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COMMUNICATION

Enhanced Production of Iranian Kefir Grain Biomass by Optimization and Empirical Modeling of Fermentation Conditions Using Response Surface Methodology

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Abstract This study focused on the optimization of key process parameters for maximizing kefir grain biomass yield using statistical methodology. A response surface methodology (RSM) was developed to describe the effects of whey lactose and yeast concentrations, temperature and pH on increases in kefir grain biomass using cheese whey as a fermentation medium. Initially, one factor at a time was applied to evaluate the effect of different nitrogen sources. The results showed that the concentration of yeast extract significantly influenced the biomass increase. Then, a 2^4 full-factorial central composite design was used to optimize the process conditions. By using multiple regression analysis, the experimental data were fitted to a second-order polynomial model. RSM analysis indicated good correlation between experimental and predicted values. The most suitable combination of variables for higher biomass increase (76.13%) was 88.4 and 21.3 g/l, 5.2, and 20 °C for concentration of whey lactose, concentration of yeast extract, pH, and temperature, respectively. At these optimal conditions, biomass increased by 81.34%, which was close to the amount predicted by the model.

Keywords Kefir grain · Process optimization · Microbial biomass · Biomass yield · Central composite design · Productivity improvement

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Introduction

Traditionally, kefir has been prepared by fermenting fresh milk with kefir grains, a symbiotic culture starter containing a spectrum of lactic acid bacteria, yeasts, and acetic acid bacteria in the form of small, yellowish-white, irregularly shaped grains that increase in size during the fermentation process. These grains are able to use lactose; thus, whey rich in lactose can be applied as a culture medium. Considering their potential applications, to the best of our knowledge, no successful attempt has been made to optimize the propagation of kefir grains in whey. This study was based on the belief that by using statistical techniques for optimization, further increases in biomass could be attained. Thus, the main objective of this research was to investigate the individual and the interactive effects of the various concentrations of whey lactose, yeast extract, pH, and temperature on kefir grain biomass increase.

Materials and Methods

Starter Culture and Fermentation Medium

Fresh kefir grains, used as the starter culture in this study, were obtained from a household (Tehran, Iran). The grains were kept in skimmed milk at room temperature (25±1 °C) for short periods, and the medium was exchanged for fresh skimmed milk daily to maintain the grains' viability. In order to increase the kefir grain biomass, the grains were activated (Tramšek and Goršek 2007). Cheese whey was obtained from Sahar Company (Ghazvin, Iran) and deproteinazed, as described in a previous

study (Khodaiyan et al. 2008). The liquid was diluted or concentrated as necessary. Whey lactose was determined by using the HPLC (Shimadzu, Model UV-3100, Kyoto, Japan) according to the method of Jeon et al. (1984).

Fermentation Experiments

To further enhance kefir grain propagation, a number of different sources (yeast extract, peptone, malt extract, ammonium nitrate, ammonium chloride, ammonium sulfate, and urea at a concentration of 1%, *w/v*; Merck, Darmstadt, Germany) were added to the cheese whey in Erlenmeyer flasks, while other factors were kept constant. After choosing that, the flasks were inoculated with 1.5% (*w/v*) kefir grain and incubated at specific pH and incubation temperatures (20, 25, or 30 according to the matrix design) without shaking for 120 h. Temperature fluctuations in the culture liquid were reduced by wrapping flasks with aluminum foil. All the experiments were performed in triplicate.

Determination of Wet Weight of Kefir Grains

After incubation, the kefir grains were separated from the fermented culture medium with a household sieve,

Table 1 Coded levels and actual values of the independent variables in central composite design and experimental results of kefir grain biomass increase

