.\ h^{-1}$ (assay 8) to 2.48 $g.L^{-1}.\ h^{-1}$ (assay 15). It was speculated that, for ethanol production, yield, and productivity, the best results lie at a common point among all these ranges.

Statistical analyses were performed using Statistica® 10.0 software. The quality of the fit of the model was expressed by the coefficient of determination R^2, by the statistical significance of the regression, and by Fisher's 'F' test. Equations (3)–(5) present the coded second order model, which describes the ethanol concentration, yield, and productivity as a function of the independent variables (analyzed factors), temperature, pH, and lactose concentration, within the studied range. The model was validated by analysis of variance (Table 5). It is noted that the factors and their interactions were significant, with *p*–value < 0.01. The *p*–value shows how much the terms of the equation

Table 4
Central composite rotational design 2^3 (real and coded values) to maximize the ethanol production, yield, and productivity by *Kluyveromyces marxianus*.

Run	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	+1 (34.7)	+1 (5.6)	+1 (66.0)	28.3	94	2.36
2	+1 (34.7)	+1 (5.6)	−1 (54.0)	20.8	84	1.73
3	+1 (34.7)	−1 (4.4)	+1 (66.0)	18.53	67	1.54
4	+1 (34.7)	−1 (4.4)	−1 (54.0)	14.88	73	1.24
5	−1 (31.3)	+1 (5.6)	+1 (66.0)	15.56	56	1.30
6	−1 (31.3)	+1 (5.6)	−1 (54.0)	14.45	67	1.20
7	−1 (31.3)	−1 (4.4)	+1 (66.0)	15.90	55	1.33
8	−1 (31.3)	−1 (4.4)	−1 (54.0)	12.64	58	1,05
9	+1.68 (36.0)	0 (5.0)	0 (60.0)	24.93	94	2.08
10	−1.68 (30.0)	0 (5.0)	0 (60.0)	19.78	77	1.64
11	0 (33.0)	+1.68 (6.0)	0 (60.0)	19.70	75	1.65
12	0 (33.0)	−1.68 (4.0)	0 (60.0)	14.50	56	1.21
13	0 (33.0)	0 (5.0)	+1.68 (70.0)	27.08	84	2.26
14	0 (33.0)	0 (5.0)	−1.68 (50.0)	20.60	87	1.72
15	0 (33.0)	0 (5.0)	0 (60.0)	29.78	98	2.48
16	0 (33.0)	0 (5.0)	0 (60.0)	29.40	97	2.45
17	0 (33.0)	0 (5.0)	0 (60.0)	28.80	98	2.40

X_1 = Temperature (°C); X_2 = Initial pH; X_3 = Lactose ($g.L^{-1}$); Y_1 = Ethanol ($g.L^{-1}$); Y_2 = Ethanol yield; Y_3 = Ethanol productivity ($g.L^{-1}.h^{-1}$).

are reliable (see Table 6).

The coefficient of determination–R^2 (93%, 90%, and 93%) describes well the behavior of the evaluated responses. According to Chauhan and Gupta (2004), a model that has a R^2 value higher than 75% can be accepted. In the present study, the models describe ethanol production, yield, and productivity. The high values of R^2 show a strong correlation between observed and predicted values, indicating good reliability of the mathematical model in predicting the behavior of *K. marxianus*. The quality of R^2 obtained in this study was higher than that obtained in other studies on cheese whey (Arora et al., 2017; Dragone et al., 2011; Sansonetti et al., 2010).

