J. Microbiol. Biotechnol. (2013), 23(8), 1084–1091
http://dx.doi.org/10.4014/jmb.1212.12043

jmb

Statistical Optimization of Medium Components for Milk-Clotting Enzyme Production by *Bacillus amyloliquefaciens* D4 Using Wheat Bran-an Agro-Industry Waste

Weibing Zhang[1], Xiaoling He[2,3], Hongna Liu[1], Huiyuan Guo[2], Fazheng Ren[1,2*], Weidong Gao[1], and Pengcheng Wen[1]

[1]*College of Food Science and Technology Engineering, Gansu Agricultural University, Lanzhou 730070, China*
[2]*Key Laboratory of Functional Dairy, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China*
[3]*Dingxi Center for Disease Control and Prevention, Dingxi 74300, China*

Received: December 20, 2012
Revised: Arpil 6, 2013
Accepted: May 1, 2013

First published online
May 16, 2013

**Corresponding author*
Phone: +86-931-7631201;
Fax: +86-931-7631201;
E-mail: ganbz@gsau.edu.cn

pISSN 1017-7825, eISSN 1738-8872

In this paper, two statistical methods were applied to optimize medium components to improve the production of the milk-clotting enzyme by *Bacillus amyloliquefaciens* D4. First, wheat bran juice, skim milk powder, and Na_2HPO_4 were shown to have significant effects on D4 enzyme production using the Plackett–Burman experimental design. Subsequently, an optimal medium was obtained using the Box–Behnken method, which consisted of 3.31 g/l of skim milk powder, 5.0 g/l of sucrose, 0.1 g/l of $FeSO_4 \cdot 7H_2O$, 0.1 g/l of $MgSO_4 \cdot 7H_2O$, 0.1 g/l of $MnSO_4 \cdot 2H_2O$, 0.1 g/l of $ZnSO_4 \cdot 7H_2O$, 1.52 g/l of Na_2HPO_4, and 172.45 g/l of wheat bran juice. With this optimal medium, the milk-clotting enzyme production was remarkably enhanced. The milk-clotting enzyme activity reached 3,326.7 SU/ml after incubation of 48 h, which was 1.76-fold higher than that of the basic medium, showing that the Plackett–Burman design and Box–Behnken response surface method are effective to optimize medium components, and *B. amyloliquefaciens* D4 possessed a high rennet-producing capacity in the optimal medium.

Keywords: Optimization, enzyme production, *Bacillus amyloliquefaciens* D4, response surface methodology

Introduction

The quality of cheese is significantly dependent on the type of milk-clotting enzyme, which is important in the processes of milk coagulation and cheese maturation [16]. Conventionally, calf rennet has been the most widely used coagulant in cheese-making, which is obtained from the stomach of an unweaned calf. The worldwide increase of cheese production and the reduced supply of calf rennet have led to a search for novel sources of rennet [7].

Milk-clotting enzymes obtained from microorganisms have received particular attention owing to their diverse properties, relative ease of preparation, and reduced cost. Various fungal proteases have been widely used as milk coagulants, such as rennet derived from *Mucor pusillus*, *Mucor miehei*, and *Endothia parasitica* [19]. Numerous bacteria have been suggested as promising microbial rennet producers, such as *Bacillus licheniformis* [1, 27], *Bacillus subtilis* [10, 11, 17], *Bacillus sphaericus* [12, 18], *Bacillus subtilis natto* [9], *Myxococcus xanthus* [6, 20], *Enterococcus faecalis* [25], and *Bacillus polymyxa* [15].

It is widely known that microbial enzyme production is greatly influenced by many factors, especially medium composition and cultivation conditions. The conventional strategy for medium optimization is the one-variable-at-a-time approach, which is time consuming, expensive, and incapable of detecting the true optimum, owing to the interactions among the multiple factors involved. A statistical experimental design involving the Plackett–Burman design and Box–Behnken response surface methodology (RSM) can eliminate the limitations of the single-factor optimization process and has been successfully applied in many areas of

biotechnology, such as production of lipase [3], xylanase [2], and tannase [5]. However, there are few studies on the optimization of medium components for microbes to produce milk-clotting enzymes *via* the Plackett–Burman design and Box–Behnken RSM [11, 22].

Wheat bran, an agro-industrial residue, contains cellulose material, starch, crude protein, trace elements, and other certain ingredients, which can be used as carbon and nitrogen sources to promote the growth of microorganisms and milk-clotting enzyme production through solid-state fermentation [21, 23, 24, 26]. However, there are few reports on the utilization of wheat bran juice for microbes to produce milk-clotting enzymes by submerged fermentation [10, 13, 14]. Therefore, we used this material for milk-clotting enzyme production by *B. amyloliquefaciens* D4 under submerged fermentation.

