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Research Article

Response Surface Methodology to Optimize a Bioprocess for Kefiran Production

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Abstract

Kefiran is an edible biopolymer formed by a galactose and glucose chain in nearly equal proportions. This biopolymer has important applications in the pharmaceutical and food industries. This is produced by the action of an acid-lactic bacteria and yeasts consortium on lactose present in the kefiran granule. In the present work, kefiran concentration in the fermentation broth was optimized by the application of the response surface methodology in a central composite design of thirteen experiments. Temperature and whey powder (WP) content were the analysed dependent variables. Among the 14 suggested optimal temperature and WP conditions, it was selected 25°C for temperature and 44.1% (w/w) for WP as optimal conditions to perform further model validation experiments. Under these conditions, the quadratic model regarding kefiran concentration displayed 209.72 ± 9.77 mg Glu/mL after 48 h of culture. The obtained response surface model was further validated with three additional experiments by using these optimal conditions for temperature and WP content described above. The validation result was 216.06 ± 14.40 mg Glu/mL suggesting that experimental and theoretical models have not significant differences ($p \leq 0.05$). Kefiran isolation process was carried out from five 100 mL batches each, yielding 3.1 ± 1.3 g/L of kefiran in the culture supernatant.

Keywords

Kefiran; Whey powder; Kefir granules; Central composite design

Introduction

In Zone 1 of Ecuador, that includes Carchi, Imbabura, Esmeraldas and Sucumbíos provinces, agriculture and livestock occupies an important part of the economic and labor activity, being Carchi province the most productive standing out with the 4.8% of the national fresh milk production, with a daily 79.8 m^3 production [1]

One third of the region's dairy production is dedicated to the production of cheese [2], generating from 10 litres of whole milk, 1 kg of cheese and around 9 litres of whey [3,4].

Whey retains a large part of the milk nutrients, mainly lactose so that, this by product has been considered a high polluting product, reaching a biological oxygen demand (BOD5) of 30-50 kg/m^3 [5].

Some solutions to this lactose pollution problem have been proposed [4-7]. One of them is the use of microorganisms capable of bio-transforming in higher added value substances, the lactose present in whey [6,8,9].

Kefir is a slightly acidic fermented milk, viscous and somewhat effervescent, with a certain alcohol content, which is obtained by the action of a consortium of lactic acid bacteria (LAB), fungi, and yeast known as kefir granules [10,11].

Kefiran is the major polysaccharide in the granule. This product is an edible biopolymer, formed by glucose and galactose units in approximately equal proportions [12,13]. Different authors worldwide [14-16] have shown several beneficial properties of kefiran that make it attractive for example in drugs formulation [15,17] and in food preservation [18] so, its production attracts great interest in producing this edible and biodegradable biopolymer [19].

The Response Surface Methodology (RSM) is a powerful tool used in experiments design widely used in the industry [20-22]. This technique allows optimizing certain experimental response and finding independent variables bets combination that allows to get good final response of the bioprocess [23]. The central-composite design (CCD) is one of the most popular RSM arrangements and is based on distributing the experiments around a central point and around this central point the rest of the experiments are equidistantly distributed around it [22].

The objective of this work is to find through a central-composite design approach, the best combination for culture temperature and whey powder (WP) content in order to maximize the kefiran concentration in the culture supernatant.

Materials and Methods

Culture conditions

Fresh kefir granules were supply by an Ecuadorian commercial company "Yogurt-Kombucha-Tibicos en Ecuador" (Quito, Pichincha, Ecuador, www.kefir.ec). The granules were kept in fresh and pasteurized milk at 4-8°C, replacing it every two days. In each experiment, 100 g of medium were inoculated with 3.73% (w/w) of kefir granules as reported by others researchers [24,25]. Before inoculation, the granules were washed with enough sterile deionized water.

At all points, dissolved solids were maintained at 14% Brix, according other similar reported studies [25].

The WP contents were 38.5-77.0% (w/w) according to the design conditions [26]. To maintain 14% Brix in each variant, defined amounts of a glucose solution at 77% (w/v) were added.

Additionally, the medium was supplemented with a ten-times concentrated (10X) salt solution formed by 1% (w/v) of KH_2PO_4, 5%

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(w/v) $MgSO_4$ and 1% (w/v) $(NH_4)_2SO_4$. The variants were adjusted to pH 6.8, by using 98% (v/v) H_2SO_4 or 0.1 M NaOH as needed.

The experiments were conducted during 48 h in an oscillating shaker at 100 rpm. The temperature was controlled in a range between 20-36°C, according to the values suggested by the design run.