In addition, $F_{calculated}$ of the model was 3.74, 1.97, and 4.46 times higher than the $F_{tabulated}$ values for ethanol production, yield, and productivity, respectively. It is known that, for the Fisher test, the more distant the value of $F_{calculated}$ than the value of $F_{tabulated}$, the more significant (*p*–value) are the factors analyzed and the better is the quality of R^2. Thus, with a coefficient of determination higher than 80% and almost all significant factors, it was possible to construct the contour curves presented in Figs. 1–3.

$$Ethanol\ (g.\ L^{-1}) = 29.52 + 2.39x_1 - 3.13x_1^2 + 1.90x_2 - 5.00x_2^2 + 1.94x_3 - 2.60x_3^2 + 1.78x_1x_2 + 0.85x_1x_3 \quad \text{(Eq. 5)}$$

$$Yield\ (\eta) = 98.23 + 8.10x_1 - 6.18x_1^2 + 5.86x_2 - 13.27x_2^2 - 1.10x_3 - 6.18x_3^2 + 3{,}5x_1x_2 + 2.25x_1x_3 + 1.00x_2x_3 \quad \text{(Eq. 6)}$$

$$Productivity\ ethanol\ (g.\ L^{-1}.\ h^{-1}) = 2.46 + 0.2x_1 - 0.26x_1^2 + 0.15x_2 - 0.41x_2^2 + 0.16x_3 - 0{,}22x_3^2 + 0.15x_1x_2 + 0.07x_1x_3 \quad \text{(Eq. 7)}$$

Were: x_1: Temperature; x_2: Initial pH and x_3: Lactose.

From the mathematical models obtained, it was possible to construct the contour curves to better explain the interactions of two factors based on the evaluated response. Strong interactions were indicated with smaller circles. The smaller was the area of a circle, the higher was the predicted maximum value. By overlapping the figures, it is possible to obtain a region that satisfies the response to reach the maximum value as provided by equations (5)–(7).

Evaluating Figs. 1–3, for maximum ethanol production, yield, and productivity, the initial pH should be in the range of 4.8–5.3, the lactose concentration from 61.0 to 65.0 $g.L^{-1}$, and the temperature from 32.5 to 35.0 °C.

3.1. Validation of experiments

Based on the statistical analysis results, tests were performed in triplicate to confirm the predictions of the mathematical models. The optimal condition used for *Kluyveromyces marxianus* was lactose concentration of 60 $g.L^{-1}$ (level 0), temperature of 33 °C (level 0), and initial pH of 5 (level 0).

Fig. 4 shows the mean results of the kinetics of ethanol production, yield, and productivity, during model validation in the culture medium.

It was observed that the relative deviations of the experimental results from the predicted results were lower than 10% for ethanol production and yield, indicating a reliability of the model (Arora et al., 2017). Only productivity values showed a deviation of more than 10%, but positive, underestimating the values predicted by the equation. Therefore, both models were confirmed as reliable.

The ethanol production and yield responses in the model validation test were very close to the predicted values, 27.61 $g.L^{-1}$ and 98%, respectively. In general, comparing the results obtained in the validation experiments, it was speculated that conducting alcoholic fermentation under conditions favoring maximum production was more advantageous in terms of the analyzed responses, since it was possible to obtain better yield and productivity.

Ghanadzadeh and Ghorbanpour (2012) found that, for ethanol production using *K. marxianus* in cheese whey, the process variables (42 $g.L^{-1}$ lactose concentration, 5.3 pH, and 30.8 °C temperature) allowed to optimize the production of ethanol with production of 25 $g.L^{-1}$ and yield of 89.28%, with almost complete consumption of lactose. In our work, it was possible to obtain 27 $g.L^{-1}$ of ethanol production.

Dragone et al. (2011), in the optimization of the process using cheese whey by *K. fragilis*, obtained an ethanol production of 55.9 $g.L^{-1}$, a yield of 69.04%, and 1.27 $g.L^{-1}$. h^{-1} of productivity. The low yield is due to the excess concentration of lactose (150 $g.L^{-1}$) at 30 °C. In our study, with *K. marxianus*, it was possible to obtain a higher yield of 98.23% and productivity of 2.46 $g.L^{-1}$. h^{-1}, with a lower initial concentration of lactose (60 $g.L^{-1}$). Sansonetti et al. (2010), under optimization conditions similar to those used in the present study for ethanol production by *K. marxianus* in whey ricotta, obtained 84% ethanol yield with production of 21.29 $g.L^{-1}$, at 32.3 °C, pH 5.4, and 47 $g.L^{-1}$ lactose concentration.

