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Optimizing and validating the production of ethanol from cheese whey permeate by *Kluyveromyces marxianus* UFV-3

Raphael H.S. Diniz [a], Marina Q.R.B. Rodrigues [b], Luciano G. Fietto [b], Flávia M.L. Passos [a], Wendel B. Silveira [a,*]

[a] Departamento de Microbiologia, Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brasil
[b] Departamento de Bioquímica e Biologia Molecular, Universidade de Federal de Viçosa, Viçosa, Minas Gerais, Brasil

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ABSTRACT

The purpose of this study was to optimize the production of ethanol from cheese whey permeate using *Kluyveromyces marxianus* UFV-3. We used the response surface methodology (RSM) with a central composite rotational design (CCRD) to evaluate the effects of pH (4.5–6.5), temperature (30–45 °C), lactose concentration (50–250 g l^{-1}), and cell biomass concentration (A_{600} 2–4). We performed 29 fermentations under hypoxia in cheese whey permeate and seven fermentations for the validation of the equation obtained via RSM. Temperature was the most significant factor in optimizing ethanol production, followed by pH, cell biomass concentration and lactose concentration. The conditions for producing ethanol at yields above 90% were as follows: temperature between 33.3 and 38.5 °C, pH between 4.7 and 5.7, cell biomass concentration between A_{600} 2.4 and 3.3, and lactose concentration between 50 and 108 g l^{-1}. The equation generated from the optimization process was validated and exhibited excellent bias and accuracy values for the future use of this model in scaling up the fermentation process.

1. Introduction

Cheese whey is the main byproduct from the dairy industry and is composed of approximately 93% water, 5% lactose, 0.9% protein, 0.3% fat, 0.2% lactic acid, vitamins, and mineral salts (González-Siso, 1996). In the production of 1 kg of cheese, approximately 10 kg of whey is generated, and it is estimated that the total volume of cheese whey produced worldwide surpasses 160 million tons per year, representing approximately eight million tons of lactose (OECD-FAO, 2008). Approximately 50% of all whey produced is discarded prior to any treatment and causes extensive environmental damage, mainly due to its high biochemical oxygen demand (BOD) of between 50,000 and 60,000 mg l^{-1} of O_2 (González-Siso, 1996). Several industries recover a portion of the whey proteins via ultrafiltration for use in food supplements or in other milk products. However, cheese whey permeate resulting from this process still contains approximately 85–95% of the whey lactose, the carbohydrate mainly responsible for its high BOD (Vienne and Stockar, 1985). Therefore, there is strong incentive for the development of a process for cheese whey permeate treatment that can produce a biotechnological product from the lactose (González-Siso, 1996). In recent decades, research on ethanol production (e.g. from permeate) has been driven by the growing demand for cleaner, more renewable energy sources (Rana et al., 2013). In addition to biofuel, the ethanol produced from permeate is used in the food, beverage, pharmaceutical, and cosmetic industries, due to its potability (Guimarães et al., 2010).

Among the few microorganisms able to ferment lactose is the yeast *Kluyveromyces marxianus*. *K. marxianus* stands out for its high metabolic diversity and its substantial degree of intraspecific polymorphism, traits that are reflected by the various environments from which it has been isolated (Lane et al., 2011). In addition to lactose fermentation, *K. marxianus* has other desirable attributes for industrial fermentation processes, such as thermotolerance, a high growth rate, and metabolizing capacity, and often ferments a wide variety of carbohydrates, such as pentoses, hexoses, and disaccharides (Lane and Morrissey 2010). *K. marxianus* UFV-3, isolated from cheese factories in Southeastern Brazil, is able to convert the lactose in cheese whey into ethanol at high yields under conditions of highly concentrated cheese whey permeate and low oxygen levels (Silveira et al., 2005). This strain's fermentative behavior is mainly due to its increased expression of key enzymes involved in lactose metabolism (Diniz et al., 2012). However, other factors that may affect the fermentative capacity of *K. marxianus* UFV-3, such as temperature, pH, substrate concentration, and cell biomass concentration, have not been established for this yeast. Recently, *K. marxianus* has been used for characterizing and optimizing empirical models for biological systems. These models allow us to study the effects of numerous independent variables (e.g. temperature and pH) that may or may not interact with each other or act on a dependent response variable

* Correspondence to: Avenida PH Rolfs, s/n, Campus Universitário, CEP 36571-000, Viçosa, Minas Gerais, Brazil. Tel.: +55 31 3899 1943; fax: +55 31 3899 2573.
E-mail addresses: wendel.silveira@ufv.br, raphael.diniz@ufv.br (W.B. Silveira).

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of interest, such as fermentation yield (Uncu and Cekmecelioglu, 2011). The response surface methodology (RSM) is a combination of mathematical and statistical functions for obtaining empirical models for the development, improvement, and optimization of processes using composite experimental designs (Myers and Montgomery, 1995). Thus, the purpose of this study was to define the optimal conditions for the production of ethanol by *K. marxianus* UFV-3 from cheese whey permeate using the RSM and central composite rotational design (CCRD). The effects of four independent factors (temperature, pH, lactose concentration, and cell biomass concentration) were analyzed with respect to ethanol yield from lactose consumption (response variable).