B. amyloliquefaciens D4, which can producing milk-clotting enzyme, has been isolated from yak grazing soil in the north-eastern Tibetan Plateau [14]. The objective of the present work was to attempt to optimize the medium components with a statistical optimization strategy to increase milk-clotting enzyme production by *B. amyloliquefaciens* D4 using wheat bran juice. The Box–Behnken RSM was used to optimize key nutrients screened *via* the Plackett–Burman design in the study.

Materials and Methods

Strain and Chemicals

B. amyloliquefaciens D4 (CGMCC 3290) was isolated from yak grazing soil from the north-eastern Tibetan Plateau and deposited in the China General Microbiological Culture Collection Center (Beijing, China). The strain was propagated at 37°C on lysogeny broth (LB) agar slants (1.0% (w/v) peptone, 1.0% (w/v) beef extract, 0.5% (w/v) NaCl, and 2.0% (w/v) agar, pH 7.2), and subcultured every 30 days. The chemicals used in this study were of analytical grade.

Preparation of Seed Culture

The strains obtained from the LB agar slants were inoculated into 5 ml of seed culture medium, composed of beef extract (10 g/l), peptone (3 g/l), and NaCl (5 g/l) in a test tube, and incubated at 37°C at pH 7.2 for 24 h with shaking at 170 rpm. After incubation, a 1 ml aliquot of the bacteria was inoculated into 100 ml of basal fermentation medium in a 250 ml flask and incubated for 24 h at 37°C at pH 7.2 with shaking at 170 rpm. The culture broth was used as seed culture for the later experiments. The basal fermentation medium was prepared as follows: 2 g of skim milk powder, 5 g of sucrose, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot 2H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, and 0.5 g of Na_2HPO_4 were dissolved in 1,000 ml of wheat bran juice (wheat bran juice: 100 g of wheat bran in 1,000 ml of distilled water, boiled for 10 min, and filtered through gauze).

Batch Fermentation

Initial optimizations were conducted in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium, containing different concentrations of medium components that were tested according to the experimental statistical design. These flasks were inoculated with 2% seed culture and incubated at 37°C for 48 h on a rotary shaker at 170 rpm. After 48 h of fermentation, the crude enzyme solution was obtained by centrifugation at 8,000 ×*g* for 10 min. All fermentations were carried out in triplicate and the results represent the average of the three trials.

The optimal medium compositions identified above were confirmed using a 10 L fermentor (GUJS-10L, Zhenjiang East Biotech Equipment and Technology Co., Ltd) filled with 8 L of culture medium, along with 0.02% antifoaming agent. After sterilization (121°C for 15 min), the medium was cooled to the scheduled temperature (37°C) and inoculated with 5% seed culture. The fermentor was operated at 37°C for 72 h. During the fermentation, the agitation rate and air flow rate were controlled manually to maintain a certain range of dissolved oxygen percentage (10–60%). Moreover, aeration helps keep a positive pressure to 0.05 MPa. Samples were taken for assay of milk-clotting activity.

Experimental Design and Data Analyses

The optimization of medium constituents to improve the milk-clotting activity of *B. amyloliquefaciens* D4 was carried out in two stages. Firstly, we investigated eight variables, including four inorganic components, in the basic medium. The variables having the most significant effect on milk-clotting activity were identified using a two-level Plackett–Burman design. Each variable was represented at two levels (high and low), which were denoted by (+1) and (-1), respectively (Table 1). The total number of trials carried out according to the Plackett–Burman design was k+1, where k is the number of variables (medium components). Here, eight assigned variables and three unassigned variables, commonly referred to as dummy variables, employed to evaluate the standard error (SE) of the experiment, were screened in 12 experimental designs. Table 1 lists the concentrations of each factor used in the experimental designs, whereas Table 2 represents the design algorithm. The statistical software package Minitab ver. 14.11 (Minitab Inc., USA) was used to analyze the experimental design.

Secondly, Box–Behnken RSM was used to optimize the screened components to enhance the milk-clotting activity. Each factor in the design was studied at three different levels (-1, 0, and +1). The variable concentrations and experimental design are shown in Table 4. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_{ij} + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear offset, β_{ii} is the squared offset, β_{ij} is the interaction effect,

Table 1. Values and effect estimates of the Plackett–Burman design.

