CI&CEQ

Available on line at
Association of the Chemical Engineers of Serbia AChE
www.ache.org.rs/CICEQ

Chemical Industry & Chemical Engineering Quarterly 18 (2) 273–282 (2012) CI&CEQ

A. SHAHRAVY[1,2]
F. TABANDEH[1]
B. BAMBAI[1]
H.R. ZAMANIZADEH[2]
M. MIZANI[2]

[1]Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[2]Faculty of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

SCIENTIFIC PAPER

UDC 60:602.6:579.864

DOI 10.2298/CICEQ110709004S

OPTIMIZATION OF PROBIOTIC *Lactobacillus casei* ATCC 334 PRODUCTION USING DATE POWDER AS CARBON SOURCE

This study was conducted to optimize culture conditions for economic production of a probiotic bacterium, Lactobacillus casei ATCC 334, in which palm date powder was applied for the first time as a low-cost main carbon source. The effect of eleven factors on bacterial growth was investigated using the Taguchi experimental design, and three factors including palm date powder, tryptone and agitation rate were found to be the most significant parameters. The optimum conditions including date powder concentration, 38 g/L; tryptone concentration, 30 g/L; and an agitation rate of 320 rpm were determined by response surface methodology of Box-Behnken. A third-order polynomial model was suggested to predict the design space following which the predicted values were validated experimentally. The maximum log value of the viable cells in the optimized alternative medium was 9.97 at 24 h of incubation which was comparable to that obtained in the complex and expensive MRS medium (10.06).

Keywords: probiotics; response surface methodology (RSM); dairy products; functional food; Taguchi method.

Currently, the production of functional foods containing probiotic bacteria such as lactobacilli is gaining increasing significance [1]. These bacteria enhance the microbial safety and offer organoleptic, technological, nutritional, or health benefits to the consumer [2]. One of the lactobacilli being used as probiotic is *L. casei* [3]. As a consequence of the large-scale production of fermented foods incorporated with probiotics, the industrial production of these bacteria is becoming more important [2,4].

Among the factors that should be considered in the choice of growth medium are costs, ability to produce a large number of cells, and the harvesting method [5]. Regarding nutrient requirements and growth conditions, studies on new medium development for enhanced biomass production could lead to more economical probiotic production [6]. Since the fruit (date) of the date palm (*Phoenix dactylifera*) contains high amounts of sucrose as well as reducing sugars (total sugars of 44-88%), especially glucose and fructose [7], it may be a potentially convenient and inexpensive substitute for the glucose required by the lactobacilli. The effectiveness of yeast extracts and potato extracts has been evaluated to promote the growth of lactic acid bacteria (LAB) such as *L. casei* EQ28, *L. rhamnosus* R-011, *L. plantarum* EQ12 and *Streptococcus thermophilus* R-083 by automated spectrophotometry [8]. The growth kinetics of several LAB (*L. fermentum, L. reuteri, L. acidophilus* and *L. plantarum*) that have potential to be used as probiotics in cereal-based substrates such as wheat, barley and malt have been reported [9]. The malt medium supported the growth of all strains more than barley and wheat media due to its chemical composition, and it was found that cereals were suitable substrates for the growth of potentially probiotic LAB [9]. Furthermore, the growth of *L. paracasei* ssp. *paracasei* on tofu whey as an alternative substrate was studied using automated spectrophotometry [10]. It was shown that Tofu whey, which is a low-cost by-product, could effectively be used as a cost effective substrate for the production of starters.

Correspondening authors: F. Tabandeh and B. Bambai, Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14965-161, Tehran, Iran.
E-mails: taban_f@nigeb.ac.ir (F. Tabandeh); bambai@nigeb.ac.ir (B. Bambai)
Paper received: 9 July, 2011
Paper revised: 3 December, 2011
Paper accepted: 11 January, 2012

Screening designs are economical experimental plans that focus on determining the relative significance of many main factors. The Taguchi method is a fractional factorial experimental design applied in an empirical study to find the most significant factors in a process. It requires fewer experiments and filters out some effects based on statistical variation [11]. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response of interest is influenced by several variables and the objective is to optimize this response [12]. RSM has been employed to investigate the optimization of medium composition for the production of probiotic microorganisms, *L. casei* YIT 9018 and *L. rhamnosus* [4, 5]. Optimization of carbon and nitrogen sources as well as growth factors for the production of an aquaculture probiotic, *Pseudomonas* MCCB 103, has also been carried out using RSM [13]. Moreover, kefir candy composition has been, recently, optimized by RSM and sequential quadratic programming [14].

RSM has been frequently used for medium optimization of lactic acid bacteria; however, to our knowledge, Taguchi and RSM statistical methods have not been employed concomitantly to screen the most important factors of a set of medium conditions and optimize their levels for the biomass production of a probiotic lactobacillus strain, where date powder is the main carbon source. A study on the individual and interactive effects of parameters in the suggested simple medium will help in efforts to optimize the mass production of the probiotic microorganism. The objectives of this study were to determine the most significant variables among the culture parameters in the production of *L. casei* ATCC 334 through the Taguchi design of experiment and to then find their optimum levels using RSM.