2. Materials and methods

2.1. Yeast strain and maintenance

The yeast used in this study, *K. marxianus* UFV-3, was isolated from cheese factories in southeastern Brazil and has been stored and maintained in the culture collection at the Laboratory of Microorganism Physiology, BIOAGRO, of the Federal University of Viçosa, Minas Gerais, Brazil. *K. marxianus* UFV-3 was kept frozen at −80 °C in medium containing 50% glycerol. The starting inoculum for fermentation was prepared by adding 1% (w/v) of the biomass stored at −80 °C into YNB (Yeast Nitrogen Base) medium (Sigma®, St. Louis, USA) supplemented with 2% lactose and cultured under agitation (200 rpm) at 37 °C for 18–24 h. After this period, the active cells were centrifuged (3000*g* for 5 min), washed three times with distilled water, and then inoculated into the fermentation medium.

2.2. Fermentation media

Cheese whey permeate (CWP) obtained from a dairy factory in the region (Indústria Maroca & Russo, Cotochés, Minas Gerais, Brazil) was dried and pulverized in a pilot plant of the Department of Food Technology, Federal University of Viçosa, Minas Gerais, Brazil. The permeate powder was reconstituted with distilled water to lactose concentrations ranging from 50 to 250 g l^{-1}. Permeate was sterilized by filtration (0.22 μm pore size) and added to the culture medium. The YNB medium was prepared according to the manufacturer's instructions. Lactose (Sigma®, St. Louis, USA) was separately sterilized when necessary at 121 °C for 20 min. The *C:N* ratio was maintained at 10:1 when the cells were cultured in YNB medium, with ammonium sulfate, $(NH_4)_2SO_4$, used as a nitrogen source. All media were buffered using citrate-phosphate buffer (100 mmol l^{-1} citrate, 200 mmol l^{-1} Na_2HPO_4) at predetermined pH values ranging from 4.5 to 6.5.

2.3. Fermentation conditions

The fermentations were performed in 50 ml test tubes containing 20 ml of fermentation medium, and the tubes were sealed with silicone plugs to reduce oxygen permeability. The test tubes were kept in a water bath for 144 h without agitation. Different combinations of lactose concentration, initial cell biomass concentration, temperature, and pH were used in this study (Table 1). All culturing was performed in hypoxic conditions under nitrogen gas (99.9%, v v^{-1}) following a 15 min purge after initial cell biomass inoculation. Samples were taken from all of the fermentations every 24 h to determine cell growth, lactose consumption, and ethanol production. The pH was measured at the end of each experiment to test the effectiveness of the buffer used.

2.4. Cell growth and the relationship between absorbance at 600 nm (A_{600}) and dry cell biomass concentration (g l^{-1})

To analyze cell growth, a BECKMAN DU 600 spectrophotometer was used at 600 nm wavelength. One unit of A_{600} was found to be equivalent to 0.507 g l^{-1} of dry cell biomass of *K. marxianus* UFV-3 (Diniz et al., 2012).

2.5. Primary metabolite analysis

Samples taken during the various fermentations were centrifuged at 13,200*g* for 5 min, and the supernatants were collected and frozen at −20 °C. To determine the levels of lactose, ethanol, and glycerol, 20 μl of supernatant from the samples was applied to a high performance liquid chromatography (HPLC) system (HP 1050 M Hewlett Packard 1050 series, HP 1047 A detector, using a BIO-RAD Aminex HPX-87 H column (300 × 7.8 mm^2)) with 5 mmol l^{-1} H_2SO_4 eluent, a flow rate of 0.7 ml min^{-1}, and a column temperature of 25 °C.

2.6. Determining fermentation parameters

Since the maintenance coefficient and maintenance yield were fixed at zero, the ethanol production by lactose consumed, designated as response factor (RF) and the fermentative parameters ethanol yield with cell mass concentration ($Y_{E/X}$) and volumetric productivity (Q_p) were determined:

$$RF = [(E_f - E_i)/(L_i - L_f)]/4 \quad (1)$$

$$Y_{E/X} = (E_f - E_i)/X_m \quad (g\ g^{-1}) \quad (2)$$

$$Q_p = (E_f - E_i)/h \quad (g\ l^{-1}\ h^{-1}) \quad (3)$$

where E_i is the initial ethanol concentration (g l^{-1}), E_f is the final ethanol concentration (g l^{-1}), L_i is the initial lactose concentration (g l^{-1}), L_f is the final lactose concentration (g l^{-1}), X_m is the average cell biomass concentration in the medium (g l^{-1}), and h is the time (h).

The theoretical ethanol yield is 0.538 g per 1 g of lactose consumed.

2.7. Experimental design and validation of methods

The design of this study consisted of two steps: (i) a preliminary analysis of the factors that influence the fermentative behavior of *K. marxianus* UFV-3 in synthetic YNB medium and (ii) the determination of the effects of these factors on the fermentation process in CWP and a subsequent optimization and validation of the process's operating conditions.

