RESEARCH ARTICLE

Optimization of fermentation medium components for producing chitin deacetylase by *Bacillus subtilis* H9

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Chitin was the second largest resource in nature, and chitosan obtained by deacetylation from chitin has a broad application prospect. Biodegradation of natural chitin by chitin deacetylase (CDA) is green and efficient compared with the commonly used chemical methods, but low CDA production limited its application. Herein, the fermentation medium of *Bacillus subtilis* H9 (Bac H9) producing CDA was optimized by single-factor experiments and response surface methodology to increase the yield of CDA. Corn flour, yeast extract, and $MgSO_4$ were determined as carbon sources, nitrogen sources, and metal salts, respectively, by single-factor experiments, while α - D - glucose pentaacetate (αGP) was used as the enzyme inducer. Plackett-Burman design found that yeast extract, $MgSO_4$, and chitin had significant effects on CDA production. The optimized fermentative medium was composed of 13.4 g/L yeast extract, 7.5 g/L corn flour, 0.94 g/L $MgSO_4$, 3.4 g/L chitin, and 5 g/L αGP. Under these conditions, the CDA activity of Bac H9 reached 27.02 U/mL, which was 3.91 times higher than that before optimization. The results were of great significance for the industrial preparation of chitosan by enzymatic method.

Keywords: chitin deacetylase; fermentation medium optimization; response surface methodology.

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Introduction

Chitin is a polysaccharide composed of N-acetyl-glucosamine (GlcNAc) units connected by β-1, 4 glucoside bonds [1]. It is divided into three crystalline forms including α-chitin, β-chitin, and γ-chitin [2]. The natural production every year reaches about 100 billion tons, and it is the second largest natural organic compound after cellulose [3, 4]. Chitin is found mainly in insects, yeast, and fungal cell walls, as well as in crustaceans [5, 6]. Chitin is not easily utilized because it is insoluble in water, dilute acid, alkali, and organic solvents, which greatly limits its application and causes a lot of waste.

Chitosan is the product of partial deacetylation of chitin [7], a linear heteropolymer of GlcNAc and glucosamine (GlcN, D) units connected by β-1, 4 glucoside bonds [8], which has better solubility, good degradability [9, 10], and biocompatibility [11] with good hemostatic property [12], antibacterial property [8], antioxidant property, moisturizing property, and molding property [13]. The conversion of chitin to chitosan can be achieved by chemical methods with NaOH to participate in deacetylation [14]. This is a relatively uncontrolled process because the participation of heated alkali solution causes the reaction to be violent [15], resulting in the unstable molecular weight of the product, poor

uniformity [16], and alkaline wastewater produced in this process. The process of enzymatic preparation of chitosan is green and controllable, and the physical and chemical properties of the production are uniform, which can meet the requirements of biology, medicine, and other fields. Chitin deacetylase (CDA, E.C. 3.5.1.41) is part of the carbohydrate esterase 4 family, which can catalyze the deacetylation of insoluble chitin and has broad substrate specificity [17, 18]. Since it was originally discovered by Araki *et al*. from *Mucor rouxii* of *Zygomycetes* [19], researchers have found CDA in fungi [20, 21], bacteria [22, 23], insects [24], and crustaceans [25]. However, the current research on CDA still has drawbacks such as instability, low enzyme activity and production. There is no suitable CDA for industrial application [26].

This study explored and optimized the enzyme-producing conditions of *Bacillus subtilis* H9 (Bac H9) to improve the yield of CDA by single-factor experiments and Box-Behnken design, which provided the technical basis for the industrial production of CDA by an enzymatic method in the future.

Materials and methods

CDA production with Bac H9

Bacillus subtilis H9 (Bac H9) was screened from naturally fermented shrimp shells in the Yellow Sea near Yantai, Shandong, China and was maintained in the Strains Preservation and Cultivation laboratory of Yantai University (Yantai, Shandong, China). The basic medium for CDA production included 10 g/L beef extract, 10 g/L glucose, 10 g/L chitin, 5 g/L NaCl, and 3 g/L α - D - glucose pentaacetate (αGP) at pH 7.0. The media were sterilized at 121℃ for 20 minutes before use. Both chitin and αGP were obtained from McLean Biochemical Technology Co., Ltd, Shanghai, China. The seed liquid at the logarithmic growth phase was inoculated into the fermentation medium at a rate of 10% ($V_{seed\ liquid}$: V_{medium}). After cultivated at 28℃ for 48 h in an MQD-B3R oscillating Incubator (Shanghai Minquan Instrument Co., Ltd., Shanghai, China), the fermentation medium was centrifuged at 8,000 rpm for 10 min. Supernatant was considered as crude enzyme used to determine CDA activity.