MATERIALS AND METHODS

Chemicals and microorganism. All chemicals were of analytical grade and obtained from Merck, Darmstadt, Germany (unless mentioned otherwise) except date powder which was a commercial product obtained from a production facility from Jam Co., Iran and employed in this study. The reducing sugar concentration in the date powder was 50 g/L. The microorganism used in this study was isolated from a commercial probiotic yoghurt drink and screened using MRS broth and agar plates. It was identified as *L. casei* according to morphological and biochemical tests. These findings were further confirmed with 16S rRNA sequencing [15]. The results demonstrated over 99 % identity with *L. casei* ATCC 334 according to homology obtained from the BLAST sequence comparisons.

Stock cultures of the isolate were stored at -70 °C in 30 % (v/v) glycerol. For inoculum preparation, the stock culture was subcultured into a 250 mL Erlenmeyer flask containing 50 mL of de Man, Rogosa, Sharpe (MRS) broth and incubated for 24 h at 37 °C on a stirrer at 50 rpm to obtain an initial optical density of 4.0±0.1 at 600 nm (ca. 1.0×10^{10} CFU/mL).

Media composition and fermentation. The cultivation media used in the screening stage consisted of date powder, tryptone, yeast extract, $MgSO_4$, K_2HPO_4, CH_3COONa, $MnSO_4$, and Tween-80. The amount of each component added was varied depending on the conditions of each cultivation experiment (Table 1). The date powder used in this study was first dissolved in determined amounts of water to obtain a date powder solution. This solution was then autoclaved, centrifuged and the supernatant added to the sterilized cultivation media as main the carbon source under sterile conditions prior to inoculation. The initial pH of each medium and the date powder solution were separately adjusted to the required levels with either 2 M NaOH or 2 M sodium acetate before autoclaving. At the screening stage, cultivation experiments were conducted using 15 mL Eppendorf tubes containing 10 mL volumes of cultivation medium. Two sizes of inoculum, at 2 and 7% (v/v), were used according to the requirements of each experiment. The tubes were incubated for 24 h at 37 °C in an incubator (Innova 4300, New Brunswick Scientific, USA) either in static conditions or with orbital shaking at 200 rpm. The initial pH was set at two different values (5 and 7) without pH control during the cultivation. The selection of these pH levels was based on preliminary experiments [15].

The constant medium compositions used in the RSM optimization stage consisted of (g/L): $MgSO_4$, 0.2; and yeast extract, 5. The amounts of date powder and tryptone differed according to the conditions of each experiment. The initial pH was adjusted to 5 (as a result of the screening stage) and pH was not controlled during the fermentation. The center-point medium used in the validation experiments consisted of (g/L): $MgSO_4$, 0.2; yeast extract, 5; date powder, 35 and tryptone, 25. The MRS broth (Merck) contained (g/L): K_2HPO_4, 2.0; $MgSO_4$, 0.2; $MnSO_4$, 0.04; glucose, 20; yeast extract, 4; peptone from casein, 10; meat extract, 8; diammonium hydrogen citrate, 2; CH_3COONa, 5 and Tween-80, 1 mL/L. During cultivation, culture samples (2 mL) were withdrawn for analysis at determined time intervals. The medium was inoculated with a 7% (v/v) concentration of inoculum derived from the screening stage. The flasks were in-

Table 1. Taguchi's design of experiment treatment combinations and responses

Run	A	B	C	D	E	F	G	H	J	K	L	Response (log_{10} (CFU/mL))
1	5(-1)[a]	5(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	5(-1)	0(-1)	2(-1)	9.16±0.01
2	5(-1)	5(-1)	0(-1)	0(-1)	0(-1)	5(+1)	0.05(+1)	1(+1)	7(+1)	200(+1)	7(+1)	9.26±0.01
3	5(-1)	5(-1)	5(+1)	0.2(+1)	2(+1)	0(-1)	0(-1)	0(-1)	7(+1)	200(+1)	7(+1)	9.43±0.01
4	5(-1)	20(+)	0(-1)	0.2(+1)	2(+1)	0(-1)	0.05(+1)	1(+1)	5(-1)	0(-1)	7(+1)	9.37±0.00
5	5(-1)	20(+)	5(+1)	0(-1)	2(+1)	5(+1)	0(-1)	1(+1)	5(-1)	200(+1)	2(-1)	9.41±0.03
6	5(-1)	20(+)	5(+1)	0.2(+1)	0(-1)	5(+1)	0.05(+1)	0(-1)	7(+1)	0(-1)	2(-1)	9.28±0.03
7	25(+1)	5(-1)	5(+1)	0.2(+1)	0(-1)	0(-1)	0.05(+1)	1(+1)	5(-1)	200(+1)	2(-1)	9.79±0.01
8	25(+1)	5(-1)	5(+1)	0(-1)	2(+1)	5(+1)	0.05(+1)	0(-1)	5(-1)	0(-1)	7(+1)	9.54±0.00
9	25(+1)	5(-1)	0(-1)	0.2(+1)	2(+1)	5(+1)	0(-1)	1(+1)	7(+1)	0(-1)	2(-1)	9.50±0.00
10	25(+1)	20(+)	5(+1)	0(-1)	0(-1)	0(-1)	0(-1)	1(+1)	7(+1)	0(-1)	7(+1)	9.64±0.02
11	25(+1)	20(+)	0(-1)	0.2(+1)	0(-1)	5(+1)	0(-1)	0(-1)	5(-1)	200(+1)	7(+1)	9.78±0.04
12	25(+1)	20(+)	0(-1)	0(-1)	2(+1)	0(-1)	0.05(+1)	0(-1)	7(+1)	200(+1)	2(-1)	9.80±0.01

