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Optimization of alkaline protease production from *Bacillus licheniformis* D5 in submerged fermentation

Dandan Yang[1,2,4], Jiewei Tian[2,3], Chuanlun Yang[2], Xinqing Zhang[2], Baojun Gao[2], Jielei Liu[2], Qingmin Feng[2], Nannan Guo[2], Dongmei Pan[2], Xinli Liu[1,4] & *Deqiang Zhu[1,4]

[1]State Key Laboratory of Biobased Material and Green Papermaking, Qilu University of Technology, Shandong Academy of Sciences, Jinan - 250 353, China

[2]Chambroad Chemical Industry Research Institute Co, Ltd, Binzhou, China

[3]Key Laboratory of Leather Chemistry and Engineering, Ministry of Education and College of Light Industry, Textile and Food Engineering, Sichuan University, Chengdu, China

[4]Shandong Provincial Key Laboratory of Microbial Engineering, School of Bioengineering, Qilu University of Technology, Shandong Academy of Sciences, Jinan - 250 353, China

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Enzymes are important raw material for the detergent industry. In order to develop enzymes for detergents, *Bacillus licheniformis* D5, a strain with high alkaline protease production, was screened in this study. The alkaline protease production was optimized using response surface methodology (RSM). The optimized fermentation medium (*w*/*v*) consisted of soybean cake flour (4.0%), peanut cake flour (2.69%), beef paste (0.95%), maltodextrin (4.0%), glucose (2.23%), KCl (0.1%), KH_2PO_4 (0.2%), $K_2HPO_4 \cdot 12H_2O$ (0.40%), $CaCl_2$ (0.04%), $MgSO_4 \cdot 7H_2O$ (0.04%), Tween-80 (0.05%) and serine (0.05%). The fermentation level increased by 2.12-fold compared with the control. The culture temperature was 33°C, and the initial pH was 10. The fed-batch fermentation was performed in a 30 L fermenter. The fermentation level was increased by 47.4% (from 43,587 U/mL to 60,830 U/mL), and the fermentation time was shortened from 78 h to 54 h. The results demonstrated significantly improved fermentation level, reduced fermentation cost, simplified large-scale production of alkaline protease, and thereby promote application of alkaline protease in detergent industry.

Keywords: Detergent industry, Response surface methodology (RSM)

Protease is a complex cluster of protein hydrolases[1] which accounts for 60% of the enzymes[2]. It is widely used in various industries, including baking, brewing, hair removal, cheese making, detergents, production of digestive aids, meat tenderization and silver recovery from photographic film[3,4]. Alkaline protease, which is active in the neutral to alkaline pH range, either has a serine center (Asp-His-Ser)-serine protease or a metal ion requirement for catalysis-metallo protease[5]. It shows a broad range of applications in the pharmaceutical, leather, laundry, food and waste-processing industries[6]. Alkaline protease can hydrolyze almost all kinds of protein stains, such as blood, sweat stains, milk stains, etc.; can release protein-coated stains or stains; and show good synergistic detergency with surfactants[7]. It accounts for 50% of global enzyme production, with an estimated value of $6.3 billion by the end of 2020[8], and more than two thirds of alkaline proteases are used in the laundry industry[9].

The most commonly used protease source in the industry is the microorganisms[10]. Mining protease from microorganisms is significant owing to its high stability, high yield, and other industrial properties[11]. Various microorganisms could produce alkaline protease, including bacteria, yeasts and molds[12]. Bacterial proteases are the most important enzymes because of their stability and wide application[13]. Alkaline proteases used in the detergents are produced by Bacillus-like microorganisms, and their variants which are alkali- and surfactant-resistant and selective for a wide range of peptide hydrolysis[9]. *Bacillus licheniformis*[14], *Bacillus subtilis*[15,16] and *Bacillus pumilus* are commonly used for the production of alkaline protease[17-20]. In addition, several other types of *Bacillus* have been reported, including *Bacillus amyloliquefaciens* [21], *Bacillus safensis* [22] and *Bacillus cereus* [23,24].

*Correspondence:
E-Mail: zdq0819@yeah.net

Culture condition and culture nutrition are closely related to cell growth and metabolic activity of bacteria, and thus affect the yield of products by microbiological fermentation[25]. In common optimization methods, the classical "one-variable-at-a-time" is commonly used to optimize the output parameters[26]. However, this method shows disadvantages, such as time consumption and increased experimental data set requirement[27], and the optimum parameters cannot be detected[28]. The response surface methodology (RSM) is an ideal method for optimization of the production bacterial enzymes[29,30]. RSM uses complex statistical and mathematical techniques with multiple optimization methods, including Box–Behnken designs and central composite design (CCD)[31]. In this study, we have made an attempt for optimization of large-scale production of alkaline protease using *Bacillus licheniformis* D5 strain adopting response surface methodology.