They observed that, as in the present study, higher concentration of lactose and higher temperature leads to a drastic decrease in the product formation and substrate utilization. At high temperatures, the deleterious effect on ethanol yield can be attributed to denaturation of ribosomes, enzymes, and changes in membrane fluidity. High lactose concentrations may lead to stress induced by increased external osmolarity, disturbing the osmotic gradient across the plasma membrane (Dragone et al., 2011; Ghanadzadeh and Ghorbanpour, 2012).

3.2. Cultivation in bioreactors

The process was rescaled to verify the fermentation behavior using the optimized condition, evaluating the evolution of ethanol production, yield, productivity, conversion rate, and other factors, in different volumes, as described in Table 7.

As can be observed, the ethanol production in the 1 L and 21 L fermenters were comparable, being 28 $g.L^{-1}$ and 27.80 $g.L^{-1}$, respectively, followed by 6 L fermenter, with 27.51 $g.L^{-1}$ ethanol production. This variation between 6 L and 21 L fermenters may have occurred due to yeast adaption with some metabolites, such as organic acids and glycerol, produced to balance the pH of the medium, which can lead to a reduction in the production of ethanol (Yadav et al., 2014; Suhaimi et al., 2012; Dorta et al., 2006).

In the 36 L assay, the ethanol production decreased by around 9.25% and the maximum ethanol production time was increased to 16 h, when compared to the fermentation in 1 L assay, with a

Table 5
Analysis of variance for response function with all effects – ANOVA test.

	Variation source	Quadratic Sum	Degrees of Freedom	Quadratic Mean	*p*–value
Ethanol ($g.L^{-1}$)	x_1	77.94	1	77.94	0.003
	x_1^2	109.99	1	109.99	0.002
	x_2	49.15	1	49.15	0.004
	x_2^2	279.92	1	279.92	0.001
	x_3	51.10	1	51.10	0.004
	x_3^2	76.10	1	76.10	0.003
	x_1x_2	25.28	1	25.28	0.009
	x_1x_3	5.75	1	5.75	0.040
	Regression	530.43	8	66.30	
	Residual	41.23	8	5.15	
	Lack of fit	40.74	6		
	Pure error	0.49	2		
	Total	571.66	16		
Ethanol yield (η)	Variation source	Quadratic Sum	Degrees of Freedom	Quadratic Mean	*p*–value
	x_1	895.84	1	895.84	< 0.001
	x_1^2	430.01	1	430.01	0.001
	x_2	468.11	1	468.11	0.001
	x_2^2	1979.93	1	1979.93	< 0.001
	x_3	16.58	1	16.58	0.020
	x_3^2	430.01	1	430.01	0.001
	x_1x_2	98.00	1	98.00	0.003
	x_1x_3	40.50	1	40.50	0.008
	x_2x_3	8.00	1	8.00	0.039
	Regression	3611.05	9	401.23	
	Residual	386.83	7	55.26	
	Lack of fit	386.16	5		
	Pure error	0.67	2		
	Total	3997.88	16		

Cont. Table 5. Analysis of variance for response function with all effects – ANOVA test.

	Variation source	Quadratic Sum	Degrees of Freedom	Quadratic Mean	*p*–value
Ethanol productivity ($g.L^{-1}.h^{-1}$)	x_1	0.55	1	0.55	0.003
	x_1^2	0.77	1	0.77	0.002
	x_2	0.34	1	0.34	0.005
	x_2^2	1.93	1	1.93	0.001
	x_3	0.36	1	0.36	0.005
	x_3^2	0.52	1	0.52	0.003
	x_1x_2	0.18	1	0.18	0.009
	x_1x_3	0.04	1	0.04	0.041
	Regression	3.70	8	3.70	
	Residual	0.28	8	0.28	
	Lack of fit	0.28	6		
	Pure error	0.00	2		
	Total	3.98	16		