[a](-1) and (+1) are coded levels of eleven independent variables including A: date powder (g/L), B: tryptone (g/L), C: yeast extract (g/L), D: $MgSO_4$ (g/L), E: K_2HPO_4 (g/L), F: KH_2PO_4 (g/L), G: $MnSO_4$ (g/L), H: Tween-80 (mL/L), J: initial pH, K: shaking rate (rpm) and L: inoculation percent (%)

cubated at 37 °C for 24 h on a stirrer at different rates of 50, 200 and 350 rpm. The selection of these stirring rates was based on the result of the screening phase which showed that among the responses to the treatments with (200 rpm) and without shaking (0 rpm), those with shaking were higher. Thus, for the second stage, two additional lower and upper limits of the previously employed range were tested. The stirring rate employed for the center-point medium and MRS medium was 200 rpm.

Design of experiments (DOE's) and statistical analysis. At the screening stage of this study, an L12 (11^2) orthogonal array (OA) associated with the Taguchi design of experiments was used to screen the most important factors during bacterial growth among the eleven studied variables. These factors, their coded and actual levels, and also the layout of the L12 Taguchi's OA are shown in Table 1. The results were analyzed using the analysis of variance technique (ANOVA) to determine those factors with a significant effect on bacterial growth.

To find the exact optimum range of the significant factors, an optimization phase was conducted in which a Box-Behnken design for three factors at three levels was used. Based on the results from the screening phase, these factors were identified as the major variables influencing the response. Each of the variables was studied at two different levels (-1,+1) and center-point (0) which is the midpoint of each factor range. For this part, 15 combination treatments including 3 center-point runs were conducted, each in duplicate.

In our regression model, the response variable was log_{10} (CFU/mL) and candidates for the explanatory variables were linear, interaction and quadratic with respect to date powder, tryptone and agitation rate. Through the point prediction method, optimum conditions for the growth of *L. casei* ATCC 334 were found. Flask cultivation using the completely optimized medium conditions was carried out twice in order to validate the predictions.

All calculations were performed using the Design Expert® software package (version 6.0.10, Stat-Ease Inc., USA).

Analytical methods. Cell growth was determined by counting the colony forming units (CFU). For enumeration, serial dilutions of each sample were plated in duplicate onto MRS agar plates and anaerobically incubated at 37 °C for 48 h. Absorbance (*A*) was measured at 600 nm by the UV-Vis spectrophotometer (Beckman DU 520, USA). A calibration curve was established and the following equation between *A* and CFU was derived:

$$\log\left(\frac{CFU}{mL}\right) = 0.2822A + 8.8524 \quad (1)$$

This equation was used only for inoculum cell number estimation.

RESULTS AND DISCUSSION

Screening of significant factors by Taguchi DOE. The L12 OA of Taguchi experiments and the responses are shown in Table 1. The ANOVA revealed that the growth of *L. casei* ATCC 334 was greatly dependent on the culture conditions (Table 2). The contribution percent of selected factors was as follows: date powder, 72.87, tryptone, 5.87; yeast extract, 0.8; $MgSO_4$, 1.67; K_2HPO_4, 0.28; sodium acetate, 2.59; $MnSO_4$, 0.26; Tween-80, 0.0021; initial pH, 0.28; shak-

Table 2. Analysis of variance (ANOVA) for the Taguchi design of experiments

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	*F*-value	Probe>*F*
Model	1.07	6	0.18	86.51	<0.0001
A	0.79	1	0.79	381.91	<0.0001
B	0.064	1	0.064	30.78	0.0026
C	0.0087	1	0.0087	4.21	0.0955
D	0.018	1	0.018	8.73	0.0317
F	0.028	1	0.028	13.58	0.0142
K	0.17	1	0.17	79.88	0.0003
Residual	0.010	5	0.0021	-	-
Total	1.08	11	-	-	-

ing rate, 15.24 and inoculation percent, 0.13. It can be observed that at this stage, with date powder as the main carbon source, shaking rate and tryptone had the highest positive impact on bacterial growth, respectively. Five factors including K_2HPO_4, $MnSO_4$, Tween-80, initial pH and inoculation percent showed much less impact among the studied factors with the assigned variance of values. By using the Taguchi method, only 12 experiments were carried out to screen the most significant factors among 11 independent variables. This is the main advantage of fractional factorial designs such as Taguchi and Plackett-Burman; requiring fewer experiments, saving time and energy to find the effective parameters in a process.

The analysis of variance (ANOVA) for the response of $\log_{10}$ (CFU/mL) was carried out by considering the factors with contribution of more than 0.8% as suggested by the software. The calculated ratios (*F*) indicate that these factors are significant at 99% confidence limit. The ANOVA of bacterial growth has the model *F*-value of 86.51 which shows that the model is significant, and implies that the multiple correlation coefficient of R^2 is 0.99, *i.e.*, the model can explain 99% variation in the response. Also, this model has an adequate precision value of 25.723 which indicates an adequate signal and thus it can be used to navigate the design space. The model shows standard deviation, mean, C.V. and predicted residual sum of square (PRESS) values of 0.045, 9.40, 0.48 and 0.060, respectively.