Kefirán detection and quantification

For kefiran precipitation, 5 mL of culture supernatant was mixed with 5 mL of absolute ethanol at -20°C and left overnight at 4°C. The next day, the mixture was centrifuged at 1690xg for 30 min at 4°C by using a refrigerated-desk centrifuge (Sorvall ST 16 Centrifuge, Thermo-Fisher Scientific, Langenselbold, Germany). The precipitate was washed twice with deionized water to remove other insoluble substances. The re-dissolved mixture in deionized water was centrifuged under the same conditions described above. The last precipitate was dissolved up to 10 mL with deionized water and the kefiran was quantified according to the phenol-sulfuric method [27].

Statistical design to optimize the kefiran production

A central composite design of response surface methodology was executed to found the combination of temperature and WP content which maximize the kefiran concentration after 48hrs of fermentation [28]. All the experiments were planned and analysed employing the DOE-software Design-Expert 11.0.3.0 (Stat-Ease, Inc., Minneapolis, USA).

Effects of the temperature (X1) and WP content (X2) on the concentration of kefiran at the end of the fermentation are shown in Table 1.

The response variable in this case, kefiran concentration, was adjusted to a second order quadratic statistical model described by the equation below:

$$K = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{1 \le i \le j}^{2} \beta_{ij} X_i X_j + \varepsilon$$

where K is the kefiran concentration (mg Glu/mL); β0 is the average value of all effects in the model; β1 represents the effect of the factor X_1 (t, °C); β2 represents the effect of the factor X2 (WP, % (w/w)); β12 is the effect of factors X_1 and X_2 interaction; β11 represents the quadratic effect of factor X1; β22 represents the quadratic effect of X_2 factor. The ε is the component related to the random model error caused by other variability sources not taken into account in this model.

The optimal values predicted for a combination of independent variables should be corroborated in additional experiments under the same conditions that suggest an extreme value, thus validating or refuting the obtained statistical model.

Results and Discussion

Coded values corresponding to the independent variables and the response obtained by both, the quadratic model and the experimental value, are presented in Table 2.

The obtained model concerning coded variables is:

$$K = 184.520 - 15.529 \cdot X_1 - 10.215 \cdot X_2 + 4.550 \cdot X_1 X_2 - 19.235 \cdot X_1^2 + 12.065 \cdot X_2^2$$

And the final equation in terms of actual factors:

$$K = 50.6344 + 30.9161 \cdot t - 8.2715 \cdot WP - 0.6011 \cdot t^2 + 0.0651 \cdot WP^2$$

Effects of temperature (t, °C) and the WP content (WP, % (w/w)) on the kefiran concentration (mg Glu/mL) is shown in Figure 1.

ANOVA for the quadratic model suggested for K is depicted in Table 3.

As shown in Table 3, the adequate accuracy of the model is > 4, so that the model obtained for K can be used to navigate and find an optimal value within the design space. The p-value of each coded coefficient serves to demonstrate the importance of the factor within the quadratic model (Table 3). Small p-values indicates that these factors are greater than their standard error (Table 4).

The adequacy of the quadratic model for the kefiran concentration determination as a function of temperature and whey powder content can also be evaluated through the diagnostic plots (Myers, Montgomery, & Anderson-Cook, 2016) of the normal distribution of residuals (Figure 2A) and the graph of the predicted response values versus the actual response values (Figure 2B).

Fourteen equally optimal condition values were obtained where the kefiran concentration reached the maximum at the highest importance of this response (five - +) by applying a numerical optimization algorithm to the second-order regression equation for kefiran concentration (Table 5).

Table 1: Actual and codified variables from independent variables (X_1: temperature (°C) y X2: whey powder (WP) content (% (w/w)) in five suggested levels (-1.41, -1, 0, +1, +1.41).

	Code values		Actual values	
Run	**X_1**	**X_2**	**t, °C**	**WP, %(w/w)**
1	-1.41	0.00	20.0	57.8
2	-1.00	-1.00	22.3	44.1
3	-1.00	1.00	22.3	71.4
4	0.00	0.00	28.0	57.8
5	0.00	1.41	28.0	77.0
6	0.00	0.00	28.0	57.8
7	0.00	0.00	28.0	57.8
8	0.00	0.00	28.0	57.8
9	0.00	-1.41	28.0	38.5
10	0.00	0.00	28.0	57.8
11	1.00	-1.00	33.7	44.1
12	1.00	1.00	33.7	71.4
13	1.41	0.00	36.0	57.8

Table 2: Central-composite design results of experiments and experimental and predicted results obtained by the quadratic model as function of the coded variables (X1: temperature (°C) and X2: whey powder (WP) content (% (w/w)).