($p < 0.05$). X_1: Temperature (°C), X_2: Initial pH and X_3: Lactose ($g.L^{-1}$).
Ethanol R: 0.96, $F_{8;8;\ 0,95(tabulated)} = 3.44$, $F_{calculated\ (model)} = 12.87$, Ratio $F_{calculated}/F_{tabulated} = 3.74$.
Ethanol yield R: 0.95, $F_{9;7;\ 0.95(tabulated)} = 3.68$, $F_{calculated(model)} = 7.26$, Ratio $F_{calculated}/F_{\ tabulated} = 1.97$.
($p < 0.05$). X_1: Temperature (°C), X_2: Initial pH and X_3: Lactose ($g.L^{-1}$).
Ethanol productivity R: 0.96, $F_{8;8;\ 0,95(tabulated)} = 3.44$, $F_{calculated\ (model)} = 15.33$, Ratio $F_{calculated}/F_{tabulated} = 4.46$.

Table 6
Mathematical model validation responses.

	Ethanol ($g.L^{-1}$)	η (%)	Q_P ($g.L^{-1}.h^{-1}$)
Expected response	29.52	97.11	2.94
Experimental response	27.61	98.23	2.46
Model deviation	−6.92	1.14	16.33

η = Ethanol yield; **Q_P** = Ethanol production.

production of 25.81 $g.L^{-1}$ (Table 7). The time taken to achieve maximum ethanol production varies according to the amount of volume being analyzed (36 L), where the yeast conducting slower fermentation required more energy to adapt to the higher volume of fermentative medium, or by the reduction of cell viability, after several fermentation cycles (Pereira et al., 2012; Laluce et al., 2009), which reduced the production of ethanol with increase in the fermentation time (Fig. 5B).

The ethanol productivity (Q_P), was comparable in the fermenters with volume of 1 L, 6 L, and 36 L ($p < 0.05$), exhibiting maximum values of 2.73 $g.L^{-1}.\ h^{-1}$, 2.90 $g.L^{-1}.\ h^{-1}$, and 2.57 $g.L^{-1}.\ h^{-1}$ respectively. In 21 L medium, ethanol productivity was 3.11 $g.L^{-1}.\ h^{-1}$ (about 12.21% higher than that in the 1 L medium) after 8 h of fermentation, as observed in Fig. 5B, where between the times of 4–8 h an accentuated ethanol production occurred, as well as a depletion in lactose consumption was higher (Fig. 5A), which consequently led to a higher productivity of ethanol by the yeast. For the same period the

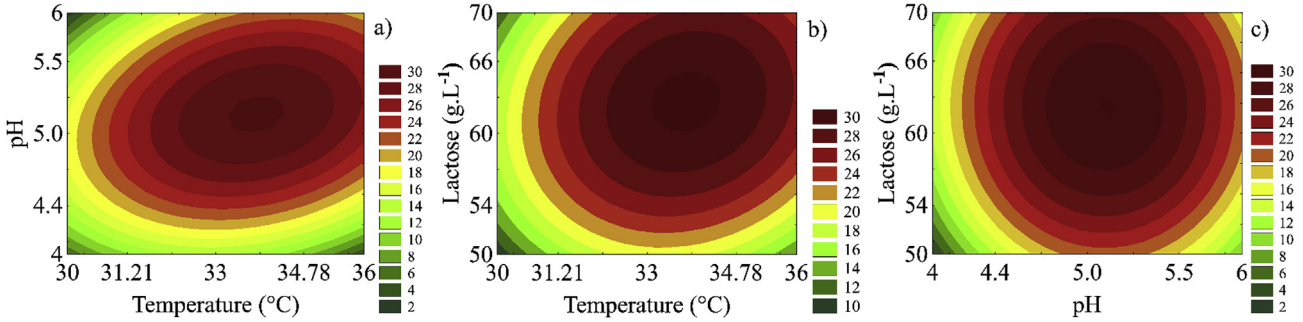

Fig. 1. Response surface of ethanol production ($g.L^{-1}$) from cheese whey powder by *K. marxianus* as a function of: (A) pH and temperature, (B) lactose concentration and temperature, (C) lactose concentration and initial pH.

