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SHORT COMMUNICATION

Statistical Optimization of Culture Conditions for Milk-Clotting Enzyme Production by *Bacillus Amyloliquefaciens* Using Wheat Bran-An Agro-Industry Waste

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Abstract In order to improve the production of the milk-clotting enzyme under submerged fermentation, two statistical methods were applied to optimize the culture conditions of *Bacillus amyloliquefaciens* D4 using wheat bran as nutrient source. First, initial pH, agitation speed, and fermentation time were shown to have significant effects on D4 enzyme production using the Plackett–Burman experimental design. Subsequently, optimal conditions were obtained using the Box–Behnken method, which were as follows: initial pH 7.57, agitation speed 241 rpm, fermentation time 53.3 h. Under these conditions, the milk-clotting enzyme production was remarkably enhanced. The milk-clotting enzyme activity reached 1996.9 SU/mL, which was 2.92-fold higher than that of the initial culture conditions, showing that the Plackett–Burman design and Box–Behnken response surface method are effective to optimize culture conditions. The research can provide a reference for full utilization of wheat bran and the production of milk-clotting enzyme by *B. amyloliquefaciens* D4 under submerged fermentation.

Keywords Optimization · Culture Conditions · Milk-clotting enzyme · Wheat bran

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Many bacteria especially several species belonging to *Bacillus* are known to produce variety of extracellular enzymes and they have a wide range of industrial applications [1]. Most commercial amylases are produced from a small subgroup of *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* [1–4].Various bacterial protease have been widely used such as *B. subtilis*, *B. licheniformis,* and *B. mojavensis* [5–7]. Xylanase from bacteria, such as *B. pumilus*, are being applied in textile processing [8]. Numerous bacteria belonging to *Bacillus* such as *B. licheniformis*, *B. subtilis* and *B. subtilis natto* have been suggested as promising microbial rennet producers [9–12].

Wheat bran, an agro-industrial residue, a cheap source of energy has high potential in the area of fermentation for the production of enzymes. Wheat bran 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 enzyme production. There are several reports describing wheat bran as potent substrate for enzyme production [12, 13]. We recently reported several bacteria producing milk-clotting enzyme isolated from the yak grazing soil in the north-eastern Tibetan Plateau. Among these bacteria, *B. amyloliquefaciens* D4 possessed high rennet-producing capacity in wheat bran juice [14, 15]. The objective of this work was to attempt to optimize the culture conditions to increase milk-clotting enzyme production from wheat bran by *B. amyloliquefaciens* D4.

The producer strain was *B. amyloliquefaciens* D4(CGMCC 3290), which was 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 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 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 fermentation medium was prepared as follows: 100 g of wheat bran in 1,000 mL of distilled water were boiled for 10 min, and filtered through gauze.

All experiments were conducted in 250-mL Erlenmeyer flasks containing fermentation medium. The initial culture conditions were as follows: initial pH 6.0, agitation speed 140 rpm, inoculum ages 4 h, fermentation time 36 h, medium volume in flask 40 mL, temperature 32 °C, inoculum size 3 %. The other different culture conditions were tested according to the experimental statistical design. After fermentation, the crude enzyme solution was obtained by centrifugation at $8000\times g$ for 10 min. All fermentations were carried out in triplicate and the results represent the average of the three trials.

The milk-clotting activity was investigated using the method of Arima 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 culture conditions having the most significant effect on milk-clotting activity were identified using a two-level Plackett–Burman design. The regression analysis of the Plackett–Burman design shows that low levels of X_5 (medium volume in flask) and X_7 (inoculum size) enhanced milk-clotting production, whereas high levels of X_1 (initial pH), X_2 (agitation speed), X_3 (inoculum ages), X_4 (fermentation time), and X_6 (temperature) resulted in high milk-clotting activity. Initial pH, agitation speed, and fermentation time were found to be statistically significant medium components with high confidence levels, but inoculum ages, medium volume in flask, temperature, and inoculum size were not.

Although the most significant variables affecting milk-clotting activity were screened by Plackett–Burman design, it was unable to predict the optimum levels of each variable. In such circumstances, we want to move rapidly to the general vicinity of the optimum levels of the variables. The method of steepest ascent was employed to find the proper direction to change the variables by increasing the initial pH, agitation speed and fermentation time to improve enzyme production. It was found that the maximum value of milk-clotting activity was reached at the third step. Then, these variables were chosen for further optimization.