To determine the effects of the four factors on ethanol production, we proposed a CCRD ($2^K + 2K + 5$, where K is the number of factors) with a total of 29 experimental units and five replicates at the central point. The 25 different experimental arrangements are listed in Table 1. The factors that were investigated, pH, temperature, lactose concentration, and cell biomass concentration, were selected due to their known effects on the production of ethanol by *K. marxianus* UFV-3 (Silveira et al., 2005; Diniz et al., 2012). The experiment was initially performed in YNB medium. After confirming the significance of the factors' effects and optimizing their operational ranges, the fermentations were performed in cheese whey permeate. The CCRD was designed using the Minitab®16.0 software, and the assays were randomized to avoid any experimental or technical bias. The fermentation process was monitored every 24 h. This experimental design allowed for the fitting of a quadratic model to estimate the response factor (RF), Eq. (1), using the factors pH, temperature, lactose concentration,

Table 1
Central composite rotational design (CCRD) conducted to optimize fermentation by *Kluyveromyces marxianus* UFV-3 in YNB medium containing lactose and cheese whey permeate. The factors evaluated were pH, temperature (T), lactose concentration (L), and cell mass concentration (C). The following fermentation parameters were analyzed: relationships between ethanol production by lactose consumed (RF), ethanol yield with cell biomass concentration ($Y_{E/X}$), and maximum volumetric productivity (Q_p). The value in parentheses in column Q_p is the time (hours) which occurred maximum productivity.

Run	pH	Temperature (°C)	Lactose concentration ($g\,l^{-1}$)	Cell mass concentration (A_{600})	YNB medium containing lactose			Cheese whey permeate		
					RF	$Y_{E/X}$ ($g\,g^{-1}$)	Q_p ($g\,l^{-1}\,h^{-1}$)	RF	$Y_{E/X}$ ($g\,g^{-1}$)	Q_p ($g\,l^{-1}\,h^{-1}$)
01	5.5	37.50	150	3.0	0.944	51.431	1.212 (24)	0.768	24.549	0.899 (48)
02	5.5	37.50	150	2.0	0.980	80.904	1.906 (24)	0.570	17.545	0.787 (24)
03	4.5	37.50	150	3.0	0.810	42.403	0.938 (72)	0.617	18.616	0.489 (48)
04	5.5	37.50	250	3.0	0.427	37.077	0.865 (24)	0.318	14.408	0.462 (24)
05	5.5	45.00	150	3.0	0.429	21.974	1.421 (24)	0.110	3.404	0.309 (24)
06	5.5	37.50	150	3.0	0.937	63.098	1.649 (24)	0.777	26.766	0.818 (48)
07	5.5	37.50	150	3.0	0.895	54.531	1.988 (24)	0.767	27.701	0.760 (48)
08	5.5	37.50	150	3.0	0.960	51.855	1.571 (48)	0.723	27.043	0.724 (72)
09	6.5	37.50	150	3.0	0.682	31.662	0.959 (48)	0.459	12.210	0.506 (24)
10	5.5	37.50	150	4.0	0.911	41.111	1.732 (24)	0.571	17.546	0.959 (24)
11	5.5	30.00	150	3.0	0.992	54.220	1.206 (48)	0.573	17.531	0.821 (24)
12	5.5	37.50	50	3.0	0.902	13.820	1.140 (24)	0.784	7.092	0.601 (24)
13	5.5	37.50	150	3.0	0.908	44.671	1.779 (24)	0.745	27.214	0.703 (48)
14	6.0	41.25	100	2.5	0.303	20.653	0.260 (72)	0.740	13.179	0.565 (24)
15	5.0	33.75	100	3.5	0.925	32.979	0.489 (48)	0.839	22.745	0.793 (48)
16	5.0	41.25	200	2.5	0.213	23.924	0.446 (24)	0.282	13.592	0.453 (48)
17	6.0	33.75	100	2.5	0.599	33.053	0.369 (120)	0.831	17.491	0.495 (24)
18	5.0	41.25	200	3.5	0.197	20.526	0.403 (48)	0.297	14.513	0.522 (24)
19	5.0	33.75	200	2.5	0.270	25.057	0.405 (24)	0.483	23.478	0.504 (72)
20	5.0	41.25	100	3.5	0.591	20.737	0.545 (24)	0.777	18.249	0.792 (24)
21	5.0	33.75	100	2.5	0.862	37.579	0.415 (72)	0.862	22.854	0.586 (48)
22	5.0	33.75	200	3.5	0.338	20.280	0.311 (120)	0.446	21.703	0.486 (24)
23	6.0	41.25	200	3.5	0.054	3.243	0.05 (120)	0.233	9.393	0.364 (24)
24	6.0	33.75	200	2.5	0.087	6.327	0.08 (120)	0.310	12.341	0.356 (24)
25	6.0	33.75	200	3.5	0.096	5.928	0.091 (96)	0.900	15.802	0.480 (24)
26	6.0	33.75	100	3.5	0.712	33.160	0.554 (72)	0.561	12.656	0.423 (48)
27	6.0	41.25	200	2.5	0.077	6.149	0.177 (48)	0.198	7.863	0.325 (24)
28	6.0	41.25	100	3.5	0.290	11.106	0.251 (72)	0.363	16.880	0.638 (48)
29	5.0	41.25	100	2.5	0.572	21.033	0.532 (48)	0.740	14.441	0.562 (24)

and cell biomass concentration, as given by:

