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Optimization of ethanol production from cheese whey powder by *Kluyveromyces fragilis* using factorial design and response surface methodology

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Abstract

The individual and combined effects of initial lactose concentration, temperature and inoculum concentration on ethanol production from cheese whey powder by *Kluyveromyces fragilis* were investigated. A 2^3 full-factorial central composite design (CCD) and response surface methodology (RSM) were employed in order to determine the optima conditions that maximize the ethanol production. Statistical analysis of results showed that, in the range studied, only the initial lactose concentration had a significant effect on ethanol production. Response surface data showed maximum ethanol production at inoculum concentration between 1 and 3 g/L and temperature between 25 and 35ºC when the initial lactose concentration was 150 g/L.

1 Introduction

The dairy industry represents an important part of the food processing industry and contributes significant liquid wastes that can be used for the production of ethanol (Ghaly and El-Taweel, 1997). Cheese whey (CW), a by-product of the cheese manufacturing process whose major components are lactose (44-52 g/L), proteins (6-8 g/L) and mineral salts (4-9 g/L), constitutes an inexpensive and nutritionally rich raw material for the production of ethanol by fermentation. However, the production of ethanol from unconcentrated CW is not economically feasible because the levels of ethanol obtained at the end of fermentation reach only about 2%, making the distillation process too expensive. Otherwise, costs are significantly reduced with the increase of lactose concentration up to about 100-120 g/L lactose (González Siso, 1996). Therefore, dry cheese whey powder (CWP) may be an attractive raw material for ethanol production. CWP is a dried and concentrated form of CW and contains lactose in addition to nitrogen, phosphate and other essential nutrients (Kargi and Ozmihci, 2006). The use of CWP instead of CW for ethanol fermentations has significant advantages such as elimination of costly ultrafiltration processes to concentrate lactose before fermentation, compact volume, long-term stability and high concentrations of lactose and other nutrients yielding high ethanol concentrations by fermentation (Ozmihci and Kargi, 2007a).

Optimization of fermentation conditions by the classical method involves changing one independent variable while fixing all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables (Adinarayana et al., 2003) and also may result in wrong conclusions (Oh et al., 1995). Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of

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factors, and searching optimum conditions of factors for desirable responses (Li et al., 2001). Although this method was successfully applied in many areas of biotechnology, there is no report in the literature describing the influence of process parameters on the production of ethanol from CWP through RSM.

The present study aimed to optimize the conditions for ethanol production from CWP through RSM designed with composite central design (CCD). Three factors were selected as process (independent) parameters: initial lactose concentration, temperature and inoculum concentration, while ethanol concentration was selected as response (dependent parameter).

2 Material and Methods

2.1 Microorganism and inoculum preparation

The yeast strain *Kluyveromyces fragilis* (Kf 1) used in this work was obtained from the culture collection of the Centre of Biological Engineering, University of Minho (Portugal) and maintained on YPD agar plates at 4ºC.

The inoculum culture was prepared by transferring a loopful of cells from a freshly grown culture (incubated at 30ºC for 30 h) into 500 mL Erlenmeyer flasks containing 100 mL sterile CWP solution (50 g/L lactose). Incubation was carried out on a rotary shaker (200 rpm) at 30ºC for 24 h.

2.2 Fermentation conditions

Batch experiments were performed in 500 mL Erlenmeyer flasks containing 100 mL of medium composed by CWP solution with initial pH adjusted to 5 with citric acid (1 M). The flasks were maintained in an orbital shaker at 150 rpm for 44 h. The initial lactose concentration, temperature and inoculum concentration were considered according to the experimental design showed in Table 1. All experiments were performed in duplicate.

2.3 CWP solutions preparation

Cheese whey powder (CWP) was kindly supplied by Lactogal (Porto/Portugal). Deproteinization was carried out by heat treatment (115ºC, 15 min) of acidified (pH 5) CWP solutions with different initial lactose concentrations. The precipitates were removed by centrifugation at 8500 rpm and 10ºC for 15 min and the supernatant was used as fermentation medium.

2.4 Analytical methods

The fermented media samples were centrifuged at 4000 rpm for 10 min and the supernatant was used to quantify the lactose and ethanol concentrations. Remaining solid was washed once with distilled water and centrifuged and then, diluted with distilled water for analysis of biomass. The biomass concentration was monitored spectrophotometrically at 600 nm and estimated from a biomass dry weight vs. absorbance calibration curve obtained previously. The lactose and ethanol concentrations in the supernatant were quantified by high-performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with a refractive index (RI) detector (Jasco 830-RI) and a Chrompack column (300 x 6.5 mm) at 60ºC, using 5 mM sulfuric acid as the eluent at a flow rate of 0.5 mL/min and a sample volume of 20 μl.