Material and Methods

Chemicals and reagents

All reagents were of analytical/microbiological grade and were obtained from commercial vendors.

Culture media

The solid screening medium (*w*/*v*) contained peptone (1.0%), yeast extract (0.5%), NaCl (1.0%), skimmed milk powder (1.0%), and agar (1.8%) at pH 10.0. The seed medium for shake-flask (*w*/*v*) contained peptone (1.0%), yeast extract (0.5%), and NaCl (1.0%) at pH 7.5. The optimized seed medium for fermenter (*w*/*v*) contained soybean cake flour (4.0%), beef paste (3.0%), glucose (3.0%), maltodextrin (1.5%), KH_2PO_4 (0.1%), $K_2HPO_4 \cdot 12H_2O$ (1.0%), $MgSO_4 \cdot 7H_2O$ (0.04%) and NaCl (0.2%) at pH 9.0. The basal fermentation medium (*w*/*v*) contained soybean cake flour (6.0%), sucrose (5.0%), glucose (2.0%), $CaCl_2$ (0.07%), $MgSO_4 \cdot 7H_2O$ (0.04%), KCl (0.3%) and Tween-80 (0.05%) at pH 10.0. The supplemental medium (*w*/*v*) contained glucose (60%) and beef paste (3%).

Isolation and characterization of alkaline protease strains.

The soil samples were collected from saline soil in Binzhou city, Shandong province. A total of 2 g of sample was obtained, and 100 mL of sterile physiological saline was added. The gradient dilution method was used to dilute the sample. The samples with 10^{-4} and 10^{-5} concentration dilutions were spread on the screening plate and cultured at 37°C for 24 h. Then, the strains with different colony morphology were chosen and inoculated on the screening plates. The strains with high PI (ratio of the diameter of the transparent zone to the diameter of the colony) were selected and further determined using the shake-flask culture. Baffled flasks (500 mL) were selected as vessels for culture, and each flask contained 100 mL of liquid medium. The culture was incubated at 33°C and 180 rpm for 54 h, and the strain with the highest enzyme activity was selected for further study. The strain was identified by molecular biology and morphological identification. The 16S rRNA gene sequence was amplified via 27F/1492R. Then, the obtained sequence of 16S rRNA gene was submitted to NCBI, and phylogenetic analysis was carried out by MEGA 7.0.26 software.

Shake-flask culture for alkaline protease production

The activated seed were scraped and inoculated into liquid medium at 33°C and 180 rpm for 15 h. Afterward, the mature seed culture (OD_{600} nm=2.8-3.0) with inoculation volume 9.0% (*v*/*v*) was incubated into the fermentation medium at 180 rpm.

Enzyme activity assay

The fermentation broth was centrifuged at 10,000×g for 5 min, and the supernatant was obtained for enzyme activity determination. In the present study, casein was used as the substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1.0 µg tyrosine per minute under the experimental conditions[32].

Influence of carbon/nitrogen sources, inorganic salts and other trace elements on alkaline protease fermentation level

Single-factor screening of carbon sources was performed, and the basal fermentation medium was used as the control. A constant level of carbon in the culture medium was maintained by replacing 5.0% sucrose in the base fermentation medium with maltose (5.0%), glucose (5.0%), lactose (5.0%), maltodextrin (5.0%), soluble starch (7.1%), corn starch (7.1%), corn flour (7.1%), bran (7.1%), and glycerin (5.0%, *w*/*v*). Similarly, single-factor screening of nitrogen sources was performed. A constant level of nitrogen was maintained in the culture medium by replacing 3.0% soybean cake powder with soybean meal (3.0%), dry corn pulp powder (3.0%), cottonseed cake powder (3.0%), peanut cake powder (2.2%), peptone (2.2%), soybean peptone (2.2%), yeast extract (2.2%), beef paste (2.2%), and corn gluten meal (2.2%, *w*/*v*). Finally, single-factor screening for inorganic salts and

other trace elements were conducted with the base fermentation medium as a control. The amounts of $MgSO_4 \cdot 7H_2O$, KCl, $CaCl_2$ and Tween-80 in the base fermentation medium were screened, and different contents of $K_2HPO_4 \cdot 12\ H_2O$, KH_2PO_4, $MnCl_2$ and serine were added and selected.

The different culture media were cultured with inoculation volume of 9.0% (*v*/*v*) at 33°C and 180 rpm for 54 h. The optimal carbon sources, nitrogen sources, inorganic salt, and other trace elements were selected according to the statistical optimization of alkaline protease production.