Factors	Medium components (g/l)	Levels		Effect estimate	SE	t-value	*p*-value	Confidence level (%)
		-1	+1					
X_1	Wheat bran juice	100	150	206.1	40.30	5.11	0.0145[a]	98.55
X_2	Skim milk powder	2	3	191.1	40.30	4.74	0.0178[a]	98.22
X_3	Sucrose	5	15	-36.1	40.30	-0.89	0.4368	56.32
X_4	$FeSO_4{\cdot}7H_2O$	0.1	1.0	-45.7	40.30	-1.13	0.3391	66.09
X_5	$MgSO_4{\cdot}7H_2O$	0.1	1.0	-55.8	40.30	-1.39	0.2600	74.00
X_6	$MnSO_4{\cdot}7H_2O$	0.1	1.0	-48.1	40.30	-1.19	0.3190	68.10
X_7	$ZnSO_4{\cdot}7H_2O$	0.1	1.0	-87.6	40.30	-2.17	0.1179	88.21
X_8	Na_2HPO_4	0.5	2	152.6	40.30	3.79	0.0323[a]	96.77

[a]Indicates model terms are significant.

and X_i is the dimensionless coded value of X_i. This equation can be used to evaluate the linear, quadratic, and interactive effects of independent variables on the dependent response.

The software Minitab ver. 14.11 was used for regression and graphical analyses of the obtained experimental data. A differentiation calculation was then employed to predict the optimum values of the various factors.

Enzyme Assay

The milk-clotting activity was investigated using the method of Arima *et al*. [4] and expressed in terms of the Soxhlet unit (SU), which is defined as the amount of enzyme required to clot 1 ml of a substrate solution (10% skim milk in 10 mM of $CaCl_2$) in 40 min at 35°C. Enzyme solution (0.5 ml) was added to 5 ml of the substrate solution containing 10% skim milk powder and 10 mM of calcium chloride and incubated at 35°C for 5 min. The mixture was mixed well and the formation time of the curd fragment was measured.

The milk-clotting activity was calculated using the following equation:

$$SU = \frac{2400 \times 5 \times D}{t \times 0.5} \quad (2)$$

where t is the clotting time (sec) and D is dilution of the enzyme solution.

Results and Discussion

Screening of important medium components

The experimental design and corresponding milk-clotting activity of eight medium components are shown in Table 2. The milk-clotting enzyme activity ranged from 1,283.9 to 2,708.5 SU/ml, indicating a strong influence of medium components on milk-clotting enzyme production.

The main effect of each variable on milk-clotting activity was estimated as the difference between the average

Table 2. Experimental design and results of the Plackett-Burman.

Trial	Variables/levels											Milk-clotting activity (SU/ml)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	Observed	Predicted
1	-1	+1	-1	+1	-1	-1	+1	+1	-1	+1	-1	2,134.6 ± 5.8	2,247.2
2	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	2,355.3 ± 6.2	2,296.4
3	-1	-1	+1	+1	+1	+1	+1	-1	-1	-1	+1	1,283.9 ± 6.8	1,279.9
4	+1	+1	-1	+1	-1	+1	-1	-1	+1	+1	-1	2,492.2 ± 7.5	2,433.3
5	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	2,218.4 ± 5.1	2,277.3
6	-1	+1	+1	-1	+1	-1	-1	-1	-1	+1	+1	1,967.2 ± 6.3	2,024.9
7	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1,884.2 ± 5.3	1,826.5
8	-1	-1	+1	+1	-1	+1	-1	+1	-1	-1	+1	1,868.1 ± 5.9	1,872.1
9	+1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	2,223.5 ± 6.1	2,336.1
10	-1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	2,243.5 ± 7.2	2,130.9
11	+1	+1	+1	+1	+1	-1	-1	+1	+1	+1	+1	2,708.5 ± 5.3	2,650.8
12	+1	-1	-1	+1	+1	-1	+1	-1	+1	-1	-1	1,856.2 ± 6.3	1,860.2

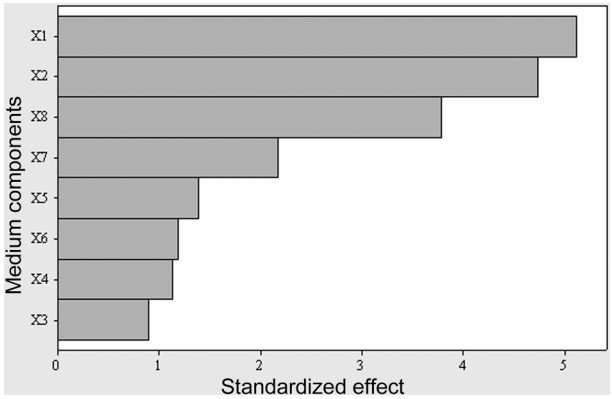

Fig. 1. Pareto plot for Plackett–Burman parameter estimates for medium components.