$$RF = \beta_0 + \Sigma_{i=1}{}^{4}\beta_{ii}X_i + \Sigma_{i=1}{}^{4}\beta_{ii}X^2{}_i + \Sigma_{i=1}{}^{3}\Sigma_{j=i+1}{}^{4}\beta_{ij}X_iX_j \quad (4)$$

where β_0 is the constant; x_i are the variables in natural scale (not coded); β_i (i=1–4) and β_{ii} are the coefficients corresponding to linear and quadratic terms, respectively; and β_{ij} (i and j=1–4) are the second-order interaction coefficients. The data were analyzed using the F test for regression (analysis of variance), and the polynomial model, Eq. (4), was fitted based on the significance (α=0.05) of the coefficients according to the t-test. Statistical analyses were performed using the Minitab® 16.0 software. The quality of the model fit was expressed by the coefficient of determination, R^2 and by the statistical significance of regression and of lack-of-fit from the analysis of variance. In addition, the model for the fermentation of cheese whey permeate was validated through bias and accuracy factors (Baranyi et al., 1999), as described in the following equations:

$$\text{Bias factor } (F_B): \quad F_B = 10^{(\Sigma \log(P/O)/n)} \quad (5)$$

$$\text{Accuracy factor } (F_A): \quad F_A = 10^{(I \log(P/O)I/n)} \quad (6)$$

The bias factor is related to the reliability of predicting the response, and values lower than 1 indicate reliable models. The Accuracy Factor examines whether the model has adequate accuracy, and acceptable values should be close to 1. This value is appropriate for comparing two or more models (Baranyi et al., 1999). The model was validated under conditions in which it was possible to obtain RF values close to 1, thus favoring maximum ethanol yield. The following fermentation conditions were used for validating the equation: temperature, 37 °C; pH, 5.4; cell biomass concentration, A_{600} 3.4; and lactose concentration, 94 $g\,l^{-1}$.

3. Results and discussion

Initially, the factors that affect the fermentative behavior of *K. marxianus* UFV-3 and the functional ranges of their values were investigated in synthetic YNB medium. Table 1 shows the experimental conditions for the fermentations performed and the RF that corresponds to the relationship between ethanol yield and lactose consumed, Eq. (1). In addition to the RF, other fermentation parameters were calculated, such as ethanol produced per gram of cell mass ($Y_{E/X}$) and maximum volumetric productivity (Q_p). In preliminary analyses performed in minimal medium, the RF values ranged from 0.054 to 0.992, indicating that the process can be optimized within the range studied for each fermentation factor. In most of the experiments, the Q_p was higher at 24 and 48 h of fermentation than at the other periods evaluated. This result indicates that the process is efficient in the early stages of fermentation, which is desirable for industrial fermentation processes. Analysis of the $Y_{E/X}$ values revealed that the factors evaluated influence the fermentative metabolism of *K. marxianus* UFV-3, as the $Y_{E/X}$ values variation were 24.94 fold under certain conditions. A quadratic model was fitted (p-value=0.001, R^2=86.46%) for this preliminary analysis, in which the factors temperature (T) and pH exhibited significant linear and quadratic coefficients and lactose concentration (L) exhibited only significant quadratic:

$$RF_{(syntheticmedium)} = -11.766^* + 0.461^*(T) + 1.698^*(\text{pH}) + 0.001(L) - 0.006^*(T^2) - 0.163^*(\text{pH}^2) - 0.000^*(L^2) \quad (7)$$

All significant coefficients are marked with an asterisk. The results of the ANOVA, t-test and F test used in fitting the model,

Eq. (7), are summarized in Table 2. The high RF values (close to 1) from some conditions used in the preliminary analysis using minimal medium containing lactose suggested that similar conditions may also result in high RF values in cheese whey permeate (CWP). Fig. 1 shows the relationship between the RF values predicted by the fitted model, Eq. (7), and the values obtained in the fermentations. The R value (correlation coefficient) was 0.93 (Fig. 1a) for the linear relationship between the predicted and observed values in synthetic medium, superior to that observed in other studies on the optimization of ethanol production from CWP (Aktaş et al., 2006; Dragone et al., 2011; Sansonetti et al., 2010).

After testing the significance of the factors that affect the fermentation process in minimal medium and confirming their range of values, a new CCRD was conducted to obtain an empirical model for optimizing the fermentation conditions in CWP. Table 1 shows the combinations of the levels of factors tested and the respective responses obtained. As in the synthetic medium, all of the fermentations in CWP were performed under hypoxic conditions similar those under which most industrial fermentations are performed.

The RF values in the CWP ranged from 0.2 to 0.9 (Table 1) and the $Y_{E/X}$ values were more homogeneous than synthetic medium with 8.14-fold variation. As observed in the synthetic medium, the Q_p was higher in the permeate particularly at 24 and 48 h of fermentation. In some cases, the RF values obtained in the present study with *K. marxianus* UFV-3 were higher than those found with other *K. marxianus* strains (Guimarães et al., 2010). However, the lack of standardization among bioreactors makes identifying the real differences between strains difficult, indicating a need for constructing validated empirical models for comparing fermentative capacities between different yeast strains.