Run	Coded variables				Uncoded variables				Kefir grain biomass increase (%)	
	X_1	X_2	X_3	X_4	Whey lactose (g/l)	Yeast extract (g/l)	pH	Temperature (°C)	Experimental[a]	Predicted
1	−1	−1	−1	−1	40	6	4.5	20	29.00±1.41	33.61
2	1	−1	−1	−1	80	6	4.5	20	51.00±4.47	40.64
3	−1	1	−1	−1	40	18	4.5	20	61.33±2.35	56.11
4	1	1	−1	−1	80	18	4.5	20	62.00±6.59	63.15
5	−1	−1	1	−1	40	6	6.5	20	55.30±7.37	49.35
6	1	−1	1	−1	80	6	6.5	20	52.33±4.00	56.38
7	−1	1	1	−1	40	18	6.5	20	60.00±2.82	61.63
8	1	1	1	−1	80	18	6.5	20	65.66±5.65	68.67
9	−1	−1	−1	1	40	6	4.5	30	29.33±0.23	27.44
10	1	−1	−1	1	80	6	4.5	30	35.33±4.83	34.48
11	−1	1	−1	1	40	18	4.5	30	28.33±6.07	32.87
12	1	1	−1	1	80	18	4.5	30	40.66±3.29	39.91
13	−1	−1	1	1	40	6	6.5	30	41.00±2.12	43.18
14	1	−1	1	1	80	6	6.5	30	44.33±5.89	50.22
15	−1	1	1	1	40	18	6.5	30	36.66±3.77	38.39
16	1	1	1	1	80	18	6.5	30	39.00±4.24	45.43
17	−2	0	0	0	20	12	5.5	25	49.33±2.35	51.87
18	2	0	0	0	100	12	5.5	25	67.66±6.83	68.94
19	0	−2	0	0	60	0	5.5	25	43.00±7.07	50.05
20	0	2	0	0	60	24	5.5	25	67.33±2.35	67.76
21	0	0	−2	0	60	12	3.5	25	24.00±3.77	29.06
22	0	0	2	0	60	12	7.5	25	58.33±3.29	50.32
23	0	0	0	−2	60	12	5.5	15	38.35±2.82	42.57
24	0	0	0	2	60	12	5.5	35	20.33±3.29	13.16
25	0	0	0	0	60	12	5.5	25	55.66±5.18	61.93
26	0	0	0	0	60	12	5.5	25	57.33±6.59	61.93
27	0	0	0	0	60	12	5.5	25	63.56±6.85	61.93
28	0	0	0	0	60	12	5.5	25	62.66±4.94	61.93
29	0	0	0	0	60	12	5.5	25	67.31±6.62	61.93
30	0	0	0	0	60	12	5.5	25	56.66±2.12	61.93
31	0	0	0	0	60	12	5.5	25	63.33±2.12	61.93

[a] Values are mean ± SD of three replications

washed with cold water, dried on the filter paper, and weighed on an electronic balance (AND series HR-200, USA) with a precision of 0.0001 g. The results were expressed as mean ± SD.

Experimental Design and Statistical Analysis

A total of 31 experimental runs with combinations of concentrations of whey lactose, yeast extract, pH, and temperature were conducted by central composite design. Analysis of variance (ANOVA) and graphical representations of the data were calculated and analyzed using the Design Expert statistical software package (trial version 7.1.6, Stat-Ease Inc., Minneapolis, USA).

The results of the experimental design were fitted by a second-order polynomial equation in order to correlate the response to the independent variables:

$$Y = C_{k0} + \sum_{i=1}^{4} C_{ki}x_i + \sum_{i=1}^{4} C_{kii}x_i^2 + \sum_{i<j=2}^{4} C_{kij}x_i x_j \qquad (1)$$

where Y stands for the percentage of kefir grain biomass increase; C_{k0},C_{ki},C_{kii}, and C_{kij} represent regression coefficients; and x_i and x_j are the coded independent factors. The quality of the fit of the polynomial model was expressed by the coefficients of determination R^2 and $R_{adj}{}^2$. Adequate precision compares the range of the predicted values at the design points to the average prediction error.

Kefir Grain Composition

The grains' water content was determined by drying them at 100 °C to constant weight. Polysaccharide content was measured by the phenol–sulfuric acid method (Dubois et al. 1956). Protein content was determined by the Kjeldahl method (AOAC 1990).

Results and Discussion

Screening of Nitrogen Sources

The results of kefir grain biomass as results of using different nitrogen sources were 55.22%, 31.44%, 45.88%, 19.11%, 7.22%, 6%, and 25% for yeast extract, peptone, malt extract, ammonium nitrate, ammonium chloride, ammonium sulfate, and urea, respectively. On the basis of that, yeast extract was chosen as a significant nitrogen source.