X1, wheat bran juice; X2, skim milk powder; X3, sucrose; X4, $FeSO_4 \cdot 7H_2O$; X5, $MgSO_4 \cdot 7H_2O$; X6, $MnSO_4 \cdot 2H_2O$; X7, $ZnSO_4 \cdot 7H_2O$; and X8, Na_2HPO_4.

measurements at the high level (+1) and low level (-1) of each factor (Table 1). If the sign of the effect of a tested variable was positive, its influence on milk-clotting activity was greater at a high level; whereas, if the sign was negative, its influence was greater at a low level. The regression analysis presented in Table 1 shows that low levels of X_3 (sucrose), X_4 ($FeSO_4 \cdot 7H_2O$), X_5 ($MgSO_4 \cdot 7H_2O$), X_6 ($MnSO_4 \cdot 7H_2O$), and X_7 ($ZnSO_4 \cdot 7H_2O$) enhanced milk-clotting production, whereas high levels of X_1 (wheat bran), X_2 (skim milk powder), and X_8 (Na_2HPO_4) resulted in high milk-clotting activity. The Pareto chart in Fig. 1 ranks the significance of the medium components based on the results obtained by the Plackett–Burman design. The results were in agreement with previous reports describing wheat bran as a better substrate for milk-clotting enzyme production by *Aspergillus oryzae* [24], *Rhizomucor* spp. [21], *Absidia ramose* [23], and *Mucor miehei* [26]. In studies on milk-clotting enzyme production by *Rhizomucor* [21] and *Mucor miehei* [26], high concentrations of skim milk powder resulted in greater enzyme production. Enhancement in the presence of Mg^{2+} and Fe^{2+} has been reported for *Amylomyces rouxii* [28], and $CaCl_2$ exhibited a synergistic effect on enhanced milk-clotting enzyme production by *Aspergillus niger* [8]. In our previous study, metal ions (Zn^{2+}, Mg^{2+}, and Mn^{2+}) significantly reduced the milk-clotting activity of *B. amyloliquefaciens* D4, whereas Ca^{2+} and Na^{+} significantly increased its activity [13].

The variable confidence levels above 95% in the Plackett–Burman design were selected and further optimized. According to Table 1, wheat bran juice, skim milk powder, and Na_2HPO_4 were found to be statistically significant

Table 3. Coded and real values of the variables in the Box–Behnken design.

Coded value (level)	Real value of variables		
	Wheat bran juice (g/l)	Skim milk powder (g/l)	Na_2HPO_4 (g/l)
-1	100	1.5	1
0	150	3	2
+1	200	4.5	3

medium components with high confidence levels, but sucrose, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$ were not. The above results indicated that the Plackett–Burman design is a powerful tool to identify factors that significantly influence milk-clotting activity.

Optimization of Medium Components

Three significant independent variables [X_1 (wheat bran), X_2 (skim milk powder), and X_8 (Na_2HPO_4)] were selected and further optimized using the Box–Behnken design to determine their optimal concentrations. The coded and real values of the variables at various levels are given in Table 3. In the Box–Behnken design, X_3 (sucrose), X_4 ($FeSO_4 \cdot 7H_2O$), X_5 ($MgSO_4 \cdot 7H_2O$), X_6 ($MnSO_4 \cdot 7H_2O$), andX_7 ($ZnSO_4 \cdot 7H_2O$) were set at their low levels of 5.0, 0.1, 0.1, 0.1, and 0.1 g/l, respectively.

Table 4 shows the Box–Behnken experimental design and the obtained milk-clotting activity of the individual components. *Via* multiple regression analysis on the experimental data in Table 4 using the Minitab ver. 14.11 software, the following second-order polynomial equation was obtained:

$$Y = 3{,}183.37 + 427.16(A) + 84.02(B) - 104.49(C) - 107.8(AB) - 235.88(AC) - 93.1(BC) - 577.75(A^2) - 193.57(B^2) - 237.35(C^2) \quad (3)$$

where Y is the predicted response and A, B, and C are the coded values of wheat bran juice, skim milk powder, and Na_2HPO_4, respectively.