Although, based on ANOVA, yeast extract was not a significant factor, and its higher level was shown to have a slightly better effect on bacterial growth. Therefore, at the optimization stage, the higher level of yeast extract (5 g/L) was applied to the medium composition. The proposed amount of $MgSO_4$ in the MRS medium was 0.2 g/L, which was also considered as a constant constituent of the medium composition. Sodium acetate was not used in the optimization medium since it was shown to have a negative effect on bacterial growth. On the basis of ANOVA, there is no difference between the levels of K_2HPO_4, $MnSO_4$, Tween-80, pH and inoculation percent in this case; therefore, the lower levels (-1) of these factors were selected.

Briefly, the optimum conditions obtained from the screening stage were as follows (g/L): date powder, 25; tryptone, 20; yeast extract, 5; $MgSO_4$, 0.2; and a shaking rate of 250 rpm at the initial pH of 5 and inoculation percent of 7 without K_2HPO_4, $MnSO_4$, Tween-80 and sodium acetate. Under these conditions a response of 9.964 for $\log_{10}$ (CFU/mL) was obtained.

Considering the significance of date powder, tryptone and shaking rate, and the increase in bacterial growth as the coded level of these factors shifted from (-1) to (+1), they were selected as the main variables in the optimization stage, to determine the exact optimum point for the growth conditions (Table 3). Also, regarding date powder and tryptone, for the same reason that bacterial growth was increased at the higher tested level, two additional upper levels were chosen for the second phase in order to assess the level up to which an increase in the amount of these components would improve the response. For this reason, the upper level of date powder and tryptone at the first stage was selected as the lower level in the second phase. Here, a narrower range of response was investigated so as to find the exact level of each factor.

Optimization using the Box-Behnken response surface methodology

i) Developing a regression model

The Box-Behnken design was used to find the exact suitable levels of the three mentioned variables for bacterial growth of *L. casei* ATCC 334. Results of the Box-Behnken design for studying the optimum conditions of the three mentioned variables are presented in Table 3.

The polynomial equation that defines the predicted response (Y) in terms of the independent variables (X_1, X_2 and X_3) was as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{123}X_1X_2X_3 + b_{112}X_1^2X_2 + b_{113}X_1^2X_3 + b_{122}X_1X_2^2 + b_{133}X_1X_3^2 + b_{223}X_2^2X_3 + b_{233}X_2X_3^2 + b_{111}X_1^3 + b_{222}X_2^3 + b_{333}X_3^3 \quad (2)$$

The data was fitted with a third-order polynomial function (Eq. (3)). The analysis of variance (Table 4) indicated that the model terms of X_1, X_2, X_3, X_3^2, X_1X_2, X_2X_3, $X_1^2X_3$ and $X_1X_2^2$ were significant ("probe>F" less than 0.05). Other terms were eliminated since they had p-values above 0.1 and also caused a decrease in the adjusted R^2 of the model. Therefore, the simplified response surface model for bacterial growth (Y) as $\log_{10}$ (CFU/mL) in terms of the coded factors was expressed as follows:

$$Y = +9.81 + 0.036X_1 + 0.050X_2 + 0.067X_3 - 0.054X_3^2 - 0.041X_1X_2 + 0.034X_2X_3 - 0.034X_1^2X_3 + 0.024X_1X_2^2 \quad (3)$$

where X_1, X_2 and X_3 are date powder (g/L), tryptone (g/L) and agitation rate (rpm), respectively. The model F-value is 57.37 implying that the model is significant. The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient of R^2 is 0.98, *i.e.*, the model can explain 98% variation in the response. It should be noted that an R^2 value greater than 0.75 indicates the goodness of fit of the model (16). The adjusted R^2 and predicted R^2 values are 0.96 and 0.92, respectively. Also, the model has an "adequate precision value" of 24.967; suggesting that the model can be used to navigate the design space. An "adequate precision value" of > 4 is an essential prerequisite for a model to be a good fit (Box and Draper, 1982). The model showed standard deviation, mean, C.V. and PRESS values of 0.014, 9.78, 0.14 and 6.262E-0.003, respectively. The "lack of fit" F-value of 0.55 implies that this value is not significant relative to the pure error, which is appropriate. The satisfactory correlation between the experimental and predictive values indicated the good fit of the response surface model described by Eq. (3). It should be considered that the polynomial model is a reasonable approximation of the true functional relationship in a relatively small region of the entire space of the independent variables.

ii) Optimum point of the factors

The estimated optimum point was found using the point prediction method. The corresponding actual levels to the optimum point were as follows: date powder, 38 g/L; tryptone, 30 g/L, and agitation rate, 320 rpm. Under these conditions, a maximum log value of 9.909 was predicted for the viable cells, being in the range of 9.89-9.93 with a confidence limit of 95%. A validation test would ascertain these findings. In an optimization study through central composite design, optimum concentrations of yeast extract, glucose and vitamin, together with pH for maximum growth of a probiotic bacterium, *L. rhamnosus* ATCC 7469, formerly known as *L. casei* subspecies *rhamno-*