2.5 Experimental design

In order to optimize process parameters, a central composite design (CCD) with three factors at three levels was performed. Experimental results of the CCD were fitted with a second-order polynomial equation by a multiple regression analysis. The quadratic model for predicting the optimal point was expressed as follows:

$$Y = C_0 + \sum_{i=1}^{3} C_i x_i + \sum_{i=1}^{3} C_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j<i} C_{ij} x_i x_j \quad (1)$$

where Y is the response (ethanol concentration), C_0, C_i, C_{ii}, and C_{ij} are constant coefficients, and x_i and x_j are the coded independent factors. The independent factors were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (2)$$

where x_i is the coded value of the ith independent variable, Xi the real value of the ith independent variable, X_0 the original value of the ith independent variable at the center point, and ΔXi the step change value (50 for initial lactose concentration, 5 for temperature and 1 for inoculum concentration).

The quality of the fitted polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was checked by the F-test. The significance of the regression coefficients was tested by t-value. Results were analyzed by the Experimental Design Module of the Statistica 5.0 software (Statsoft, USA). The model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variable.

3 Results and Discussion

A central composite design for the three factors (initial lactose concentration, temperature and inoculum concentration), each at three levels and three replicates at the center (to account for pure internal error), was applied for optimizing ethanol production in shake flasks. The design matrix for these factors in the optimization runs is noted in Table 1.

The results presented in Table 1 for ethanol production were subjected to analysis of regression and analysis of variance (ANOVA) (Table 2). After applying the ANOVA statistical test, it was found that, in the range studied, only the linear and quadratic effect of initial lactose concentration had a significant effect on ethanol production at 95% confidence level. The lack-of-fit test was insignificant (p = 0.066), and the regression equation obtained presented a R^2 value of 0.968 (R^2 > 0.75 indicates the aptness of the model), suggesting that a second-order model accurately represents the data in the experimental region studied, explaining 96.8% of the variability in the response. The following regression equation was obtained:

$$Y = 27.5 + 17.9\, x_1 + 3.8\, x_1^2 \quad (3)$$

where Y is the ethanol concentration (response) and x_1 is the initial lactose concentration (coded independent factor). The significance of each coefficient was determined by Student's t-test and p-values, which are listed in Table 2. The larger the magnitude of the t-value and smaller the p-value, more significant is the corresponding coefficient.

Table 1. Coded levels (C) and real values (R) of the variables in central composite design: lactose (X_1, g/L); temperature (X_2, ºC); inoculum (X_3, g/L); ethanol (Et); ethanol per biomass yield factor ($Y_{P/X}$); ethanol yield factor ($Y_{P/S}$).

Run	Design matrix						Experimental results		
	X_1		X_2		X_3		Et (g/L)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)
	C	R	C	R	C	R			
1	-1	50	-1	25	-1	1	12.7	3.21	0.25
2	-1	50	-1	25	+1	3	13.4	3.39	0.26
3	-1	50	+1	35	-1	1	12.0	2.65	0.27
4	-1	50	+1	35	+1	3	13.4	3.04	0.35
5	+1	150	-1	25	-1	1	48.2	7.49	0.34
6	+1	150	-1	25	+1	3	47.0	7.43	0.36
7	+1	150	+1	35	-1	1	48.5	7.57	0.35
8	+1	150	+1	35	+1	3	41.5	8.80	0.28
9	-1	50	0	30	0	2	10.3	2.22	0.22
10	+1	150	0	30	0	2	55.9	8.01	0.37
11	0	100	-1	25	0	2	28.8	4.78	0.33
12	0	100	+1	35	0	2	24.5	4.51	0.28
13	0	100	0	30	-1	1	32.4	4.91	0.33
14	0	100	0	30	+1	3	28.8	4.62	0.29
15	0	100	0	30	0	2	26.1	3.96	0.30
16	0	100	0	30	0	2	25.6	3.98	0.30
17	0	100	0	30	0	2	23.8	3.68	0.29

Table 2. Analysis of variance (ANOVA) and coefficient estimates for second-order model.