Mathematical Modeling

Table 1 shows the design matrix for these factors in the experimental runs. ANOVA analysis (Table 2) was per-

Table 2 ANOVA analysis and statistical parameters of the model

Analysis of variance (ANOVA)					
Source	Sum of squares	*df*	Mean square	*F* value	*P* value
Model	5,396.46	8	674.56	22.42	<0.0001
X_1	296.95	1	296.95	9.87	0.0047
X_2	470.64	1	470.64	15.64	0.0007
X_3	667.98	1	667.98	22.54	<0.0001
X_4	1,296.83	1	1,296.83	43.11	<0.0001
X_2X_3	104.55	1	104.55	3.48	0.0757
X_2X_4	291.56	1	291.56	9.69	0.0051
X_3^2	673.62	1	673.62	22.39	0.0001
X_4^2	1,757.87	1	1,757.87	58.43	<0.0001
Residual	661.83	22	30.08		
Lack of fit	546.23	10	34.14	1.77	0.2474
Pure error	115.60	6	19.27		
Cor Total	6,058.29	30			
Statistical parameters for the model					
SD		5.48	R^2	0.8908	
Mean		49.18	$R_{Adj}{}^2$	0.8510	
C.V.%		11.15	$R_{Pred}{}^2$	0.7096	
PRESS		1,759.34	Adeq precision	18.780	

PRESS (predicted residual sum of squares)

formed to check the adequacy of the suggested models and identify the significant factors. A second-order polynomial model is shown in Eq. 2 in coded form:

$$Y = 58.90 + 3.52X_1 + 4.43X_2 + 5.31X_3 - 7.35X_4 - 2.56X_2X_3 - 4.27X_2X_4 - 4.80X_3^2 - 7.77X_4^2 \quad (2)$$

where Y is the experimental response and X_1, X_2, X_3, and X_4 correspond to the independent variables of whey lactose, yeast extract, pH, and temperature, respectively.

ANOVA

The F value of 1.77 for the lack of fit implies that it is not significant relative to the pure experimental error, suggest-