The regression coefficients and the analysis of variance presented in Tables 5 and 6 indicate the high significance of the model. The highest R^2 value (0.98) was also in good agreement with the experimental results and theoretical values predicted by the model. Our results revealed that linear and quadratic terms of wheat bran juice and Na_2HPO_4 had a significant effect on the milk-clotting activity production ($p < 0.05$). Simultaneously, the square of skim milk powder and interactive terms of wheat bran juice and Na_2HPO_4 were also significant.

Table 4. Design and results of the Box–Behnken design.

Trial	Variables/levels			Milk-clotting activity (SU/ml)	
	A: wheat bran juice (coded value)	B: skim milk powder (coded value)	C: Na_2HPO_4 (coded value)	Observed	Predicted
1	-1	0	-1	1,876.1 ± 7.5	1,809.7
2	0	-1	-1	2,601.6 ± 5.8	2,679.8
3	-1	-1	0	1,804.9 ± 6.8	1,793.1
4	-1	0	1	1,991.4 ± 6.5	2,072.5
5	1	-1	0	2,860.1 ± 5.7	2,862.9
6	0	-1	1	2,726.3 ± 6.3	2,657.1
7	1	1	0	2,803.6 ± 7.2	2,815.4
8	1	0	1	2,388.7 ± 6.9	2,455.1
9	1	0	-1	3,216.9 ± 5.9	3,135.8
10	0	1	-1	2,964.8 ± 5.2	3,034.1
11	-1	1	0	2,179.6 ± 6.6	2,176.7
12	0	1	1	2,717.1 ± 7.1	2,638.9
13	0	0	0	3,191.9 ± 6.3	3,183.4
14	0	0	0	3,176.8 ± 6.4	3,183.4
15	0	0	0	3,181.4 ± 5.9	3,183.4

Table 5. Analysis of variance for the Box–Behnken design experiments.

Term	Coefficient	Standard error	t Ratio	*p*-Value
Intercept	3,183.37	54.29	58.636	< 0.0001
X_1	427.16	33.25	12.849	<0.0001
X_2	84.02	33.25	2.527	0.053
X_3	-104.49	33.25	-3.143	0.026
$X_1 X_2$	-107.80	47.02	-2.293	0.070
X_1X_3	-235.88	47.02	-5.017	0.004
X_2X_3	-93.10	47.02	-1.980	0.105
X_1^2	-577.75	48.94	-11.806	< 0.0001
X_2^2	-193.57	48.94	-3.956	0.011
X_3^2	-237.35	48.94	-4.850	0.004

From equations derived by differentiation of Eq. (3), the optimal values of A, B, and C in the coded units were found to be 0.4491, 0.2084, and -0.4841, respectively. Correspondingly, we obtained the maximum point of the model, which was 172.45 g/l of wheat bran juice, 3.31 g/l of skim milk powder, and 1.52 g/l of Na_2HPO_4, respectively. The maximum predicted value of milk-clotting activity was 3,313.7 SU/ml.

Using the Minitab software, contour curves described by the regression model were constructed and are shown in Figs. 2–4. The shape of the corresponding contour plot indicates whether the mutual interactions between the independent variables are significant. Fig. 2 depicts the effects of wheat bran juice (A) and Na_2HPO_4 (C) concentrations on milk-clotting enzyme production by keeping a constant concentration of skim milk powder (B). The elliptical nature of the contour plots indicates that the mutual interactions between the independent variables A and C were significant, indicating that the effect of wheat bran juice on milk-clotting activity is dependent on the concentration of skim milk powder. The contour curves shown in Fig. 3 are based on the independent variables wheat bran juice (A) and skim milk powder (B), while the third independent variable, Na_2HPO_4 (C), was kept at an optimal level. A two-dimensional contour plot with respect to wheat bran juice and skim

Table 6. Statistical significance of the response equation.

Source	Degrees of freedom	Sum of squares	Mean square	F-value	*p*-Value
Regression	9	3,346,332	371,815	42.05	< 0.0001*
Residual	5	44,211	8,842		
Total	14	3,390,544			

R^2 = 98.7%; R^2(adj) = 96.3%.