			Kefiran (K) (mg Glu/mL)	
Run	**X1**	**X2**	**Model**	**Actual**
1	-1.41	0.00	168.01	188.15
2	-1.00	-1.00	207.64	190.45
3	-1.00	1.00	178.11	159.30
4	0.00	0.00	184.52	208.20
5	0.00	1.41	194.20	208.20
6	0.00	0.00	184.52	162.00
7	0.00	0.00	184.52	175.00
8	0.00	0.00	184.52	187.40
9	0.00	-1.41	223.10	234.80
10	0.00	0.00	184.52	190.00
11	1.00	-1.00	167.49	160.60
12	1.00	1.00	156.16	147.65
13	1.41	0.00	124.09	129.65

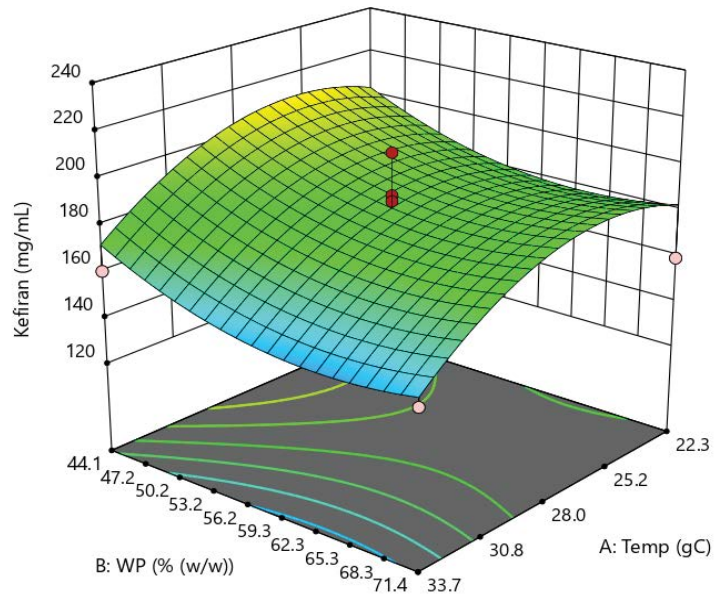

Figure 1: 3D-plots of the response surface showing the combined effect of temperature (°C) and whey powder (WP) content (% w/w) on the kefiran concentration (mg Glu/mL).

Table 3: ANOVA for the second order model related to the kefiran concentration as a function of temperature and whey powder (WP) content.

		Kefiran (*K*) (mg Glu/mL)			
Source	**df**	**Sum of square**	**Mean square**	**F Value**	**p-value Prob>F**
Model	4	6840.85	1710.21	4.850	0.028
X_1-Temp	1	1929.18	1929.18	5.483	0.047
X_2-%WP	1	834.73	834.73	2.371	0.162
X_1^2	1	2573.81	2573.81	7.310	0.027
X_2^2	1	1012.62	1012.62	2.870	0.128
Residual	7	2818.39	352.30		
Lack of Fit	3	1621.55	405.39	1.350	0.388
Pure Error	4	1196.85	299.21		
Corr. Total	12	9659.25			
Std. Dev.		18.77			
Mean		180.11			
C.V. %		10.42			
R^2		0.7082			
Adj R^2		0.5623			
Adeq. Prec.		8.505			

Table 4: Regression coefficients, confidence intervals ($p < 0.05$) and standard error of the quadratic regression model for kefiran concentration.

		Kefiran (K) (mg Glu/mL)				
Factor	Coeff. Estimate	df	Std. Error	95% CI Low	95% CI High	VIF
Intercept	184.52	1	8.39	165.16	203.88	
X_1-Temp	-15.53	1	6.64	-30.83	-0.23	1.00
X_2-%WP	-10.21	1	6.64	-25.52	5.09	1.00
X_1^2	-19.24	1	7.12	-35.65	-2.82	1.02
X_2^2	12.07	1	7.12	-4.35	28.48	1.02

Other authors also reported an optimum temperature equal to 25°C [29,30] while others reported nearby values. For example, values of 24°C have been reported 30°C andup to 33°C [19,20,31],

Figure 3 shows one of the optimal values obtained (condition No.2 in Table 5, X1* = -0.531 and X2* = -1.003) that maximizes the kefiran concentration model (Kmáx = 209.72 ± 9.77 mg Glu/mL).

Table 5: Optimal conditions of the independent variables that maximize the quadratic regression model for the kefiran concentration.

No.	T*, °C	WP*, %(w/w)	Kefiran (*K*) (mg Glu/mL)	Std. Error (mg Glu/mL)
1	24.808	44.100	209.535	9.801
2	**24.995**	**44.100**	**209.717**	**9.769**
3	25.242	44.100	209.895	9.739
4	25.377	44.100	209.961	9.727
5	25.459	44.100	209.990	9.722
6	25.596	44.100	210.021	9.715
7	25.662	44.100	210.029	9.713
8	25.715	44.100	210.030	9.711
9	25.783	44.100	210.028	9.710
10	25.891	44.100	210.012	9.709
11	26.007	44.100	209.979	9.708
12	25.717	71.400	189.544	9.711
13	25.785	71.400	189.541	9.710
14	26.276	71.400	189.356	9.712

To validate the suggested model, three similar experiments were performed using the second optimal suggested point (Table 5). The results obtained (216.06 ± 14.40 mg Glu/mL, n = 3) confirm the accuracy of the regression model for the kefiran concentration (Figure 4).