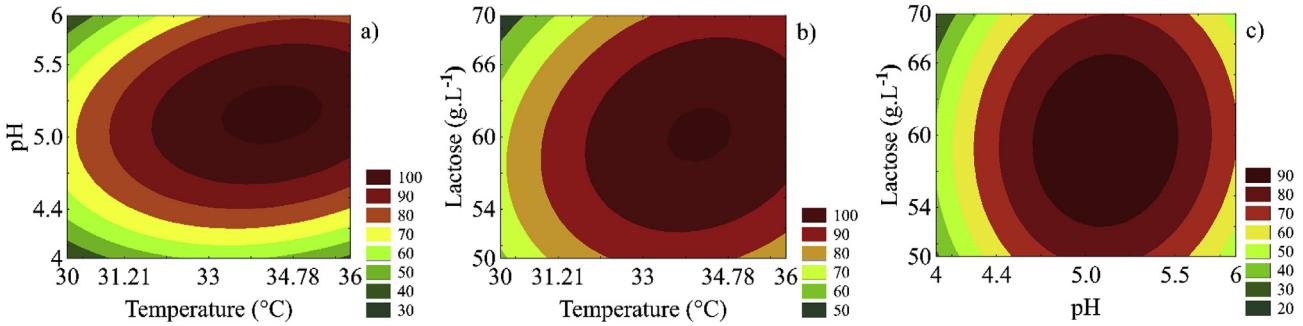

Fig. 2. Response surface for ethanol yield (η %) from cheese whey powder by *K. marxianus* as a function of: (A) pH and temperature, (B) lactose concentration and temperature, (C) lactose concentration and initial pH.

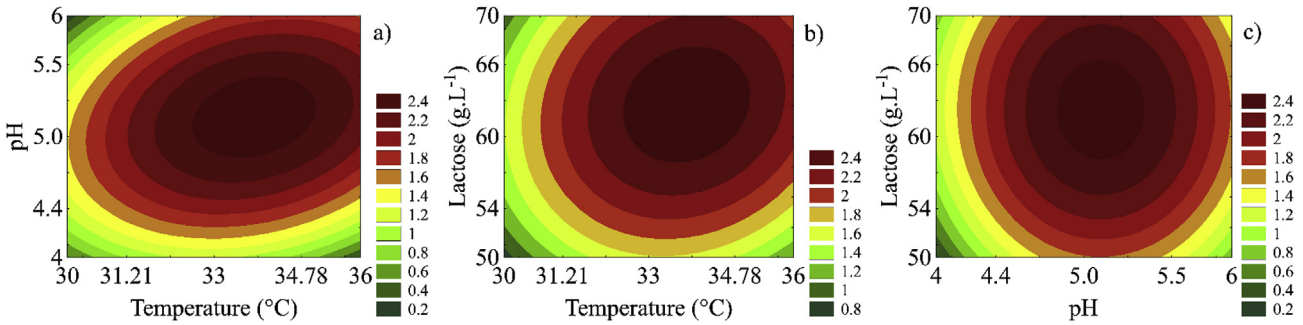

Fig. 3. Response surface for ethanol productivity (Q_P $g.L^{-1}.h^{-1}$) from cheese whey powder by *K. marxianus* as a function of: (A) pH and temperature, (B) lactose concentration and temperature, (C) lactose concentration and initial pH.

productivity in the medium of 36 L was lower (Fig. 5A and B).