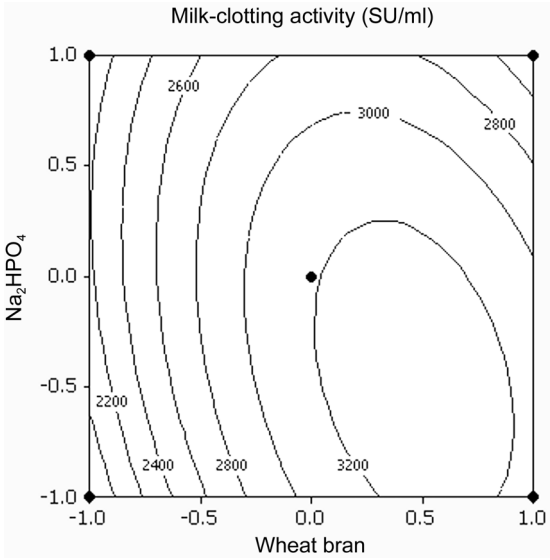

Fig. 2. Contour plots showing the effects of wheat bran juice and Na_2HPO_4 on milk-clotting activity.

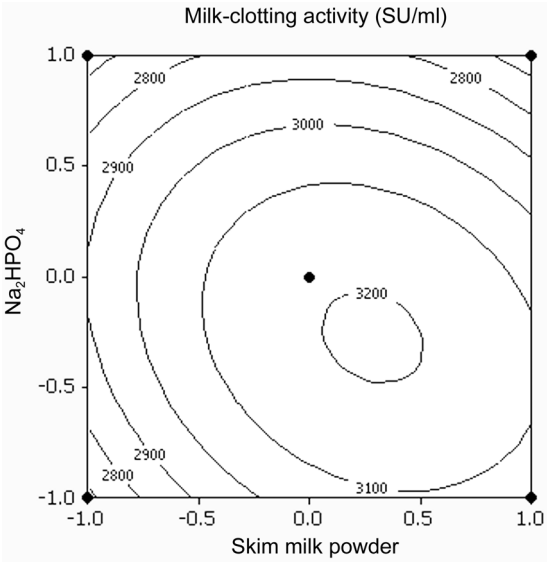

Fig. 4. Contour plots showing the effects of skim milk powder and Na_2HPO_4 on milk-clotting activity.

milk powder showed a clear elongated running diagonal line on the plot, suggesting that wheat bran juice and skim milk powder were interdependent, or that they significantly influenced milk-clotting activity. Fig. 4 denotes the effects of skim milk powder (B) and Na_2HPO_4 (C), while maintaining a fixed wheat bran juice (A) concentration of 172.45 g/l. The isoresponse contour of milk-clotting activity showed an approximate rounded ridge running diagonally on the plot, implying that skim milk powder (B) and Na_2HPO_4 (C) concentration were slightly interdependent.

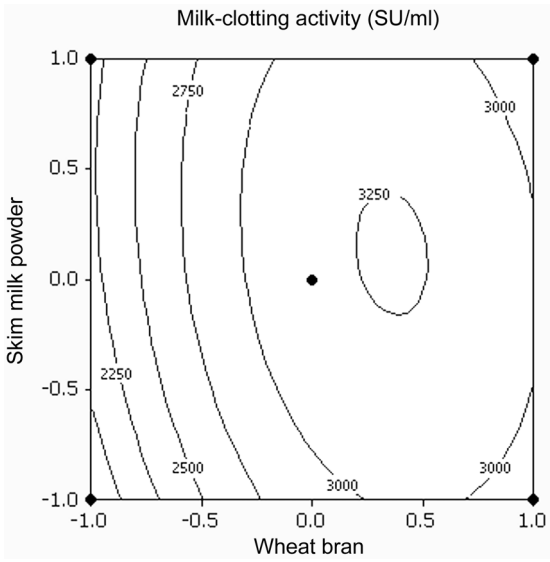

Fig. 3. Contour plots showing the effects of wheat bran juice and skim milk powder on milk-clotting activity.

Experimental Validation of the Optimized Medium Components

In order to confirm the predicted results of the model, batch fermentation in a 10 L fermentor was carried out in the statistically optimized medium. Fig. 5 shows the batch profile of milk-clotting enzyme poduction in the 10 L fermentor. The milk-clotting enzyme activity of 3,326.6 SU/ml in the optimized medium was achieved after 48 h of cultivation, which was very close to the predicted value of

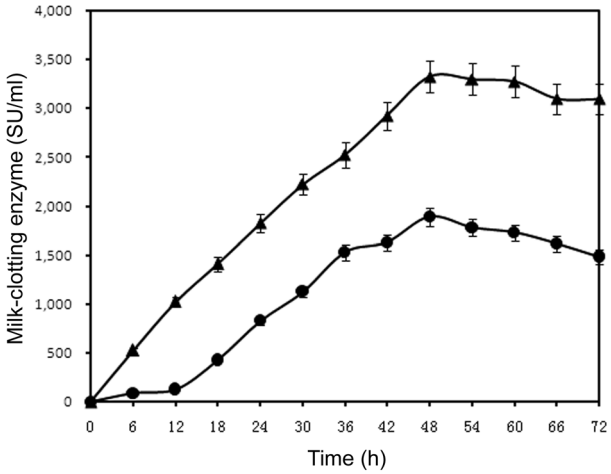

Fig. 5. A comparison of milk-clotting enzyme activity between the statistical optimized medium (▲) and the basic medium (●). Error bars represent the standard deviation of triplicate experiments.