Table 3. Box-Behnken's design of experiment treatment combinations and responses

Run	Coded levels			Actual levels			Actual response ($\log_{10}$ (CFU/mL))	Predicted response ($\log_{10}$ (CFU/mL))
	X_1[a]	X_2[b]	X_3[c]	X_1	X_2	X_3		
1	-1	-1	0	25	20	200	9.66±0.03	9.66
2	+1	-1	0	45	20	200	9.87±0.01	9.86
3	-1	+1	0	25	30	200	9.85±0.02	9.84
4	+1	+1	0	45	30	200	9.89±0.05	9.88
5	-1	0	-1	25	25	50	9.69±0.01	9.69
6	+1	0	-1	45	25	50	9.75±0.00	9.76
7	-1	0	+1	25	25	350	9.75±0.06	9.75
8	+1	0	+1	45	25	350	9.82±0.01	9.82
9	0	-1	-1	35	20	50	9.68±0.04	9.67
10	0	+1	-1	35	30	50	9.70±0.01	9.71
11	0	-1	+1	35	20	350	9.74±0.01	9.74
12	0	+1	+1	35	30	350	9.90±0.08	9.91
13	0	0	0	35	25	200	9.80±0.01	9.81
14	0	0	0	35	25	200	9.79±0.03	9.81
15	0	0	0	35	25	200	9.81±0.00	9.81

[a] X_1: date powder concentration, g/L; [b] X_2: tryptone concentration, g/L; [c] X_3: agitation rate, rpm

Table 4. Analysis of variance (ANOVA) for the Box-Behnken design of experiments

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	*F*-value	Probe>*F*	Significance
Model	0.085	8	0.011	57.37	<0.0001	Significant
X_1	5.041E-003	1	5.041E-003	27.10	0.0008	
X_2	0.020	1	0.020	108.60	<0.0001	
X_3^2	0.018	1	0.018	95.10	<0.0001	
X_{12}	0.012	1	0.012	67.01	<0.0001	
X_{23}	6.724E-003	1	6.724E-003	36.15	0.0003	
$X_1^2 X_3$	4.624E-003	1	4.624E-003	24.86	0.0011	
$X_1X_2^2$	2.312E-003	1	2.312E-003	12.43	0.0078	
Residual	1.200E-003	1	1.200E-003	6.45	0.0347	
Lack of fit	1.488E-003	8	1.860E-004			
Pure error	5.300E-004	4	1.325E-004	0.55	0.7098	Not significant
Total	9.580E-004	4	2.395E-004			

sus, were found [4]. The optimum conditions for growth of this bacterium were pH, 6.9; vitamin solution, 12.8 mL/L; glucose, 50.1 g/L, and yeast extract, 60.0 g/L. Other ingredients of their investigated medium included (1/L): $MgSO_4$, 0.2 g; K_2HPO_4, 2.7 g; $MnSO_4$, 0.05 g and Tween-80, 1 mL. The counts of viable cells in the optimized medium increased by 3.45 and 4.08 fold, when compared to those of the MRS and center-point media, respectively; however, due to the high amounts of glucose and yeast extract and also presence of the above mentioned salts, there would still be concerns from an economical point of view. A two-stage optimization study on the probiotic *Pseudomonas* MCCB 103 was recently conducted [13]. Carbon and nitrogen sources and growth factors, such as amino acids and vitamins, were screened initially in a mineral medium for increased biomass production and then, the selected ingredients were further optimized by a full-factorial central composite design of the response surface methodology. The optimized medium contained (1/L) mannitol, 20 g; glycerol, 20 g; sodium chloride, 5 g; urea, 3.3 g and mineral salts solution, 20 mL [13]. Furthermore, the optimum conditions for the growth of *L. casei* YIT 9018 have been obtained using a central composite design of response surface methodology where the medium consisted of tryptone, yeast extract, glucose and Tween 80 [5]. The effects of medium composition and incubation temperature on the growth of *L. casei* YIT 9018 have also been assessed [5], where the estimated optimum conditions have been shown to include glucose, 15.8 g/L; tryptone, 30.4 g/L; yeast extract, 8.92 g/L and an incubation temperature of 35 °C. Their investigated medium included only two nitrogen sources of yeast extract and tryptone and a less than conventional amount of glucose as the main carbon source which imply a reduction in costs. In our study, the final optimized medium consisted generally of four components including date powder, 38 g/mL; tryptone, 30 g/mL; yeast extract, 5 g/mL and $MgSO_4$, 0.2 g/mL. Here, substitution of the more expensive carbon source of glucose with date powder is another example of cost reduction. Thus, compared to the complex MRS medium, the use of date powder instead of glucose, absence of Tween 80 and salts, *i.e.*, K_2HPO_4, $MnSO_4$, ammonium citrate, CH_3COONa, and also the use of a less expensive nitrogen source combination, such as tryptone and yeast extract instead of yeast extract, casein peptone and meat extract, causes a pronounced economical gain.

iii) Assessing factor effects with the partial-effects plot

Partial-effect functions and plots were used to assess the effect of each factor graphically in greater detail (Figure 1). The partial-effect function of a certain factor describes how the response moves as the level of that factor changes when the other factors are fixed at their optimum levels [17]. The partial-effect plot is drawn with the vertical axis representing the response (log_{10} (CFU/mL)) and the horizontal axis representing the coded levels of the factors.