For the substrate conversion to ethanol ($Y_{P/S}$), the four volumes presented similar values, with no significant difference among the assays (Table 7). The final yield in the medium of higher volume (36 L) was satisfactory, reaching 95.80% (in 8 h). These results indicated a great potential for industrial application, due to the lower time required to reach its maximum yield. The cultures in 1 L, 6 L, and 21 L volumes presented maximum yields (η) of 97.84%, 96.7%, and 96.30% respectively, after 12 h of fermentation. Koutinas et al. (2007), using a Kefir culture for ethanol production, obtained a reduction of 16% in the ethanol production and of 14.4% in the ethanol yield, as the fermentation process was scaled-up from 100 L to 300 L, after 22 h of fermentation.

It was observed that fermentations carried out at 1 L and 6 L volumes showed highest reduction in organic matter content, exhibiting 85.18% and 86.54% reduction in COD. COD reduction efficiency began to fall as the medium volume increased from 21 L, reaching 80.90% in 21 L fermenter and 78.94% in 36 L fermenter. Thus, the lowest COD reduction efficiency was just 8.78% lower than the highest efficiency.

Koutinas et al. (2007) obtained a COD reduction of 70.86% in 100 L fermenter. When the culture volume was increased to 300 L, the reduction in COD was 62.10% after 16 h of fermentation. These findings corroborated with the results in our study, where the increase in scale led to a decrease in the efficiency of COD reduction, indicating that the yeast used was more efficient in the removal of organic matter. Considering the volume of whey used for the scale-up, *K. marxianus* was efficient in the treatment of whey in large volumes, where ethanol was produced with satisfactory yields.

The scale-up of fermentation process exhibited similar results to those obtained in optimized flask scale. In this context, the experiments provided relevant data that suggests the viability of scale-up, for the utilization of large daily amount of whey effluent. The strain used in this study, *Kluyveromyces marxianus* URM 7044, showed high lactose to ethanol conversion values, higher organic load reduction, and low fermentation time to obtain best yields, revealing attractive alternative to existing industrial processes.

4. Conclusion

The use of *K. marxianus* URM 7404 to convert lactose in cheese

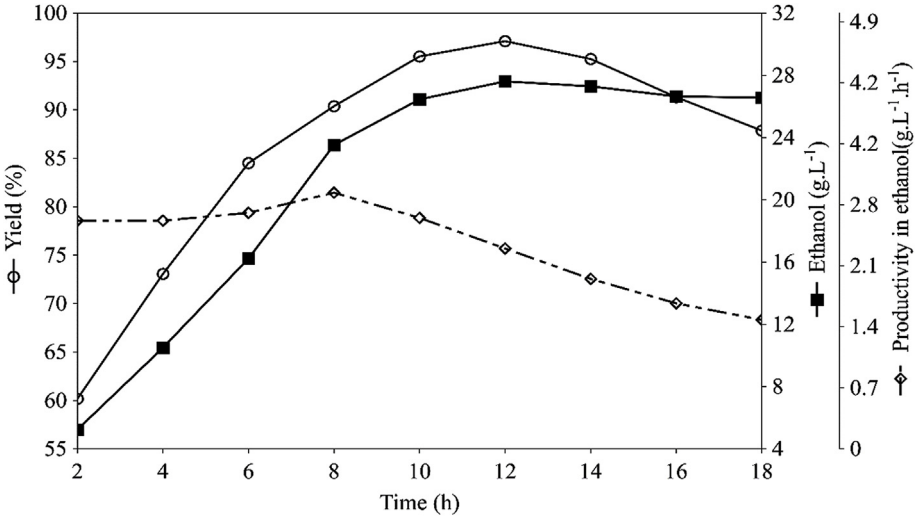

Fig. 4. Validation of the mathematical model obtained for optimizing ethanol production ($g.L^{-1}$), ethanol yield (η %), and ethanol productivity (Q_P $g.L^{-1}.h^{-1}$) from cheese whey using *K. marxianus*. Initial lactose concentration: 60 $g.L^{-1}$, temperature: 33 °C, and pH: 5.

Table 7
Parameters analyzed from the scaled-up fermentation carried out by *K. marxianus* URM 7404 under optimized conditions.