The fitted model, Eq. (8), for permeate was more complete and significant (p-value=0.000, R^2=90.10%) than that derived in synthetic medium, and the factors temperature (T), pH, and cell biomass concentration (C) showed significant linear and quadratic coefficients and lactose concentration (L) showed significant quadratic coefficients, as follows:

$$RF_{(permeate)} = -14.991^{*} + 0.505^{*}(T) + 2.060^{*}(pH) + 0.002(L) + 0.957^{*}(C) - 0.007^{*}(T^2) - 0.197(pH^2) - 0.000^{*}(L^2) - 0.165^{*}(C^2) \quad (8)$$

The R^2 value observed for this fit was higher than those obtained in different types of cheese whey used in other studies (Aktaş et al., 2006; Dragone et al., 2011; Sansonetti et al., 2010).

The RF values predicted by the fitted model, Eq. (8), showed a high linear correlation (R=0.95) with the values obtained in the fermentation trials using cheese whey permeate (Fig. 1b), which was a considerably high value for experiments on fermentation processes.

Figs. 2–4 show the effects of the different factors on RF from fermenting CWP. Fig. 2 reveals that temperature (the factor with greatest effect on the RF) interacts with pH and cell biomass concentration. In both graphs, the 33–42 °C temperature range shows the highest RF values. As the temperature approaches 45 °C, there is a trend of decreasing RF. The RF values were higher at temperatures above 30 °C. This result concurs for adopting *K. marxianus* UFV-3 in the ethanol industry, because at temperatures above 30 °C, the traditionally *Saccharomyces cerevisiae*, used in alcoholic fermentation, loose viability (Olivério et al., 2010). Because the temperature in the fermentation vats may increase by up to 10°C during the period of greatest cell metabolic activity, leading to a loss of cell viability in traditional yeasts (Ghaly and Kamal, 2004). Therefore, using thermotolerant yeasts capable of fermenting at high temperatures, such as *K. marxianus* UFV-3, is appropriate, especially considering the costs associated with the cooling process for fermentation vats. It is estimated that depending on the fermentation process, fermentations at temperatures 5 °C above 30 °C can save approximately US$ 30,000.00 annually for a medium-sized factory (Babiker et al., 2010). *K. marxianus* UFV-3 exhibits RF close to the theoretical values at temperatures up to 5 °C higher than the other *K. marxianus* strains already studied (Aktaş et al., 2006; Dragone et al., 2011; Sansonetti et al., 2010). Both pH (Fig. 2a) and cell biomass concentration (Fig. 2b) showed a wide operational range that allowed for robust RF values close to 0.8, allowing for the moderate variations that are common in fermentation processes without much loss of efficiency.

Although lactose concentration showed the lowest significance value among all of the factors studied, inter-factor analysis of the relationships lactose-cell concentrations (Fig. 3a) and lactose concentration-pH (Fig. 3b) revealed that variations in lactose concentration resulted in higher variations in the RF. RF was high

Table 2
Analysis of variance – ANOVA – of the adjusted model using the Minitab® 16.0 software for the response factor (RF) – relationship between ethanol production and lactose consumed.

Source	Medium YNB with lactose					Cheese whey permeate				
	DF	SS	MS	*F*	*p*	DF	SS	MS	*F*	*p*
Regression	6	1.156	0.193	21.290	0.000	8	1.249	0.156	20.470	0.000
Linear	3	0.909	0.073	8.040	0.001	4	0.912	0.066	8.910	0.000
Square	3	0.247	0.082	9.090	0.001	4	0.293	0.073	9.940	0.000
Residual Error	20	0.181	0.009			18	0.132	0.007		
Lack-of-Fit	8	0.091	0.011	1.52	0.249	14	0.130	0.009	19.210	0.006
Pure Error	12	0.090	0.008			4	0.002	0.000		
Total	26	1.337				26	1.337			
Term	**DF**	**Coef**	**SE Coef**	***t***	***p***	**DF**	**Coef**	**SE Coef**	***t***	***p***
pH	1	0.048	0.040	4.400	0.049	1	0.048	0.056	7.670	0.013
T	1	0.181	0.199	22.010	0.000	1	0.181	0.230	31.320	0.000
L	1	0.680	0.002	0.200	ns	1	0.680	0.007	0.940	ns
C	ns	ns	ns	ns	ns	1	0.003	0.041	5.550	0.030
pH^2	1	0.015	0.044	4.940	0.038	1	0.015	0.062	8.470	0.009
T^2	1	0.194	0.218	24.030	0.000	1	0.200	0.250	33.930	0.000
L^2	1	0.038	0.038	4.210	0.054	1	0.040	0.055	7.430	0.014
C^2	ns	ns	ns	ns	ns	1	0.043	0.044	5.950	0.025

T: temperature; L: lactose concentration; C: cell mass concentration; pH^2: pH × pH; T^2: temperature × temperature; L^2: lactose concentration × lactose concentration; C^2: cell mass concentration × cell mass concentration; ns: not significant; DF: degree of freedom; SS: square sum; MS: mean square; F: F test value; p: p-value; Coef: adjusted coefficient; SE Coef: coefficient standard deviation t: t-test value.