The most conspicuous change was observed in the response curve associated with the agitation rate; the estimated response increased rapidly until the coded level of agitation rate reached 0.89 and decreased after the coded level climbed to values higher than 0.89. With the other factors being fixed to their optimum levels, the estimated maximum and minimum responses were 9.90 and 9.74, respectively. The difference between these responses was the largest when compared to the maximum and minimum response differences of the other two factors. Therefore, it was concluded that unlike the screening stage, agitation rate was the most significant factor in the optimization phase among the three investigated

factors, with its linear effect being the most pronounced. This is probably due to easier accessibility to and better distribution of the nutrients that are provided through agitation for all the microorganisms.

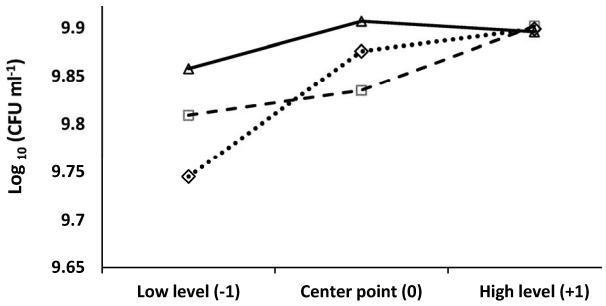

Figure 1. Partial-effect plot of date powder (△), tryptone (◇) and agitation rate (□) showing the effect of each variable at different levels.

The partial-response curve of tryptone showed the second conspicuous change; the estimated response increased gradually as the coded level of tryptone approached 0 and increased more rapidly as the coded level exceeded 0. Since the coded level of +1 was the high end of the investigated range, it was difficult to assure whether the estimated response would continue to increase as the level of tryptone exceeded. Therefore, it was suggested to conduct additional studies in the future using a wider range of tryptone concentration.

The partial-response curve of date powder showed a smaller change compared to the others as opposed to the screening stage. However, the estimated response increased gradually as the coded level of date powder shifted from -1 to +1 and decreased steadily after the coded level passed beyond 0.24. It has been reported that the partial-effect of glucose, as the main carbon source, was less important than that of the incubation temperature and yeast extract in the tryptone-yeast extract-glucose medium [5]. On the other hand, in a medium that included yeast extract, glucose and vitamins, the strongest partial-effect was related to the yeast extract concentration followed by pH [4]. Based on this study, the partial-effect of the present carbon source (date powder) was ranked after that of agitation rate and tryptone.

iv) Plotting three-dimensional response surface plots

The three-dimensional response surface graphs associated with the effects of the interaction of date powder versus tryptone, date powder versus agitation rate and tryptone *versus* agitation rate on the response were plotted (Figure 2). Figure 2a shows the response for the interactive factors, date powder and tryptone concentrations, when the agitation rate was fixed at 200 rpm. The maximum log value of the viable cells under these conditions was predicted to be 9.879, corresponding to high concentrations of both date powder (ca. 45 g/L) and tryptone (30 g/L). It can be observed that the response varies much as an interaction function of date powder and tryptone concentrations. The estimated response decreased rapidly as the actual levels of date powder and tryptone started to shift from 45 to 25 g/L and from 30 to 20 g/L, respectively. In the presence of 25 g/L tryptone, the estimated response clearly increased as the agitation rate approached its actual level of ca. 320 rpm, but decreased slightly thereafter. Under these conditions, the estimated response increased when the actual level of date powder shifted from 25 to ca. 37 g/L and then remained approximately constant as the actual level exceeded 37 g/L (Figure 2b). The reason for such responses is that agitation had mixes the culture medium and consequently increases the availability of the nutrients and mass transfer of the substrate. The maximum log value of viable cells, when the actual level of date powder was fixed at 35 g/L, was predicted to be 9.907 (Figure 2c). This corresponds to 30 g/L tryptone and an agitation rate of ca. 300 rpm. The predicted response was mainly under the influence of the agitation rate, so that it had a pronounced increase when the actual level the of agitation rate moved from 50 toward 250 rpm, but only increased slightly thereafter. However, the interaction effect of the two factors of date powder and tryptone on cell growth was the most significant. Finally, Figure 2 demonstrates the profound influence of these factors on each other, as was predicted by the model.

v) Validating the optimum points of the factors

To validate the optimum point of factors predicted by the model, a validation test in duplicate was conducted using three growth media: the MRS medium, the optimum-point medium and the center-point medium. The mean log values of the viable cells were 10.06 (MRS medium), 9.97 (optimum-point medium) and 9.80 (center-point medium), which are equal to 11.48×10^9, 9.33×10^9 and 6.31×10^9 CFU/mL, respectively. Therefore, bacterial growth increased by almost 1.5 fold when compared to the center-point using a two-stage statistical design. Moreover, the obtained response was close to the predicted range (9.89--9.93), indicating the good fit of the model. The optimum point medium investigated by Oh *et al.* was found to be approximately twice more productive than the center-point medium, and more economical than the MRS medium [5]. The maximum biomass for *L. casei* EQ28, *L. rhamnosus* R-011, *L. plantarum* EQ12,