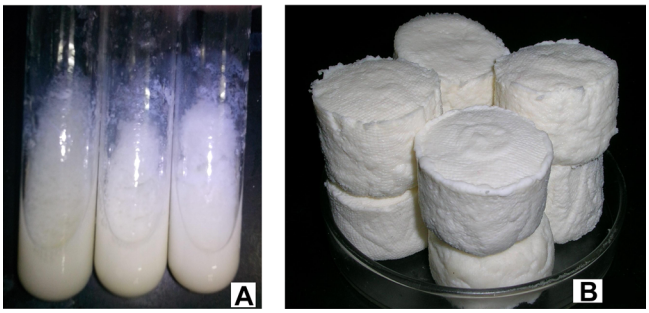

Fig. 6. Test of the milk-clotting enzyme in cheese production. (**A**) Milk clotting using samples of rennet produced by *B. amyloliquefaciens* D4. (**B**) Cheese made with milk-clotting enzyme produced by *B. amyloliquefaciens* D4.

3,313.7 SU/ml according to Eq. (3). The good correlation between these two results validates the model and the existence of an optimal value.

Using the statistical optimization method, a 1.94-fold increase in milk-clotting protease production by *Bacillus subtilis* [11] and 139% increase in recombinant cyprosin B production by transformed *Saccharomyces cerevisiae* BJ1991 [22] were achieved. In this study, a 1.76-fold increase in milk-clotting enzyme production was obtained, which indicated that the Plackett–Burman design and Box–Behnken RSM are powerful tools to identify factors and optimize the medium components for milk-clotting enzyme production by *B. amyloliquefaciens* D4.

Herein, we investigated the characterizations and applications of the milk-clotting enzyme by *B. amyloliquefaciens* D4 and found that the enzyme was a metalloprotease with a molecular mass of 58.2 kDa and was completely inactivated by heating at 55°C for 20 min. The optimum temperature and optimum pH were 65°C and 5.5, respectively [28]. Milk-clotting enzyme production by *B. amyloliquefaciens* has been successfully applied in the preparation of cheese (Fig. 6). Considering these properties, *B. amyloliquefaciens* D4 is a promising producer of microbial rennet.

Acknowledgments

This work was financed by the National Key Technologies R & D Program of China in rural areas (2011AA100903), Innovation Fund of GAU (GAU-CX1107), and National Key Technologies R & D Program of China (2012BAD28B07).