Optimization of alkaline protease production using RSM

The Plackett-Burman experiment was designed according to the results of single-factor experiments with carbon sources, nitrogen sources, inorganic salt, and other trace elements. Seven factors (soybean cake powder, peanut cake powder, beef paste, maltodextrin, glucose, $K_2HPO_4 \cdot 12H_2O$, and KH_2PO_4) were selected and designed at high and low levels (Table 1) for 12 experimental groups. The results of the assay were used to determine the three factors with the greatest effect on alkaline protease production. Then, the steepest climb experiment was designed to further select the level range of each factor. Based on the results of the steepest climbing experiment, the CCD was designed. The factors and levels of CCD are presented in Table 2. Afterward, Design Expert 8.0.6 was used to perform analysis of variance (ANOVA) on the experiment and determine the optimal value. Finally, the optimal medium was verified. During the experiment, the culture conditions were kept constant. The different culture media were cultured with inoculation volume 9.0% (*v*/*v*) at 33°C and 180 rpm for 54 h.

Table 1 — Experimental factors and levels of Plackett–Burman experiment

FactorS	Level/ (%)	
	1	1
A (Soybean cake powder)	3.0	4.0
B (Peanut cake powder)	2.0	4.0
C (Beef paste)	0.5	4.0
D (Maltodextrin)	3.0	4.0
E (Glucose)	2.0	4.0
F ($K_2HPO_4.12\ H_20$)	0.2	0.4
G (KH_2PO_4)	0.1	0.2

Table 2 — Factors and levels of central composite design

Factors	Level (%)				
	−1.682	−1	0	1	1.682
A (Peanutcake powder)	2.40	2.50	2.65	2.80	2.90
B (Beef paste)	0.87	0.90	0.95	1.00	1.03
C (Glucose)	1.87	2.00	2.20	2.40	2.54

Influence of culture conditions on alkaline protease fermentation level.

The culture conditions were determined based on the optimized medium by RSM, including initial pH, temperature and supplementary medium. The initial medium pH was set to 7.0, 8.0, 9.0, 10.0 and 11.0. The culture temperature was set to 30, 33, 35, 37 and 40°C. The effect of the supplementary medium was studied. A mixed solution containing glucose (60%, *w*/*v*) and beef paste (3%, *w*/*v*) was added as the supplementary medium, which was replenished at 24, 48, 72 and 96 h. Each experimental set was supplemented with 5 mL of the supplementary medium at a time. The experiment was kept as a single variable, and the remaining conditions were at the optimal level.

Fed-batch fermentation

According to the results of the medium and culture condition optimization, the fed-batch fermentation was performed in a 30 L fermenter with a loading volume 20 L, temperature of 33°C, and tank pressure of 0.05 MPa. Approximately, 10% of the inoculum was applied, and the seeds were cultured in an optimized medium. The dissolved oxygen level was kept at 20-30%. The initial pH was set to 6.8-7.5, and the pH was maintained at 6.2-7.4 during the fermentation process. The pH was adjusted using 20% sodium hydroxide and 10% sulfuric acid. Throughout the fermentation process, the supplementary medium was added continuously, while the concentration of reducing sugar was kept between 2.5% (*w*/*v*) and 3.0% (*w*/*v*).

Results

Isolation, characterization, and phylogenetic analysis of alkaline protease strains.

A total of 45 strains were selected and inoculated in solid screening plates for primary screening. Eight strains with large PI were selected for shake-flask re-screening. Based on the results of the re-screening test, a strain with high enzyme activity was selected. The strain was named D5, and the colony morphology (Fig. 1A) and microscopic observation (Fig. 1B) are shown in Fig. 1. The colony morphology on the screening plate was raised, rough, and irregular with rough margins. When strain D5 was magnified 400 times under a microscope, it took on the appearance of a long rod. For molecular characterization, the 16S rRNA gene was cloned and analyzed. Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for

PCR amplification. The amplified PCR products were sequenced by Jinan Shangbo Biotechnology Co. (Jinan, China). The sequence was analyzed in the NCBI server (www.ncbi.nim.nih.gov) by using the BLAST tool and submitted to GenBank. (GenBank ON. 714595). The phylogenetic tree of the isolate was constructed using the neighbour joining method (Fig. 1C). The sample showed high sequence identity with the strain (*B. licheniformis* ATCC 14580^T 98.83%[33]) that belonged to the genera of *Bacillus licheniformis*. The sample was named as *Bacillus licheniformis* D5.

Influence of carbon sources, nitrogen sources, inorganic salts, and other trace elements on alkaline protease fermentation level.