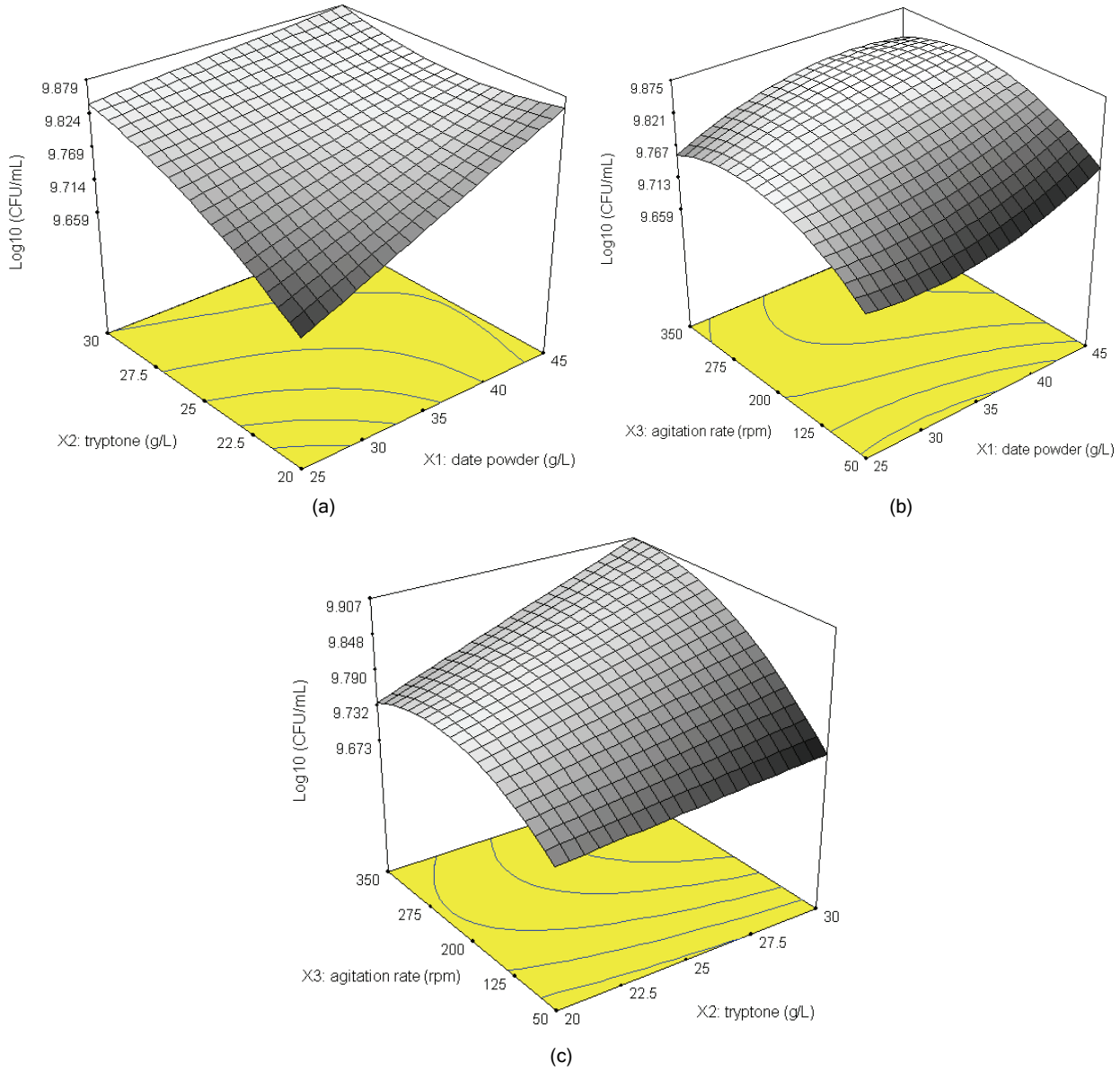

Figure 2. Three dimensional surface plots of L. casei ATCC 334 growth (log_{10} (CFU/mL)) illustrating the interactions between a) date powder and tryptone, b) date powder and agitation rate and c) tryptone and agitation rate.

and *Streptococcus thermophilus* R-083 was also obtained with a mixture of yeast and potato extracts [8]. Thus, through partial replacement of yeast extract with the less expensive 40 % potato extract, strong economical advantages could be observed. A new medium has recently been developed in which a significant 19% biomass increase of the probiotic *Pseudomonas* MCCB 103 has been obtained. Furthermore, in another study on *L. paracasei ssp. paracasei*, the additions of yeast extract, salts (phosphates, citrates, Mg and Mn), glucose as well as Tween to the Tofu whey base have resulted in cell populations as high as that obtained in the MRS broth [10]. The use of tofu whey, supplemented with less than conventional amounts of glucose and yeast extract, could lead to more cost-effective production of starters [10]. In the current study, the optimum-point medium was found to be comparable with the MRS medium and more productive than the center-point medium.

CONCLUSION

Statistical design of experiments can be employed to model the relationship between certain variables and one or more responses in process. Since the cost of culture medium has a remarkable impact on the mass production of probiotics, the optimization of growth conditions, substitution with low-price nutrient ingredients and simplification of medium are vital for their economical production. The Taguchi method

can be employed to screen the significant parameters and design a simple medium. Response surface methodology can be implemented to estimate a polynomial model representing the effect of significant factors on viable cell counts in the probiotic products, as well as to optimize the process variables. Such combination of statistical design of experiments would be useful to optimize bioprocesses.