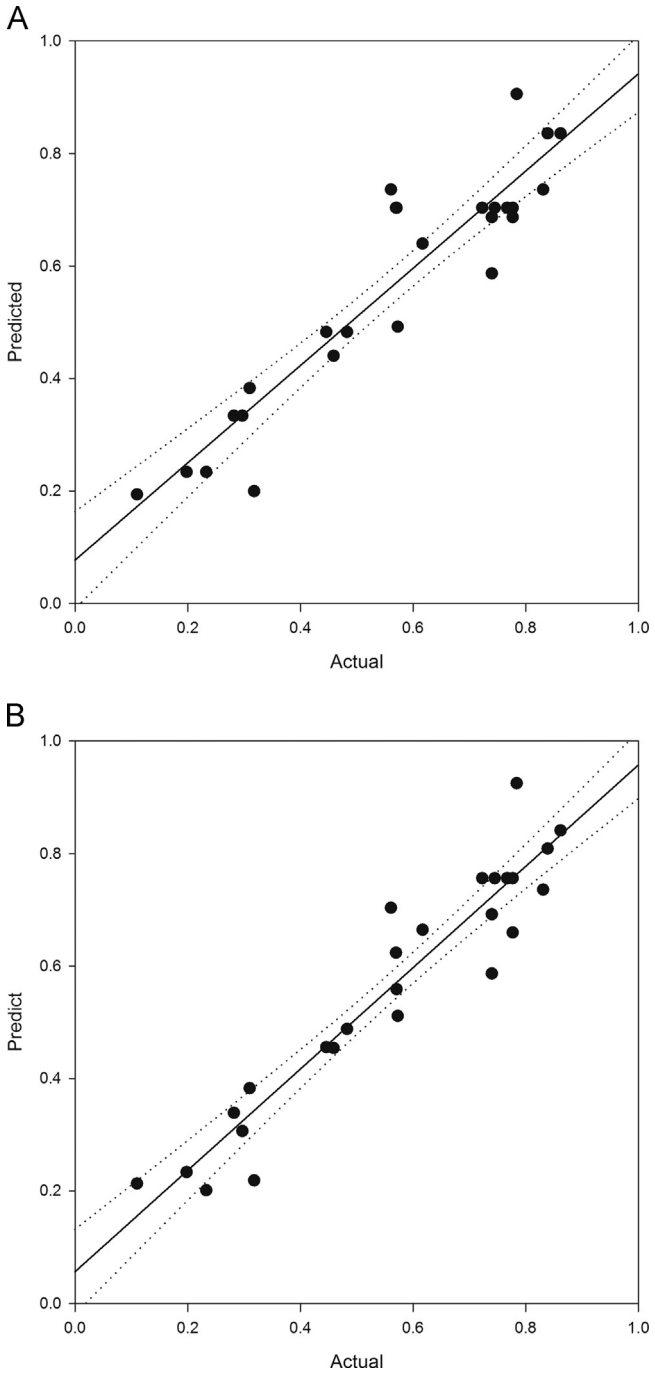

Fig. 1. Predicted values and actual values for the RF (relationship between ethanol production and lactose consumed). (A) YNB medium containing lactose and (B) cheese whey permeate. The dotted lines indicate the 95% confidence interval.

at lactose concentrations near 120 g l^{-1}. At concentrations above 120 g l^{-1}, there was a progressive decrease in the RF, reaching minimal values when the lactose concentration was above 220 g l^{-1}.

RF was higher at cell biomass concentrations lower than 3.5 A_{600} (Fig. 3a) and was high within the 4.5–6 pH range (Fig. 3b). Depending on the type of cheese that is produced, sweet or sour, the pH of the permeate/cheese whey will vary (Guimarães et al., 2010). The ability of *K. marxianus* UFV-3 to efficiently convert lactose from the CWP into ethanol in a pH range common to different types of cheese whey can be considered an advantage from an industrial perspective because there is no need for prior correction of substrate pH.