The results of single-factor experiment for the carbon sources of alkaline protease (Fig. 2A) indicated that the fermentation level of basal fermentation medium was 16,388 U/mL, and the experimental group with maltodextrin as the carbon source showed the highest fermentation level with a 7.9% increase compared with the control group, followed by maltose and glucose with 3.5 and 2.6%, respectively. Considering the high cost of maltose, maltodextrin and glucose were selected as the carbon sources for the following fermentation medium optimization. The effect of different nitrogen sources (Fig. 2B) on fermentation level varied greatly. Among the nitrogen sources, peanut cake powder and beef paste could significantly improve the alkaline protease fermentation level, and the alkaline protease fermentation level increased by 15.8 and 16.8%, respectively, compared with the control. Therefore, peanut cake powder and beef paste were selected as the nitrogen sources for the following fermentation medium optimization. Based on the results of inorganic salt and trace element screening (Fig. 2C), the optimal concentration of $MgSO_4 \cdot 7H_2O$ was 0.04%, the optimal concentration of KCl was 0.1%, the optimal concentration of $CaCl_2$ was 0.04%, and the optimal concentration of Tween-80 was 0.05%. In addition, $MnCl_2$ inhibited the enzyme activity, while the addition of 0.05% serine could increase the enzyme activity by 23.8%, and the addition of different concentrations of $K_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 had different effects on the enzyme activity. When the $K_2HPO_4 \cdot 12H_2O$ was added, 0.4% was better than 0.2%. When the KH_2PO_4 was added alone, 0.2% was better than 0.4%. As the pH of the fermentation medium changes, $K_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 can transform into each other when added to the fermentation broth. The further optimization can be done to find a suitable ratio between two types of phosphates to achieve the best results.

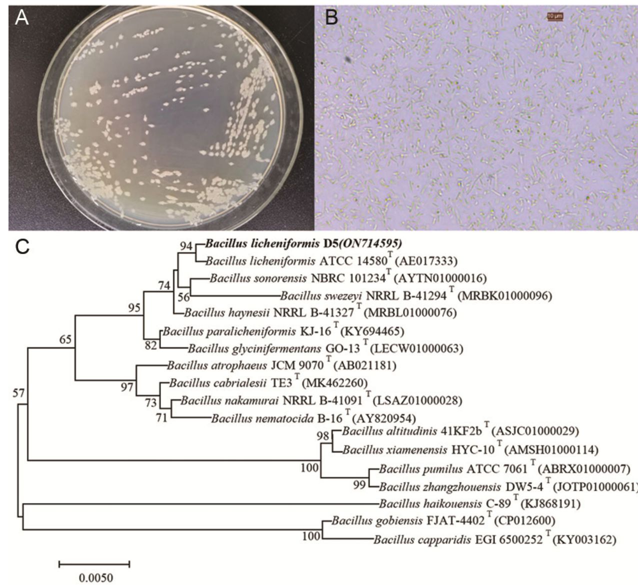

Fig. 1 — Characterization and phylogenetic study of alkaline protease strains. (A) Colony morphology on the screening plate; (B) *Bacillus licheniformi*s D5 was magnified (400X); and (C) phylogenetic tree established by the neighbor-joining method.

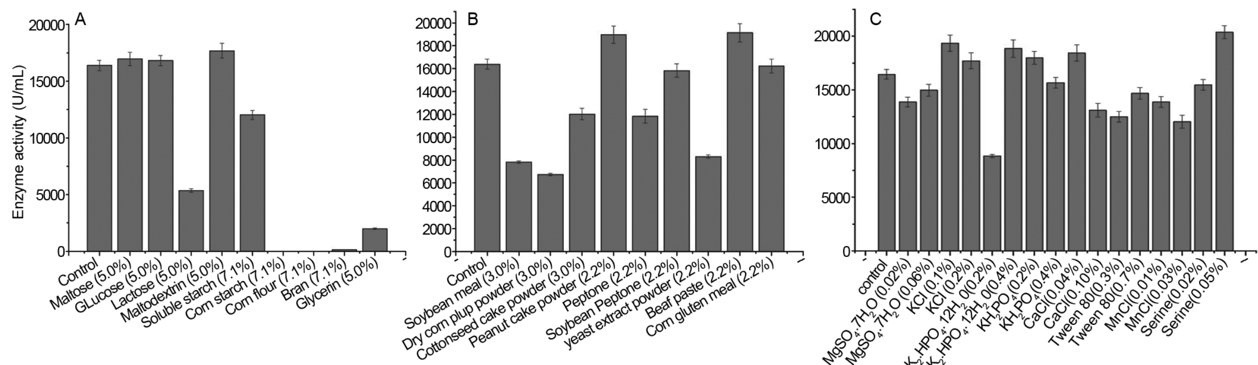

Fig. 2 — Statistical optimization of alkaline protease yield. Influence of (A) carbon sources; (B) nitrogen sources; and (C) inorganic salts, and other trace elements on fermentation level.

Determination of the most effective factors by Plackett-Burman design.