This study employed a two-phase procedure including a screening phase through Taguchi and an optimization phase through Box-Behnken DOE to develop an economical broth for the growth of *L. casei* ATCC 334. The optimum levels of the medium composition were confirmed by verification experiments, as $\log_{10}$ CFU/mL = 9.97, which was approximately 9% more than that obtained in the basal medium that contains low levels of all factors in the Taguchi design of experiments (9.16). Moreover, it was shown that the log value of viable cells in the optimized medium based on date powder was approximately equal to that in the complex and expensive MRS medium. Results showed that date powder as a low-price carbon source could be an appropriate substitute for many other carbon sources, such as glucose, in different medium compositions. The suggested simplified medium has the potential to be employed in the industrial production of different types of Lactobacilli used in dairy products.

Acknowledgment

The authors gratefully acknowledge the help of Dr. Parvin Shariati.

REFERENCES

[1] C.G. Vinderola, W. Prosello, D. Ghiberto, J.A. Reinheimer, J. Dairy Sci. **83** (2000) 1905-1911

[2] F. Leroy, L. De Vuyst, Trends Food Sci. Technol. **15** (2004) 67-78

[3] R. Fuller, Probiotics, The Scientific Basis. London: Chapman and Hall Ltd., 1992, pp. 1-8

[4] S.L. Liew, A.B. Ariff, A.R. Raha,Y.W. Ho, Int. J. Food Microbiol. **102** (2005) 137-142

[5] S. Oh, S. Rheem, J. Sim, S. Kim, Y. Baek, Appl. Environ. Microbiol. **61** (1995) 3809-3814

[6] M. Rogosa, J.G. Franklin, K.D. Perry, J. Gen. Microbiol. **24** (1961) 473-482

[7] W. Al-Shahib, R.J. Marshall, Int. J. Food Sci. Nutr. **24** (2003) 247-259

[8] H. Gaudreau, N. Renard, C.P. Champagne, D. Van Horn, Can. J. Microbiol. **48** (2002) 626-634

[9] D. Charalampopoulos, S.S. Pandiella, C. Webb, J. Appl. Microbiol. **92** (2002) 851-859

[10] L.N. Thi, C.P. Champagne, B.H. Lee, J. Goulet, Int. J. Food Microbiol. **89** (2003) 67-75

[11] D.C. Montgomery, Design and Analysis of Experiments. New York: John Wiley and Sons Inc., 1991

[12] R.H. Myers, D.C. Montgomery D.C. New York: Response Surface Methodology: Process and Product Optimization Using Designed Experiments, John Wiley & Sons, 1995

[13] R. Preetha, N.S. Jayaprakash, R. Philip, I.S. Bright Singh, J. Appl. Microbiol. **102** (2007) 1043-1051

[14] K.N. Chen, C.Y. Kuo, J.S. Shiu, M.J. Chen, J. Food Process Eng. **34** (2011) 427-443

[15] A. Shahravy, A study on probiotic yoghurt production by adding isolated probiotic bacterial strains of a certified foreign probiotic yoghurt in order to improve the quality of the product, M.Sc. Thesis, Faculty of food Science and Technology, Science and Research Branch, Islamic Azad University, Iran, 2007

[16] B.S. Everitt, Cambridge Dictionary of Statistics, CUP. ISBN 0-521-81099-x, 2002

[17] S. Rheem, The partial effects plot for graphical comparative assessment of factor effects. In: Proceedings of the International Conference on Statistical Methods and Statistical Computing for Quality and Productivity Improvement (ICSQP '95). (670-676) Seoul, Korea, 1995

[18] A.S. Walid, R.J. Marshall, R.J. Int. J. Food Sci. Nutr. **54** (2003) 247-259.

A. SHAHRAVY[1,2]
F. TABANDEH[1]
B. BAMBAI[1]
H.R. ZAMANIZADEH[2]
M. MIZANI[2]

[1]Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[2]Faculty of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran

NAUČNI RAD

OPTIMIZACIJA PRODUKCIJE PROBIOTIKA POMOĆU SOJA *Lactobacillus casei* ATCC 334 KORIŠĆENJEM PRAHA URME KAO IZVORA UGLJENIKA

U ovom radu je izvršena optimizacija uslova za ekonomičnu proizvodnjuprobiotske bakterije, Lactobacillus casei ATCC 334, tako što je prvi put kao izvor ugljenika korišćen jeftin prah urme palme. Uticaj jedanaest faktora na razvoj bakterija je ispitivan primenom Taguchijevemetode. Kao najznačajniji parametri su se pokazali prah urme palme, tripton i brzina mešanja. Optimalni uslovi: koncentracijepalmine urme u prahu (38 g/l) itriptona (30 g/l) i brzina mešanja(320 min^{-1}) određeni su metedologijom površine odziva po Box-Behnkenu.Predložen je model polinoma trećeg reda koji dobro opisuje eksperimentalne podatke. Maksimalna log vrednost vidljivih ćelija u optimizovanoj alternativnoj hranljivoj podlozi je 9,97za vreme inkubacije od 24 h, koja je uporedivaje sa vrednošću dobijenom u složenom i skupom MRS hranljivoj podlozi (10,06).

Ključne reči: probiotik; metodologija površine odziva (RSM); mlečni proizvodi; funkcionalan hrana; Taguchijeva metoda.