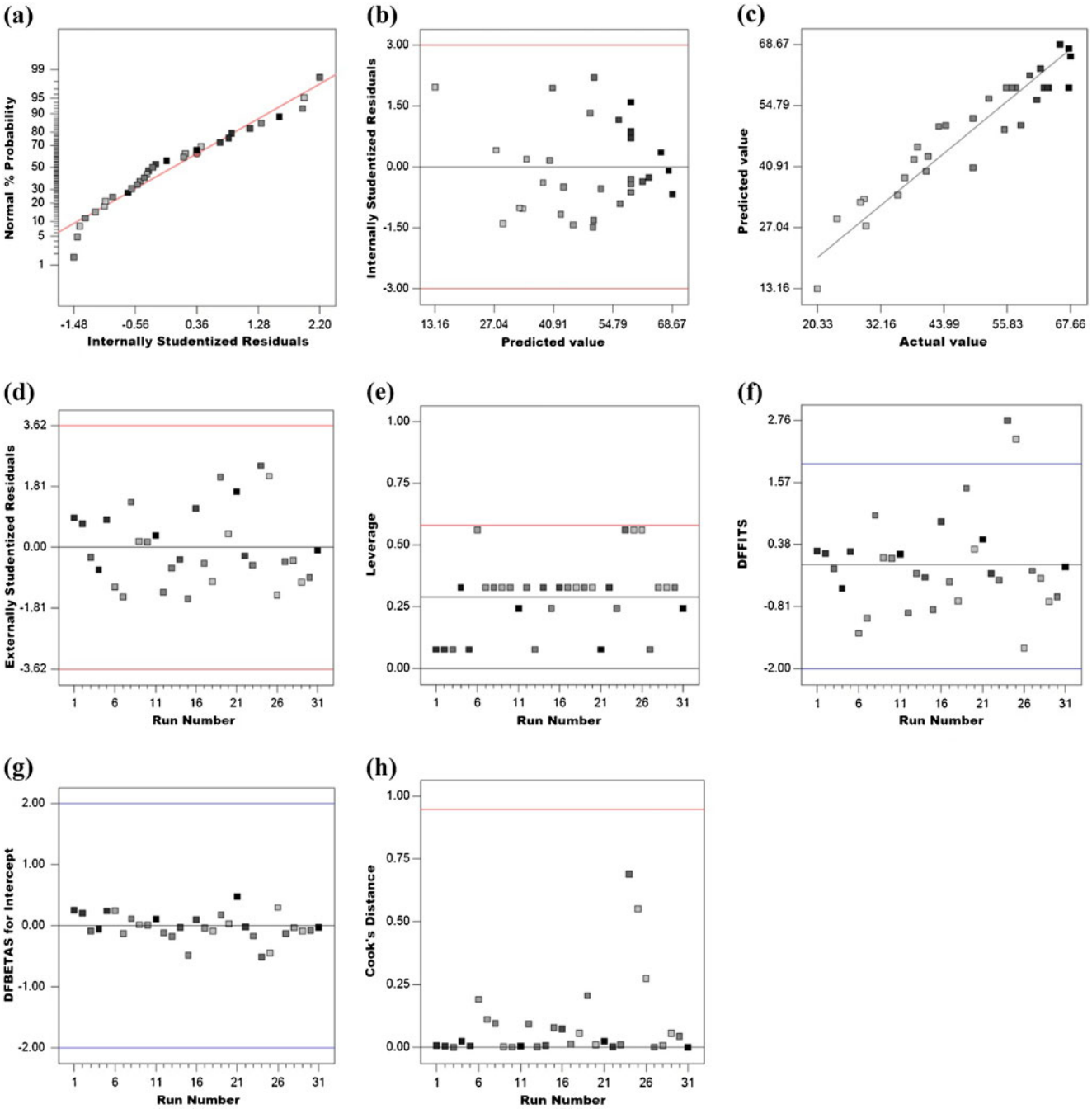

Fig. 1 Normal probability graph of internally studentized residuals (**a**), internally studentized residuals vs. predicted values plot (**b**), predicted versus actual plot (**c**), externally studentized residuals vs. run number (**d**), leverage vs. run number (**e**), difference of fits (DFFITS) vs. run number (**f**), difference in beta values (DFBETAS) vs. run number (**g**), and Cook's distance vs. run number

ing that the model correlated well with the experimental values. Statistical parameters of the model are shown in Table 2. R^2, a measure of the goodness of fit of the model was 0.898. The adjusted R^2 value (0.851) also indicated the model's goodness of fit. For the proposed model, the value of 18.78 for adequate precision indicates an adequate signal, suggesting that this model can be used to navigate the design space. Also, Table 2 shows a value of 1,759.34 for the predicted residual sum of squares, a measure of how a particular model fits each point in the design.

Model Adequacy Testing

Diagnostics

Figure 1 shows the residual and the influence plots for the experiments in this study. Figure 1a shows that the normal plot of residuals for response was normally distributed, as they lie approximately on a straight line and show no deviation of the variance. Figure 1b shows the studentized residuals versus predicted data points. The predicted and actual values also show relatively good agreement, as shown in Fig. 1c. Hence, no obvious patterns were found in the analysis of residuals.

Influence Plots

Figure 1d shows that all the data points lay within the limits. Since all leverage values were less than 1 (Fig. 1e), there are no outliers or unexpected errors in the model. Difference of fits plot (Fig. 1f), a measure of the influence of each point on the predicted value, suggested two points (corresponding to runs 24 and 25) that influence the regression equation and the response very disproportion-