Kefiran isolation process was carried out from five 100 mL batches each, yielding 3.1 ± 1.3 g /L of kefiran in the culture supernatant.

These results are slightly-higher than the 1.91 g/L of kefiran reported recently [19], and similar to the values between 1.5-3.7 g/L reported previously [32,33].

Conclusions

The optimal values for temperature and the kefiran concentrations obtained were close to those obtained by other authors. This work

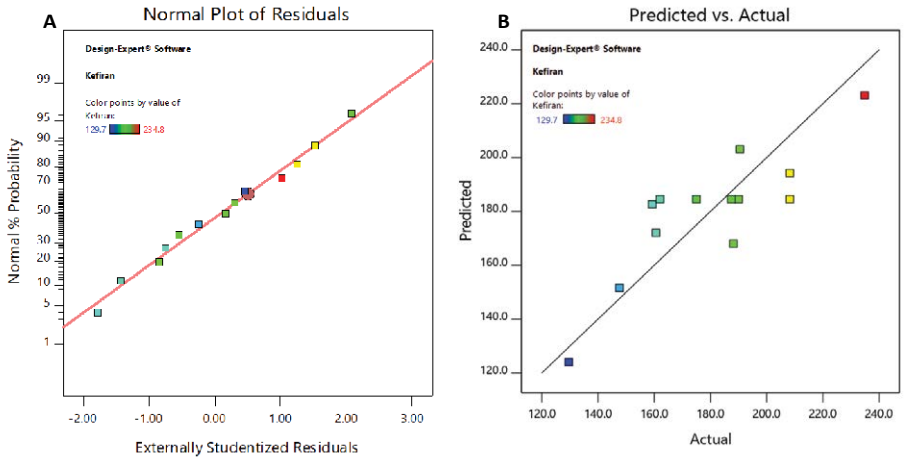

Figure 2: Diagnostics plots (A) Normal probability plots of externally Studentized residuals; (B) Predicted versus actual values of kefiran concentration (mg Glu/mL).

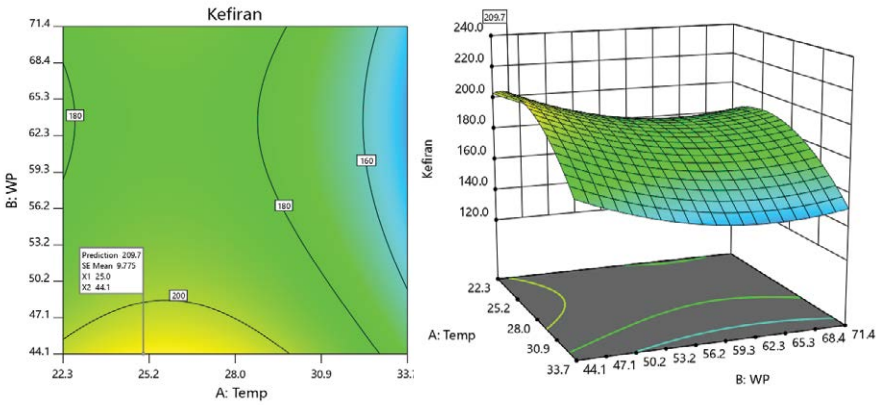

Figure 3: One of the maximum values obtained by applying the numerical optimization procedure for the kefiran concentration.

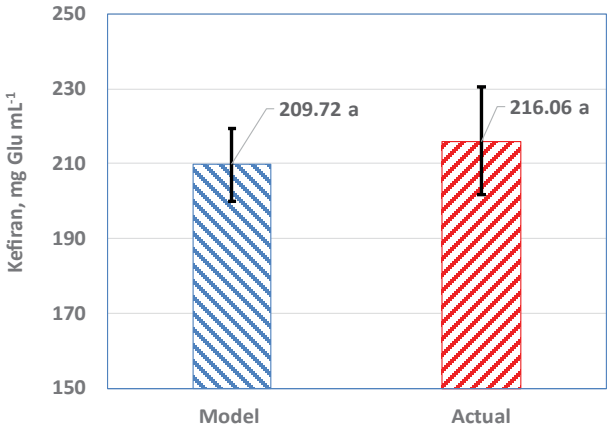

Figure 4. Validation experiments for the kefiran model. The average values were shown over the columns, bars mean standard deviations, and equal letter indicate do not significantly differences exist (n = 3, $p < 0.05$).

can contribute to establish a bioprocess that allows to produce this valuable biodegradable and biodegradable biopolymer, with potential uses in the Ecuadorian pharmaceutical and food industry.

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