Source of variation	Degree of freedom	Sum of squares	Mean square	*F*-value	*p*-value
X_1	1	3214.849	3214.849	2196.936	0.000*
X_1^2	1	39.105	39.105	26.723	0.035*
X_2	1	10.404	10.404	7.110	0.117
X_2^2	1	18.526	18.526	12.660	0.071
X_3	1	9.409	9.409	6.430	0.127
X_3^2	1	4.671	4.671	3.192	0.216
X_1X_2	1	2.531	2.531	1.730	0.319
X_1X_3	1	13.261	13.261	9.062	0.095
X_2X_3	1	3.251	3.251	2.222	0.275
Lack of fit	5	106.254	21.251	14.522	0.066
Pure error	2	2.927	1.463		
Total	16	3422.749			

Factors	Degree of freedom	Regression coefficient	Standard error	*t*-value	*p*-value
Mean/Intercept	1	27.5	0.52	53.16	0.000
X_1	1	17.9	0.38	46.87	0.000
X_1^2	1	3.8	0.74	5.17	0.035
X_2	1	-1.0	0.38	-2.67	0.117
X_2^2	1	-2.6	0.74	-3.56	0.071
X_3	1	-1.0	0.38	-2.54	0.127
X_3^2	1	1.3	0.74	1.79	0.216
X_1X_2	1	-0.6	0.43	-1.32	0.319
X_1X_3	1	-1.3	0.43	-3.01	0.095
X_2X_3	1	-0.6	0.43	-1.49	0.275

$R^2 = 0.968$

* Values significant at 95% confidence level

The relation between factors and response can best be understood by examining surface plots as a function of two factors at a time and holding the other factor at fixed level. The three-dimensional response surface curves were then plotted (Fig. 1). Figure 1 shows that increasing initial lactose concentration resulted in higher ethanol production, with maxima values (≥41.5 g/L) at maximum concentration tested (150 g/L). Previous report on ethanol production from cheese whey powder found that the ethanol concentration was proportionally increased with the increase in initial sugar concentration up to 75 g/L (Ozmihci and Kargi, 2007b). The maxima ethanol amounts produced in the present study were higher than those obtained by direct fermentation of crude (nonconcentrated) cheese whey (Zafar and Owais, 2006) or cheese whey powder (Ozmihci and Kargi, 2007b).

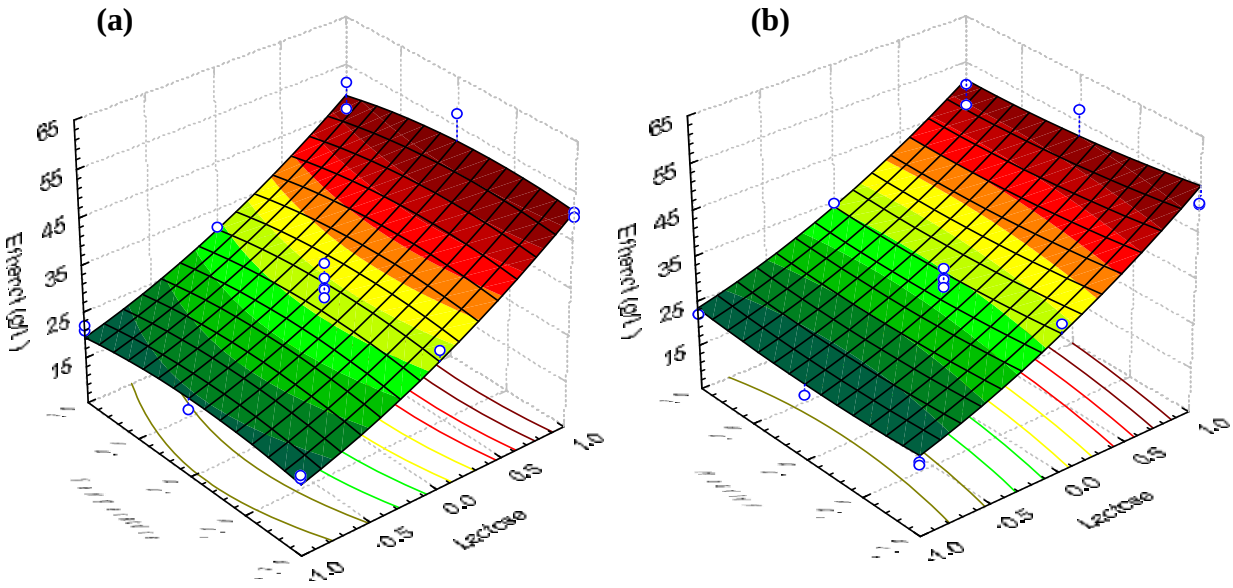

Figure 1. Response surface of ethanol production from cheese whey powder by *K. fragilis* as a function of: (a) initial lactose concentration and temperature, (b) initial lactose and inoculum concentrations.

4 Conclusions

The initial lactose concentration of cheese whey powder affected the production of ethanol by *K. fragilis* (Kf 1) (p<0.01).

The response surface analysis of the central composite design results indicated that, in the range studied, the optima conditions for maximum ethanol production (≥41.5 g/L) consisted in using an initial lactose concentration of 150 g/L, temperature between 25 and 35ºC, and inoculum concentration between 1 and 3 g/L.

The results proved the feasibility of using cheese whey powder as substrate to produce higher ethanol concentrations than those obtained by direct fermentation of nonconcentrated cheese whey.

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