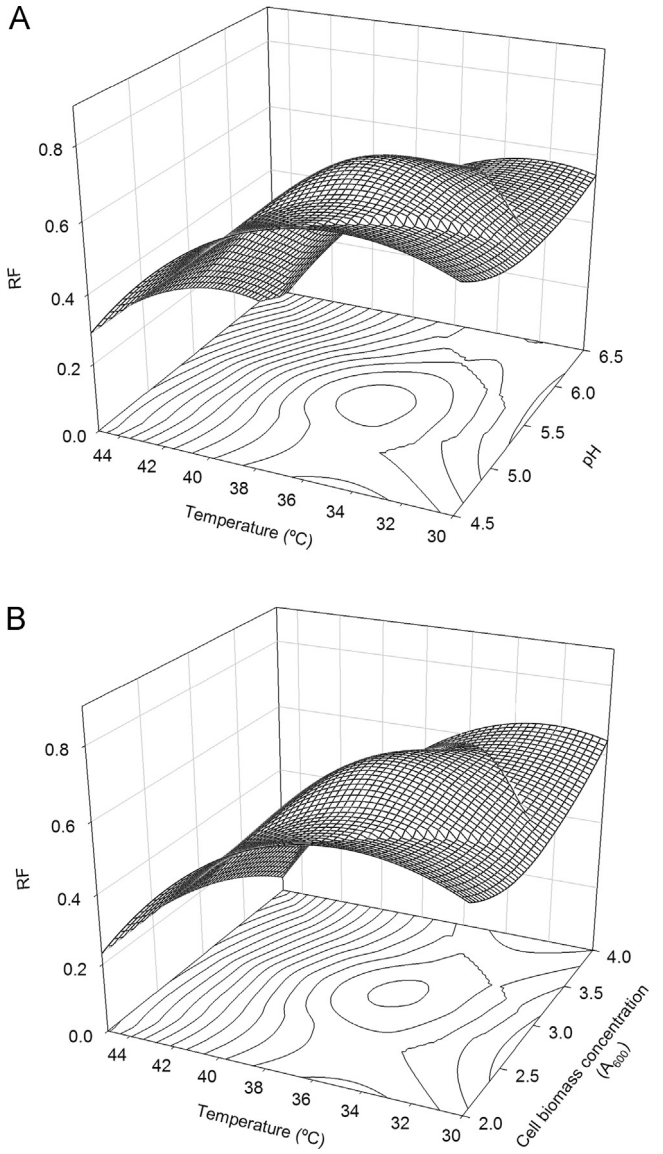

Fig. 2. Surface response for the RF as a function of temperature and pH (A) and temperature and cell biomass concentration (B) from cheese whey permeate (CWP).

Fig. 4a shows that the variation in the factors temperature and lactose concentration significantly alters the RF. Within the ranges of temperature from 33 to 38 °C and lactose concentration from 50 to 120 g l^{-1}, RF is close to the theoretical value. In contrast, at high temperatures and lactose concentrations, minimal yields are attained. In general, the different *K. marxianus* strains analyzed recently, such as MSR Y-8281, CBS 397, and *Kluyveromyces fragilis* (Kf1) (Aktaş et al., 2006; Dragone et al., 2011; Sansonetti et al., 2010), show higher RF in lactose concentrations near or below 80 g l^{-1}. RF near the theoretical values obtained by *K. marxianus* UFV-3 in CWP with a lactose concentration near 100 g l^{-1} are promising when considering the economic viability of using permeate as the raw material for producing ethanol. Economic feasibility studies show that ethanol production from cheese whey becomes economically viable when it is concentrated two-fold to achieve a lactose concentration near 100 g l^{-1} (Mawson 1994). It is to be noted that significant variations in the RF were only observed at cell biomass concentrations near A_{600} 4 and pH values near 4.5 or 6.5 (Fig. 4b). A weaker response to the cell biomass concentration factor was also observed in ethanol production from cheese whey by *K. fragilis* Kf1 (Dragone et al., 2011). Apparently,

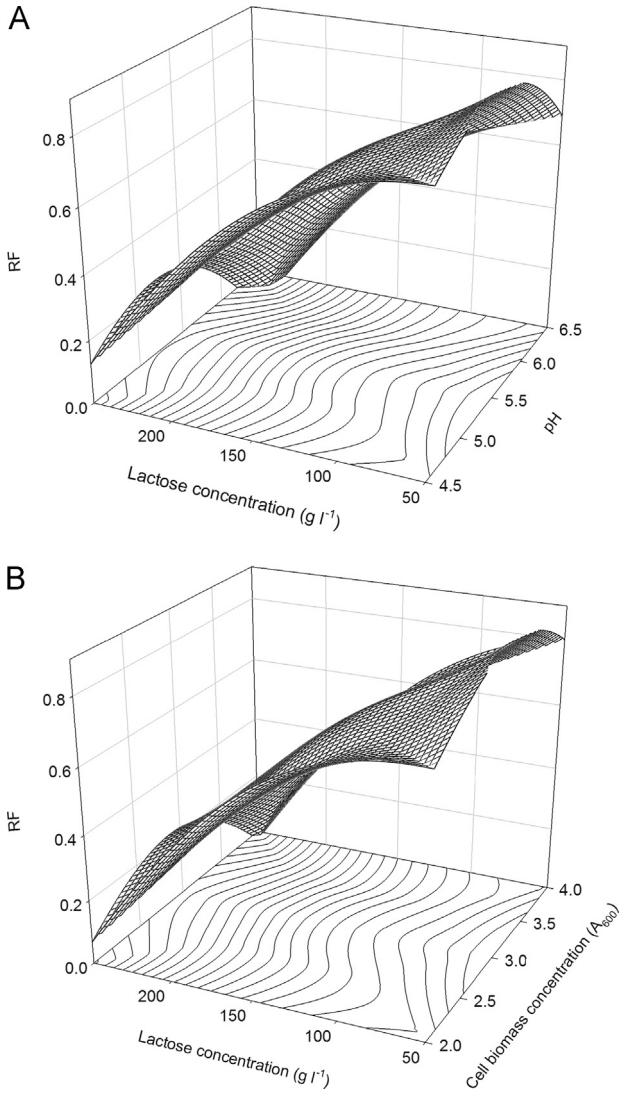

Fig. 3. Surface response for the RF as a function of lactose concentration and cell biomass concentration (A) and lactose concentration and pH (B) from cheese whey permeate (CWP).