The results of the Plackett-Burman design assay indicated that the optimal fermentation level reached 33,204 U/mL (Table 3). The fermentation level increased by 2.03-fold compared with the basal fermentation medium. The experimental results of the Plackett-Burman design yielded the following regression equation:

Enzyme activity (U/mL) = 22,422.5 − 50.83A − 3,504.33B + 2,795.00C + 192.33D − 5,404.50E − 204.00F − 395.67G

where A represents soybean cake powder, B represents peanut cake powder, C represents beef paste, D represents maltodextrin, E represents glucose, F represents $K_2HPO_4 \cdot 12H_2O$, and G represents KH_2PO_4. The model with a high probability *P* value (P=0.0038) evaluated using Fisher's test. The coefficient of determination (R^2) was 0.9777, and the adjusted R^2 (R^2_{Adj}) was 0.9387, indicating that the model was reasonable. The consequences of Plackett-Burman experiment (Table 4) indicated that the factors influencing the fermentation level are as follows: E>B>C>G>F>D>A. The significant factors (P <0.05) were glucose, peanut cake powder, and beef paste, and other factors showed no significant effect. According to the experimental results, the protease yield demonstrated a rising trend with decreasing concentration of glucose and peanut cake flour and increasing concentration of beef paste.

Optimization of the selected factors by steepest climb experiment.

Based on the Plackett-Burman experimental results, three factors, namely, glucose, peanut cake powder, and beef paste, were selected to design the steepest climbing experiment. As shown in Table 5, the fermentation level initially increased and then decreased. The best group was the experimental Group V with a fermentation level of 34,673 U/mL with 2.12-fold changed compared with the basal fermentation medium.

Optimization of the selected factors by central composite design.

The fermentation medium was optimized using a central composite design with fermentation level (Y) as the reaction value and peanut cake powder (A), beef paste (B), and glucose (C) as independent variables. The design and results of RSM are shown in Table 6. The optimal fermentation level could

Table 3 — Results of Plackett–Burman experiment

A	B	C	D	E	F	G	Enzyme activity/(U/mL)
1	1	−1	−1	−1	1	−1	19872
1	1	1	−1	−1	−1	1	28646
1	−1	1	1	−1	1	1	33204
−1	1	−1	1	1	−1	1	10665
1	1	−1	1	1	1	−1	12656
1	−1	1	1	1	−1	−1	23480
−1	−1	−1	−1	−1	−1	−1	29556
−1	−1	−1	1	−1	1	1	28644
−1	1	1	1	−1	−1	−1	27040
−1	1	1	−1	1	1	1	14630
−1	−1	1	−1	1	1	−1	24305
1	−1	−1	−1	1	−1	1	16372

Table 4 — Results of Plackett–Burman experiment

Factors	t	Prob>F	Order of importance
A (Soybean cake powder)	−50.83	0.9284	7
B (Peanut cake powder)	−3504.33	0.0027	2
C (Beef paste)	2795.00	0.0062	3
D (Maltodextrin)	192.33	0.7356	6
E (Glucose)	−5404.50	0.0005	1
F ($K_2HPO_4.12H_2O$)	−204.00	0.7205	5
G (KH_2PO_4)	−395.67	0.4978	4

Table 5 — Design and results of steepest ascent experiment

Group	Peanut cake powder (%)	Beef paste (%)	Glucose (%)	Enzyme activity (U/mL)
I	4	0.5	4	18732
II	3.7	0.6	3.6	20388
III	3.4	0.7	3.2	19014
IV	3.1	0.8	2.8	29972
V	2.8	0.9	2.4	34673
VI	2.5	1	2	34022
VII	2.1	1.1	1.6	28304

Table 6 — Design and results of response surface method

Experimental run	A	B	C	Enzyme activity (U/mL)
1	1	−1	1	31965
2	0	0	0	36150
3	1	1	−1	30785
4	0	−1.682	0	32190
5	−1.682	0	0	29525
6	0	0	−1.682	30118
7	1	−1	−1	29785
8	0	0	1.682	31598
9	−1	1	1	29045
10	−1	1	−1	27565
11	0	0	0	36150
12	0	0	0	36150
13	1.682	0	0	32745
14	0	0	0	36150
15	1	1	1	31265
16	0	1.682	0	32190
17	0	0	0	36150
18	0	0	0	36150
19	−1	−1	−1	27565
20	−1	−1	1	29045

reach 36,150 U/mL. The fermentation level increased by 2.21-fold compared with the basal fermentation medium. The results of ANOVA for the second-order response surface model were shown in Table 7. The model with a high probability *P*-value ($P < 0.0001$) was evaluated using Fisher's test. The results obtained by implementing the central composite design were analyzed using standard ANOVA, and the regression equation was as follows:

Fermentation level (U/mL) = +36,197.7 + 1,171.1A + 21.97B + 593.77C + 37.50AB - 37.50AC – 212.50BC – 2,085.04A^2 – 1,712.04B^2 – 2,182.97C^2