Three significant independent variables [X_1(initial pH), X_2(agitation speed), and X_4(fermentation time)] were selected and further optimized using the Box–Behnken design to determine their optimal levels based on the above results. In the Box–Behnken design, X_5 (medium volume in flask) and X_7 (inoculum size) were set at its low levels of 40 mL and 3 %, but X_3 (inoculum ages) and X_6 (temperature) were set at their high levels of 7 h and 37 °C, respectively. Table 1 shows the Box-Behnken experimental design and the obtained milk-clotting activity of the individual variables. Via multiple regression analysis on the experimental data using Minitab 14.11 software, the following second-order polynomial equation was obtained:

$$\begin{aligned} Y = {} & 1966.6 - 18.09X_1 - 33.58X_2 + 162.56X_4 \\ & - 187.88X_1^2 - 343.3X_2^2 - 253.63X_4^2 + 11.93X_1X_2 \\ & - 9.85X_1X_4 + 179.83X_2X_4 \end{aligned} \quad (1)$$

where Y is the predicted response and X_1, X_2, and X_4 are the coded values of initial pH, agitation speed, and fermentation time, respectively.

The regression coefficients and the analysis of the variance presented in Table 2 indicate the high significance of the

Table 1 Design and results of Box–Benhnken design

Trial	Variables/levels			Milk-clotting activity(SU/mL)	
	X_1: initial pH (coded value)	X_2: agitation speed (coded value)	X_4: fermentation time (coded value)	Observed	Predicted
1	−1	0	−1	1329.7 ± 5.5	1370.78
2	0	−1	−1	1471.5 ± 6.8	1420.51
3	−1	−1	0	1489.1 ± 7.5	1499.01
4	−1	0	1	1748.3 ± 4.5	1715.60
5	1	−1	0	1420.7 ± 8.7	1438.99
6	0	−1	1	1363.2 ± 9.5	1385.99
7	1	1	0	1405.6 ± 6.5	1395.69
8	1	0	1	1700.8 ± 3.6	1659.73
9	1	0	−1	1321.6 ± 4.1	1354.30
10	0	1	−1	1016.5 ± 7.5	993.71
11	−1	1	0	1426.3 ± 6.2	1408.01
12	0	1	1	1627.5 ± 8.5	1678.49
13	0	0	0	1928.5 ± 5.3	1966.60
14	0	0	0	1993.8 ± 7.4	1966.60
15	0	0	0	1977.5 ± 8.1	1966.60

Table 2 Analysis of variance (ANOVA) for the quadratic model

Source	d.f.	Sum of squares	Mean square	F value	Prob > F
Model	9	1.059E+006	1.177E+005	39.42	0.0004*
X_1	1	2617.26	2617.26	0.88	0.3921
X_2	1	9018.25	9018.25	3.02	0.1427
X_4	1	2.114E+005	2.114E+005	70.82	0.0004*
X_1 X_2	1	568.82	568.82	0.19	0.6807
X_1 X_4	1	388.09	388.09	0.13	0.7332
X_2 X_4	1	1.293E+005	1.293E+005	43.33	0.0012*
X_1^2	1	1.303E+005	1.303E+005	43.66	0.0012*
X_2^2	1	4.352E+005	4.352E+005	145.77	<0.0001*
X_4^2	1	2.375E+005	2.375E+005	79.56	0.0003*
Residual	5	14926.52	2985.30		
Lack of fit	3	12616.26	4205.42	3.64	0.2229
Pure error	2	2310.26	1155.13		
Total	14	1.074E+006			

R-Sq = 98.6 %

* Significant at 1 % level

model. Our results revealed that linear and quadratic terms of fermentation time had a significant effect on the milk-clotting activity production ($p < 0.01$). Simultaneously, the square of initial pH, agitation speed, fermentation time and interactive terms of agitation speed and fermentation time were also significant. From equations derived by differentiation of Eq. 1, the optimal values of X_1, X_2, and X_4 in the coded units were found to be −0.0557, 0.0378 and 0.3349, respectively. Correspondingly, we obtained the maximum point of the model, which was initial pH 7.57, agitation speed 241 rpm, fermentation time 53.3 h, respectively. The maximum predicted value of milk-clotting activity was 1994.1 SU/mL.

In order to confirm the predicted results of the model, we repeated the experiments under optimal conditions. The milk-clotting enzyme activity of 1996.9 ± 3.1 SU/mL in the statistically optimized conditions was achieved, which was 2.92-fold higher than that of the initial conditions and was only 0.14 % higher than the predicted value of 1994.1 SU/mL according to Eq. 1. The good correlation between these two results validates the model and the existence of an optimal value.

Herein, we investigated the characterizations and applications of the milk-clotting enzyme and found that the enzyme was a metalloprotease with a molecular weight 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 [15]. Milk-clotting enzyme production by *B. amyloliquefaciens* has been successfully applied in the preparation of cheese (Fig. 1). Considering these properties, *B. amyloliquefaciens* D4 is a promising producer of microbial rennet.

Fig. 1 Cheese made with milk-clotting enzyme produced by *Bacillus amyloliquefaciens* D4

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