Parameter	1 L	6 L	21 L	36 L
Initial lactose ($g.L^{-1}$)	59.68 ± 0.32[a]	60.02 ± 0.32[a]	60.31 ± 0.32[a]	60.00 ± 0.32[a]
Ethanol production ($g.L^{-1}$)	28.00 ± 0.11[a] (12 h)	27.51 ± 0.13[b] (14 h)	27.80 ± 0.10[a] (12 h)	25.81 ± 0.08[c] (16 h)
$Y_{P/S}$ ($g.g^{-1}$)	0.53 ± 0.01[a] (12 h)	0.52 ± 0.02[a] (12 h)	0.51 ± 0.03[a] (12 h)	0.50 ± 0.02[a] (10 h)
Q_P ($g.L^{-1}$ h^{-1})	2.73 ± 0.03[c] (8 h)	2.90 ± 0.02[b] (6 h)	3.11 ± 0.03[a] (8 h)	2.57 ± 0.05[d] (8 h)
η (%)	97.84 ± 0.10[a] (12 h)	96.70 ± 0.15[b] (12 h)	96.30 ± 0.09[c] (12 h)	95.80 ± 0.12[d] (8 h)
Initial COD ($mg.COD.L^{-1}$)	51.04 ± 0.26[a]	51.30 ± 0.26[a]	51.56 ± 0.26[a]	51.30 ± 0.26[a]
COD Reduction (%)	85.18 ± 0.09[b]	86.54 ± 0.010[a]	80.90 ± 0.08[c]	78.94 ± 0.011[d]

The values denote the mean of triplicate (± SD). Different letters in the same line are significantly different ($p < 0.05$).
$Y_{P/S=}$ maximum substrate to ethanol conversion, $Q_{P=}$ maximum volumetric productivity, $\eta_=$ maximum theoretical ethanolic yield, **COD** = chemical oxygen demand.

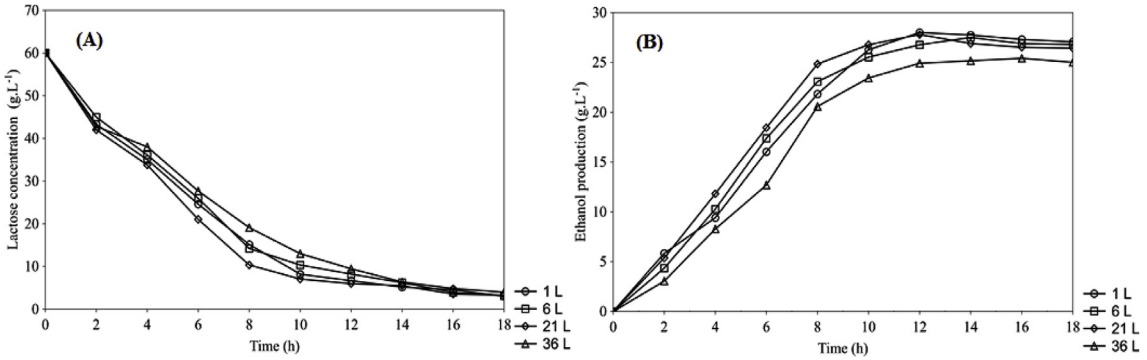

Fig. 5. Lactose consumption (A) and ethanol production (B) obtained by *K. marxianus* URM 7404 after scale-up. Initial lactose concentration of 60 $g.L^{-1}$ at 33 °C and pH 5

whey to ethanol is a promising alternative since the yields obtained were comparable to the theoretical values. These values were achieved within an optimum range of values for temperature, pH, and lactose concentration, the parameters considered crucial for the economic viability of the whey used as raw material for ethanol production. In addition, the process used in this study is effective in reducing the organic load content of this effluent.

The quadratic polynomial model developed for this coproduct has been evaluated and can be used to scale-up the fermentative processes, proving it to be a useful and powerful tool in the development of optimal fermentation conditions.

Conflicts of interest

The authors declare that they have no conflict of interest.

Submission declaration

The present work has not been published previously in any form and is not under consideration for publication elsewhere.

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