where A, B and C represent peanut cake powder, beef paste, and glucose, respectively. The coefficient of determination (R^2) was 0.9548 and the adjusted R^2 (R^2_{Adj}) was 0.9141, indicating that this model was reasonable. As shown in Table 7, the linear and squared terms of A and C had significant effect on the fermentation level ($P < 0.05$). The response surface plot of fermentation level showed the interactive effects of the peanut cake powder, beef paste, and glucose in alkaline protease production (Fig. 3). Based on Fig. 3, the enzyme activity tended to increase first and then decrease with the variation of each factor. The optimal fermentation level was located at the top of each plane, and the interaction of the factors was not significant. At the resting point, the theoretically predicted maximum fermentation level was 36,401 U/mL, and the concentrations of peanut cake powder, beef paste, and glucose were 2.69% (*w*/*v*), 0.95% (*w*/*v*), and 2.23% (*w*/*v*), respectively. The accuracy of the model was tested by verifying the best predicted medium under the same experimental conditions. The results demonstrated that the enzyme activity reached 36,502, 37,085 and 36,004 U/mL. The average value was 36,530 U/mL, which was very close to the theoretical value. Therefore, the model was reliable for the regression equation to predict and analyze fermentation level. Through a series of statistical optimization, the optimal fermentation medium (*w*/*v*) was determined to be containing soybean cake flour (4.0%), peanut cake flour (2.69%), beef paste (0.95%), maltodextrin (4.0%), glucose (2.23%), KCl (0.1%), KH_2PO_4 (0.2%), $K_2HPO_4 \cdot 12H_2O$ (0.40%), $CaCl_2$ (0.04%), $MgSO_4 \cdot 7H_2O$ (0.04%), Tween-80 (0.05%) and serine (0.05%).

Table 7 — ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F Value	Prob > F
Model	1.69E+008	9	1.88E+007	23.46	<0.0001
A	1.87E+007	1	1.87E+007	23.37	0.0007
B	6590.1	1	6590.10	8.22E-003	0.9295
C	4.82E+006	1	4.82E+006	6.01	0.0342
AB	11250.0	1	11250.0	0.014	0.908
AC	11250.0	1	11250.0	0.014	0.908
BC	3.61E+005	1	3.61E+005	0.45	0.5172
A^2	6.27E+007	1	6.27E+007	78.16	<0.0001
B^2	4.22E+007	1	4.22E+007	52.69	<0.0001
C^2	6.87E+007	1	6.87E+007	85.67	<0.0001
Residual	8.02E+006	10	8.02E+005		
Lack of Fit	8.02E+006	5	1.60E+006		
Pure Error	0.0	5	0.0		
Cor Total	1.77E+008	19			
R^2	0.9548				
R^2_{Adj}	0.9141				

[A-Peanut cake powder; B-Beef paste; and C-Glucose]

Influence of culture conditions on alkaline protease fermentation level.

The effect of temperature on alkaline protease yield is shown in Fig. 4A. The alkaline enzyme activity showed a growth trend from 30 to 33°C and a decreasing trend from 33 to 40°C. Therefore, the optimal temperature is 33°C. Similarly, with the increase in initial pH from 7.0 to 11.0, the enzyme

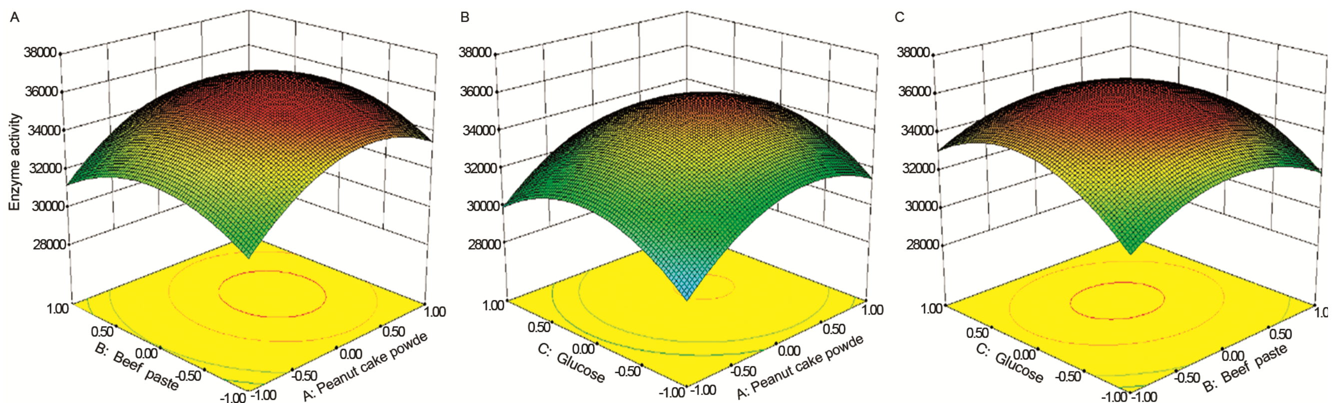

Fig. 3 — Optimization of the selected factors by central composite design. Response surface diagrams of enzyme activity indicating the interactives of (A) peanut cake powder and beef paste; (B) peanut cake powder and glucose; and (C) beef paste and glucose.