References

1. Ageitos JM, Vallejo JA, Sestelo ABF, Poza M, Villa TG. 2007. Purification and characterization of a milk-clotting protease from *Bacillus licheniformis* strain USC13. *J. Appl. Microbiol.* **103:** 2205-2213.
2. Ajay P, Khanum F. 2010. Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Bioresour. Technol.* **101:** 7563-7569.
3. Aravindan R, Viruthagiri T. 2009. Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus* MTCC 2233. *LWT-Food Sci. Technol.* **42:** 985-992.
4. Arima K, Yu J, Iwasaki S. 1970. Milk-clotting enzyme from *Mucor pusillus* var. Lindt. *Methods Enzymol.* **19:** 446-459.
5. Belur PD, Goud R, Goudar DC. 2012. Optimization of culture medium for novel cell-associated tannase production from *Bacillus massiliensis* using response surface methodology. *J. Microbiol. Biotechnol.* **22:** 199-206.
6. Carias JR, Raingeaud J, Mazaud C, Vathon G, Lucas N, Cenatiempo Y, *et al*. 1990. A chymosin-like extracellular acidic endoprotease from *Myxococcus xanthus* DK101. *FEBS Lett.* **262:** 97-100.
7. Cavalcanti MTH, Teixeira MFS, Lima FJL, Porto ALF. 2004. Partial purification of new milk-clotting enzyme produced by *Nocardiopsis sp. Bioresour. Technol.* **93:** 29-35.
8. Channe PS, Shewale JG. 1998. Influence of culture conditions on the formation of milk-clotting protease by *Aspergillus niger* MC4. *World J. Microbiol. Biotechnol.* **14:** 11-15.
9. Chwen-Jen S, Lan-Anh PT, Ing-Lung S. 2009. Milk-clotting enzymes produced by culture of *Bacillus subtilis natto. Biochem. Eng. J.* **43:** 85-91.
10. Ding ZY, Liu SP, Gu ZH, Zhang L, Zhang KC, Shi GY. 2011. Production of milk-clotting enzyme by *Bacillus subtilis* B1 from wheat bran. *Afr. J. Biotechnol.* **10:** 9370-9378.
11. Dutt K, Gupta P, Saran S, Misra S, Saxena RK. 2009. Production of milk-clotting protease from *Bacillus subtilis*. *Appl. Biochem. Biotechnol.* **158:** 761-772.
12. El-Bendary MA, Moharam ME, Ali TH. 2007. Purification and characterization of milk clotting enzyme produced by *Bacillus sphaericus*. *J. Appl. Sci. Res.* **3:** 695-699.
13. He XL, Fazheng R, Huiyuan G, Weibing Z, Xi S, Bozhong G. 2011. Purification and properties of a milk-clotting enzyme produced by *Bacillus amyloliquefaciens* D4. *Korean J. Chem. Eng.* **28:** 203-208.
14. He XL, Weibing Z, Fazheng R, Huiyuan G, Bozhong G. 2012. Screening fermentation parameters of the milk-clotting enzyme produced by newly isolated *Bacillus amyloliquefaciens* D4 from the Tibetan Plateau in China. *Ann. Microbiol.* **62:** 357-365.
15. Hittu M, Punj V. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. *Int. J. Food Microbiol.* **42:** 139-145.
16. Kumar S, Sharma NS, Saharan MR, Singh R. 2005. Extracellular acid protease from *Rhizopus oryzae*: purification and characterization. *Process. Biochem.* **40:** 1701-1705.
17. Li Y, Liang S, Zhi DJ, Chen P, Su F, Li HY. 2012. Purification and characterization of *Bacillus subtilis* milk-clotting enzyme

from Tibet Plateau and its potential use in yak dairy industry. *Eur. Food Res. Technol.* **234:** 733-741.
18. Magda AE. 2004. Formation and properties of serine protease enzyme with milk-clotting activity from *Bacillus sphaericus*. *Egypt. J. Appl. Sci.* **19:** 68-91.
19. Neelakantan S, Mohanty AK, Kaushik JK. 1999. Production and use of microbial enzymes for dairy processing: a review. *Curr. Sci.* **77:** 143-148.
20. Poza M, Sieiro C, Carreira L, Barros-Velazquez J, Villa TG. 2003. Production and characterization of the milk-clotting protease of *Myxococcus xanthus* strain 422. *J. Ind. Microbiol. Biotechnol.* **30:** 691-698.
21. Preetha S, Boopathy R. 1994. Influence of culture conditions on the production of milk-clotting enzyme from *Rhizomucor*. *World J. Microbiol. Biotechnol.* **10:** 527-530.
22. Sampaio PN, Calado CRC, Sousa L, Bressler DC, Pais MS, Fonseca LP. 2010. Optimization of the culture medium composition using response surface methodology for new recombinant cyprosin B production in bioreactor for cheese production. *Eur. Food Res. Technol.* **231:** 339-346.
23. Sannabhadti SS, Srinivasan RA. 1977. Milk clotting enzymes from *Abisidia ramosa*. Part 1. Factors influencing production. *Ind. J. Dairy Sci.* **30:** 331-335.
24. Shata HMA. 2005. Extraction of milk-clotting enzyme produced by solid state fermentation of *Aspergillus oryzae*. *Polish J. Microbiol.* **54:** 241-247.
25. So S, Tokuda H, Koizumi T, Nakanishi K. 2004. Purification and characterization of an extracellular proteinase having milk-clotting activity from *Enterococcus faecalis* TUA2495L. *Food Sci. Technol. Res.* **10:** 44-50.
26. Thakur MS, Karanth NG, Nand K. 1990. Production of fungal rennet by *Mucor miehei* using solid state fermentation. *Appl. Microbiol. Biotechnol.* **32:** 409-413.
27. Trevor MD, Lancelot P. 1982. Production and immobilization of a bacterial milk-clotting enzyme. *J. Dairy Sci.* **65:** 2074-2081.
28. Yu PJ, Cheng-Chun C. 2005. Factors affecting the growth and production of milk-clotting enzymes by *Amylomyces rouxii* in rice liquid medium. *Food Technol. Biotechnol.* **43:** 283-288.