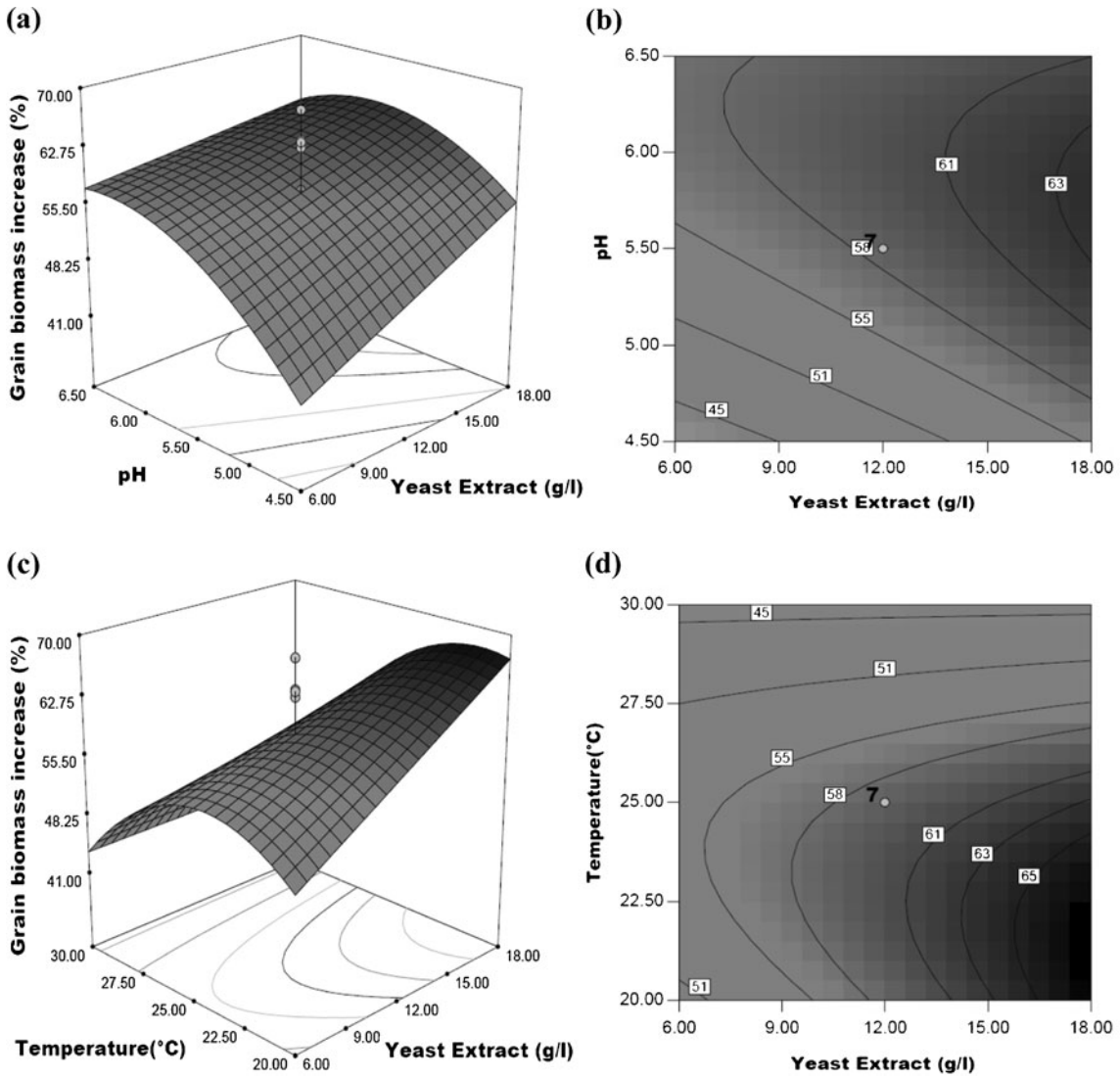

Fig. 2 Three-dimensional (3D) response surface and contour plot for pH–yeast extract concentration (**a** and **b**; whey lactose = 60 g/l, temperature = 25 °C) and for temperature–yeast extract concentration (whey lactose = 60 g/l, pH=5.5; **c** and **d**)

ately. However, difference in beta values plot (Fig. 1g) showed no undue influence of any observation on any of the regression coefficients. Since the Cook's distance values are in the determined range (shown with red line in Fig. 1h), there is no strong evidence of influential observations in these data.

Model Interpretation

Figure 2a, b contain the response surface and contour plot, showing the effect of pH and yeast extract concentration on the response at the fixed values of temperature and whey lactose concentration in their center values. The percentage grain weight increased when pH and yeast extract were increased to around 5.5 and 19 g/l, respectively, but decreased when they were increased past these levels. From the curves in Fig. 2c, d, which presents the interaction of yeast extract concentration and temperature, it is clear that maximum biomass increase was obtained at high yeast extract concentration and low temperature.

Optimization and Model Validation

The analysis of the design indicated that optimal conditions for the highest kefir grain increase (76.13%) were lactose concentration of 88.4 g/l, yeast extract of 21.3 g/l, pH of 5.2, and temperature of 20 °C. The maximum grain produced experimentally was found to be 81.34%, which was clearly very close to the predicted value. Response surface methodology has been broadly discussed in the literature for optimizing different processes (Chopra et al. 2009; Tripathi and Mishra 2009). For example, Bitaraf et al. (2010) used this methodology to study the individual and interactive effects of inulin content, probiotic inoculum level, and incubation temperature on fermentation time and rheological properties of yogurt. Their results showed that the quadratic models are well adjusted to predict the experimental data. Comparison of this study's optimization results with the literature on the enhancement of kefir grain biomass using other culture media revealed that this study's results were considerably closer to experimental values than those in other investigations (Schoevers and Britz 2003; Goršek and Tramšek 2007). For example, in a study conducted by Schoevers and Britz (2003) that used kefir grains in different incubation times, the maximum biomass increase in a defined medium was around 52% (after 120 h incubation time), lower than this study's results.

Kefir Grain Composition

The chemical composition of grains after seven subcultures in whey was 81.5% water, 8.6% polysaccharide, and 7.2% protein. These values did not differ from those obtained from grains grown in milk (Goršek and Tramšek 2007).

Conclusion

The study successfully optimized the enhancement of kefir grain biomass in cheese whey by using central composite design and response surface methodology. This indicates that cheese whey can be considered as a potential culture medium for different applications, such as propagation of kefir grains, a conclusion that has until this point been lacking in the literature.

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