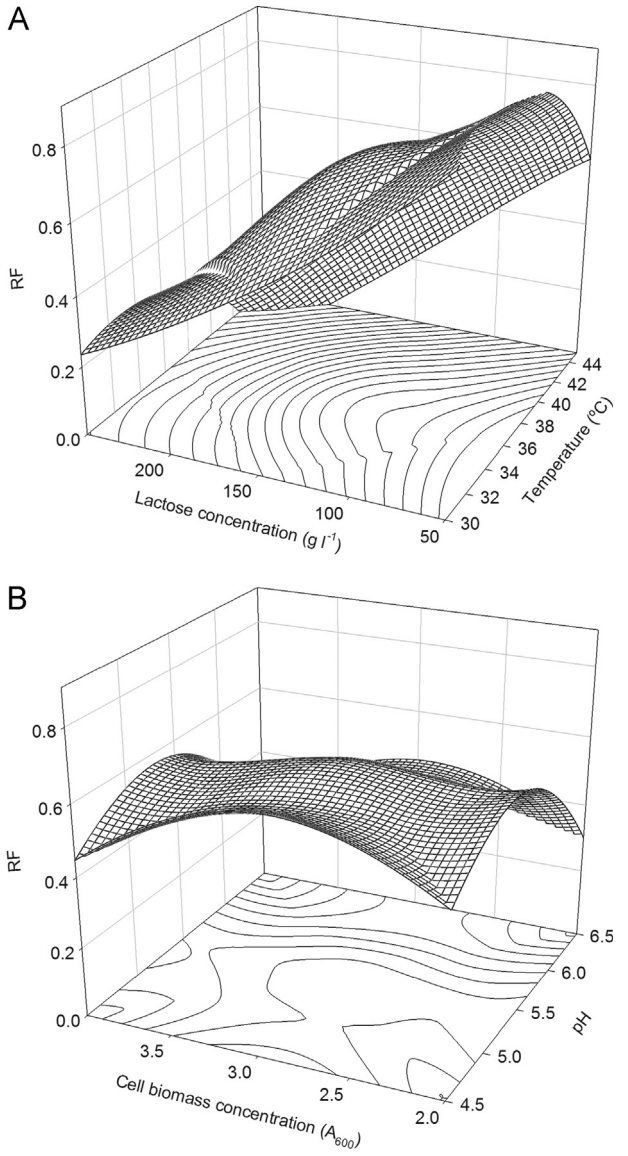

Fig. 4. Surface response for the RF as a function of temperature and lactose concentration (A) and pH and cell biomass concentration (B) from cheese whey permeate (CWP).

cell biomass concentration only affects the rate at which lactose is converted into ethanol and does not increase the conversion efficiency. In fact, recent studies with the *K. marxianus* DSMZ 7239 strain found that lower cell mass concentrations led to longer lag phases before initial ethanol production but without significant changes in ethanol yields (Christensen et al., 2011).

Despite the consensus in the scientific community regarding the potential for using *K. marxianus* under industrial conditions, there are few studies on optimizing these processes and validating the resulting optimization models. In the present study, the bias and accuracy factors were evaluated (Table 3) to test the reliability and suitability of the fitted model for predicting RF values in optimizing the fermentation parameters for CWP. Seven fermentations were performed under the following conditions: temperature, 37 °C; pH, 5.4; lactose concentration, 94 g l^{-1}; and cell biomass concentration, A_{600} 3.4. The values obtained for the bias factor (0.944) and the accuracy factor (1.060) indicate that the model is reliable and suitable for estimating the RF values of this process and that modulations of the factors pH, temperature, lactose concentration, and cell concentration all contribute to maximizing the RF in optimizing ethanol production from cheese whey permeate.

Table 3
Validation of the quadratic model (Eq. (8)) obtained for optimizing ethanol production from cheese whey permeate by *Kluyveromyces marxianus* UFV-3. The culturing conditions were as follows: temperature, 37 °C; pH, 5.4; lactose concentration, 95 g l^{-1}; and cell biomass concentration, A_{600} 3.4.

Run	Actual RF	Relation actual/predicted RF (%)	Bias factor	Accuracy factor
01	0.954	8.305		
02	0.917	4.097		
03	0.919	4.332		
04	0.969	9.933		
05	0.948	7.649		
06	0.925	4.975		
07	0.904	2.613		
			0.944	1.060

4. Conclusions

Temperature, pH, lactose concentration, and cell biomass concentration are factors that significantly affect the fermentation of cheese whey permeate by *K. marxianus* UFV-3. Optimization of the

RF values through factors that affect the fermentation process produced ethanol yields above 90%. The quadratic response model was adequately validated and may be used for guiding the scaling of the fermentation process. The ranges in values that obtained RF above 90% were as follows: temperatures between 33.3 and 38.5 °C, pH between 4.7–5.7, lactose concentrations between 50–108 g l^{-1}, and cell biomass concentrations between A_{600} 2.4–3.3.

The results indicated that using *K. marxianus* UFV-3 to convert lactose from cheese whey permeate into ethanol is promising because yields close to the theoretical value were achieved over a range of temperatures, pH values, and lactose concentrations, all of which are considered crucial to the economic feasibility of using the permeate as a raw material for ethanol production.

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