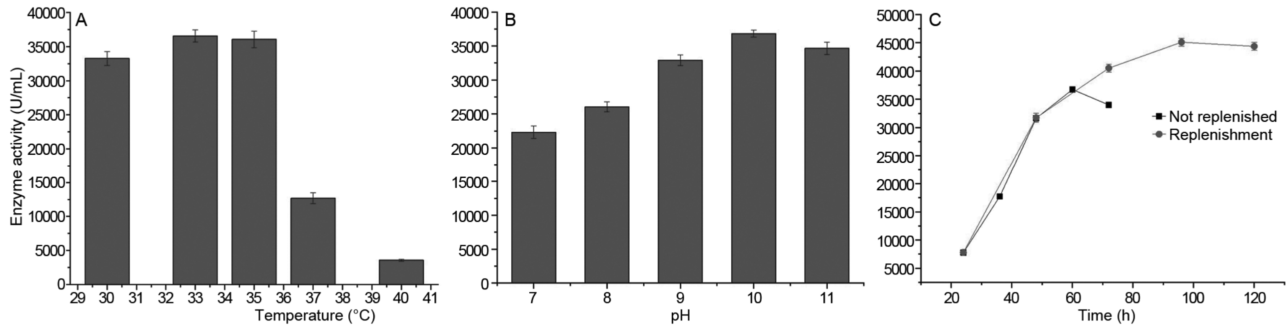

Fig. 4 — Influence of culture conditions on alkaline protease yield. (A) initial pH; (B) temperature; and (C) adding supplement on fermentation level.

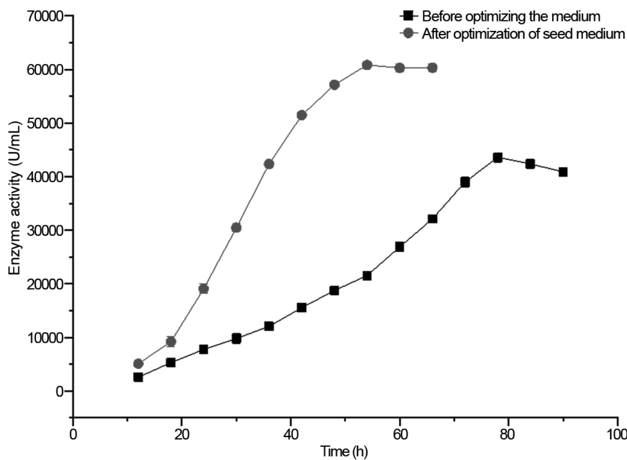

Fig. 5 — Fed-batch fermentation in 30 L fermenters.

activity increased first and then decreased with an optimal initial pH of 10.0 (Fig. 4B). When the pH was 10.0, the actual pH of the tested sterilized medium would reach 7.4. Fig. 4C shows the comparison of alkaline protease before and after replenishment. The results indicate that the fermentation time was extended from 56 h to 108 h, and the enzyme activity increased by 1.23 times from 36,740 U/mL to 45,087 U/mL after adding the supplement. This phenomenon occurred, because in the fermentation of alkaline protease, the carbon source was consumed more, and proper supplementation of carbon source was beneficial to prolong the fermentation time and improve the alkaline protease yield. Moreover, the reducing sugar concentration was maintained at 2-4% (*w*/*v*) during the shake-flask supplementation experiment.

Fed-batch fermentation.

The results in Fig. 5 show that the enzyme activity in the 30 L fermenter increased by 47.4% from 43,587 U/mL to 60,830 U/mL, and the fermentation time was shortened from 78 h to 54 h after optimizing the seed medium. Also, 4 L of the supplemental medium was consumed, and an OD_{600} maximum of 170 was reached during the fermentation process. The analysis of the reasons showed that the application of optimized seed medium had a crucial role in enzyme production, which significantly improved the adaptability, growth activity, and enzyme production capacity of the strain.

Discussion

Culture nutrition and culture condition are closely related to cell growth and metabolic activity of bacteria, and thus affect the yield of products by microbiological fermentation[34]. In order to achieve the optimal fermentation level of enzyme, it is necessary to optimize carbon source, nitrogen source, inorganic salt, and other trace elements, and the optimal cultivation conditions are also optimized. Carbon source is one of the key factors in fermentation medium. When the fermentation medium lacks a carbon source, it will limit the growth of bacteria. when it is too high, it will change the carbon metabolism pathway, increase cell osmotic pressure, and limit cell growth. In this study, *Bacillus licheniformis* D-5 shows higher enzymes production in the presence of maltodextrin, maltose, and glucose, making better use of disaccharides and monosaccharides, which has been confirmed in study Sharma *et al*.[35]. In our study, maltodextrin, containing monosaccharides, disaccharides and other oligosaccharides, is more suitable for bacterial growth, which may be related to the complex interactions between various sugars. However, using starch as carbon sources shows a poor effect on enzyme production. Therefore, a suitable combinations of various carbon sources could effectively improve enzyme production. Moreover, the application of nitrogen sources plays a crucial role

in fermentation yield. In industrial production, in addition to higher fermentation levels, the cost of raw materials is also an important factor to consider. In reported studies [21,22,36], more expensive nitrogen sources such as casein peptone are used. Although the yield of enzyme activity is considerable, the cost is relatively high and cannot be industrialized for application. In our study, peanut cake powder, which is rich in protein, amino acids, trace elements, and other nutrients, can increase enzyme production and reduce the fermentation costs of raw materials. In addition, the effects of inorganic salts and other trace elements on bacterial growth and enzyme production cannot be ignored. In our study, single factor adjustment of the concentrations of $MgSO_4 \cdot 7H_2O$, KCl, $CaCl_2$ and Tween-80 could increase enzyme production, and Cui *et al.*[37] reported similar findings. P, S, Ca, K and Mg are general biological chemicals, which are essential for bacterial growth and metabolism. Mg^{2+} is an activator of many enzymes, which can promote the catalytic action of enzymes, increase the metabolic rate of bacterial growth and increase the production of metabolic products. Tween-80 is a surfactant that could interact with the cell membrane to promote membrane permeability and promote the secretion of extracellular enzymes[38]. In addition, because serine is a nucleophilic amino acid in the structure of the alkaline protease, and the addition of serine can promote the synthesis of the enzyme. Additionally, different concentrations of K_2HPO_4 and KH_2PO_4 in the fermentation broth could supply specific phosphorus and potassium components and maintain cell osmotic pressure and buffer solution pH, which could increase the enzyme activity in a suitable range.

Response surface methodology (RSM) is often used in research on improving alkaline enzyme activity through fermentation[24,27,39].In this study too, we used RSM for fermentation cultivation and optimization. The fermentation level increased by 2.12-fold compared with the control. The 30 L fermentation tank in the present study was used for scale-up trials and was useful for production applications, while other studies[26, 40] were only able to operate at the shake-flask level. Moreover, the seed medium was optimized, which significantly increased the enzyme activity and reduced the fermentation time, thus further improving the fermentation process and making it easier for production applications, which was not involved in other studies[14,41]. In this study, the fermentation level of alkaline protease reaches 60830 U/mL, which is at a relatively high level in the current reported literature. Jayakumar *et al.*[24] reported that fermentation level of alkaline protease is 28.6 U/mL (One unit of protease activity is equivalent to the amount of enzyme needed under the given conditions of assay to release 1 µmol of tyrosine per min). Sharma *et al.*[35] reported that fermentation level of alkaline protease is 3630 µg/mL/min (One unit of protease activity was equivalent to the amount of enzyme required for releasing 1.0 µg of tyrosine/mL/min under standard assay conditions). Certainly, this study has some shortcomings, which need to be addressed in subsequent experiments. The enzyme activity level needs to be further improved, the raw material cost treatment needs to be further optimized, and more large-scale pilot fermenter experiments are needed to explore the fermentation process.

The alkaline protease application in stain removal is well studied and is used by detergent industries as a key ingredient[24]. The alkaline protease properties were determined (detailed data not provided). The alkaline protease showed great enzymatic properties, the optimal temperature range was 23-45°C, the optimal pH range was 7.0-11.0, and it showed good stability. Alkaline protease's detergency was tested by adding it into detergents such as laundry detergent and washing powder, and good application results were obtained. As a result, the alkaline protease took good application prospect and a large market in the detergent sector.

Conclusion

In order to develop enzymes for detergents, *Bacillus licheniformis* D5, a strain with high alkaline protease production, was screened and isolated in this study. We optimised the fermentation medium and culture conditions for alkaline protease production of strain D5 by single-factor experiments and RSM and observed increase in the alkaline protease fermentation level by 2.21-fold compared to the basal fermentation medium. The optimal fermentation medium (*w*/*v*) contained soybean cake flour (4.0%), peanut cake flour (2.69%), beef paste (0.95%), maltodextrin (4.0%), glucose (2.23%), KCl (0.1%), KH_2PO_4 (0.2%), $K_2HPO_4 \cdot 12H_2O$ (0.40%), $CaCl_2$ (0.04%), $MgSO_4 \cdot 7H_2O$ (0.04%), Tween-80 (0.05%), and serine (0.05%). The culture temperature was 33°C, and the initial pH was 10. In addition, after

adding the supplement, the fermentation level increased by 1.23 times from 36,740 U/mL to 45,087 U/mL. When the fed-batch fermentation in submerged fermentation was performed in 30 L fermenters, the fermentation level increased by 47.4% (from 43,587 U/mL to 60,830 U/mL), and the fermentation time was shortened from 78 h to 54 h after optimizing the seed medium. In conclusion, this study demonstrated significant improvement of fermentation level, reduced fermentation cost, simplified large-scale production of alkaline protease, and thereby promote application of alkaline protease in the detergent industry.

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Conflict of interest

Authors declare no competing interests.

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