Optimization of medium composition

The optimization of culture medium was performed by single-factor experiments. The types of carbon source, nitrogen source, and mineral salt in the basic medium were changed, respectively, at a time, and keeping other conditions unchanged. The effects of five carbon sources on CDA production were investigated including glucose, sucrose, soluble starch, lactose, and corn flour (Solarbio Science & Technology Co., Ltd., Beijing, China) based on the basic fermentation medium at a 10 g/L concentration. To test the effect of nitrogen sources on CDA production, the following nitrogen sources including peptone, corn steep powder, yeast extract, urea, glutamate, $(NH_2)SO_4$, and NH_4NO_3 were chosen to replace beef extract of the fermentation medium at a 10 g/L concentration. The influence of metal ions on CDA production was investigated including the following metal ions of NaCl, $ZnSO_4$, $MnSO_4$, $CaCl_2$, and $MgSO_4$ based on the basic fermentation medium at a 5 g/L concentration. Chitin and αGP were used as CDA inducer in this experiment, and the effects of their concentrations on CDA production were studied. The concentrations of chitin were set as 4, 6, 8, 10, 12, and 14 g/L, while the concentrations of αGP were 1, 3, 5, 7, and 9 g/L, respectively. According to the results from single-factor experiments, Plackett-Burman was designed with Design-Expert 13.0 software (StatEase, Inc., Minneapolis, MN, USA) and the test number N=12 was created to find the significant factors among yeast extract (5 - 13 g/L), corn flour (7.5 - 12.5 g/L), $MgSO_4$ (1 - 2.5 g/L), chitin (4 - 8 g/L), and αGP (5 - 9 g/L). The CDA activity was used as the response to investigate the effect on enzyme production. According to the results of the Plackett-Burman design, the concentrations of yeast extract, $MgSO_4$, and chitin were selected as three significant factors to proceed to the

Steepest ascent design. These factors took the gradient direction of response change as the direction. The step size of yeast extract was 2 g/L, which had a positive effect. $MgSO_4$ and chitin had negative effects, and the step size was 0.4 g/L and 1 g/L, respectively. After the Steepest ascent design, the levels of yeast extract (11 - 15 g/L), $MgSO_4$ (0.6 - 1.4 g/L) and chitin (3 - 5 g/L) were optimized by Design-expert with CDA activity as the response to determine the optimum.

Enzyme activity assay

CDA activity was determined as described by previous research [27]. Briefly, 1 mL crude enzyme solution was incubated with 1 mL of 200 mg/L 4-Nitroacetanilide (McLean Biochemical Technology Co., Ltd, Shanghai, China) in 3 mL of 0.05 mol/L phosphate buffer at 50℃ for 15 min, before inactivated the reaction at 100℃. After the reaction system cooled down to room temperature, the volume of reaction was replenished to 10 mL with deionized water. The absorbance of supernatant was then detected at 400 nm using TU 1810 spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) with the inactivated crude enzyme solution as control. A unit of enzyme activity was defined as the amount of enzyme required to produce 1 μg 4-Nitroaniline per hour under the above reaction conditions.

Data analysis and statistics

All tests were repeated three times, and the data was expressed as mean ± standard error. Statistical analysis was conducted by one-way ANOVA followed by LSD multiple-range test using SPSS (version 26.0) (IBM, Armonk, NY, USA). The RSM resulting data were analyzed by using Design-Expert 13.0 software (StatEase, Inc., Minneapolis, MN, USA) including statistical analysis and regression model.

Results and discussion

Effect of fermentation medium components on CDA production

The effect of carbon source on CDA production showed that, when corn flour was used as carbon source, the CDA activity reached the highest value of 7.31 U/mL (Figure 1A). The CDA activity was the lowest when sucrose was used as carbon source ($P < 0.01$). The CDA activity reached the highest 11.36 U/mL when the yeast extract was used as nitrogen source, while the CDA activity was lower when inorganic nitrogen source was used such as $(NH_4)_2SO_4$ or NH_4NO_3 ($P < 0.001$) (Figure 1B). Yeast extract was beneficial to the growth of Bac H9 and the production of CDA because it contained essential amino acids, trace elements, *etc.*, which could provide a variety of nutrients. When $MgSO_4$ was used as inorganic salt, the enzyme activity was the highest as 10.43 U/mL, while the CDA activity was the lowest when $CaCl_2$ was used as the metal ions ($P < 0.001$) (Figure 1C). With the increase of chitin amount, the CDA activity increased first and then decreased (Figure 1D). When the dosage of chitin was 6 g/L, the CDA activity was the highest as 10.31 U/mL. The CDA activity then decreased with the increase of chitin addition. The results might be due to too much chitin leading to the reduction of dissolved oxygen in the system to significantly inhibit the growth of bacteria ($P < 0.001$). Therefore, the optimal addition of chitin was determined to be 6 g/L. The CDA activity increased continuously with the increase of αGP addition (Figure 1E). When the added amount of αGP was 7 g/L, the CDA activity was up to 11.54 U/mL. However, when the amount of αGP was further increased, the CDA activity decreased ($P < 0.05$), which might be because the viscosity of the fermentation medium increased with the addition of αGP to cause the reduction of dissolved oxygen in the system to restrain the growth of bacteria. Therefore, the optimal addition amount was determined to be 7 g/L.

Plackett–Burman experiment

The influence of five factors on CDA production were determined by Plackett-Burman experiment (Table 1). The variance analysis on the design data was shown in Table 2. The results showed that the Prob > F = 0.0017, indicated that the model was significant and reliable, and the

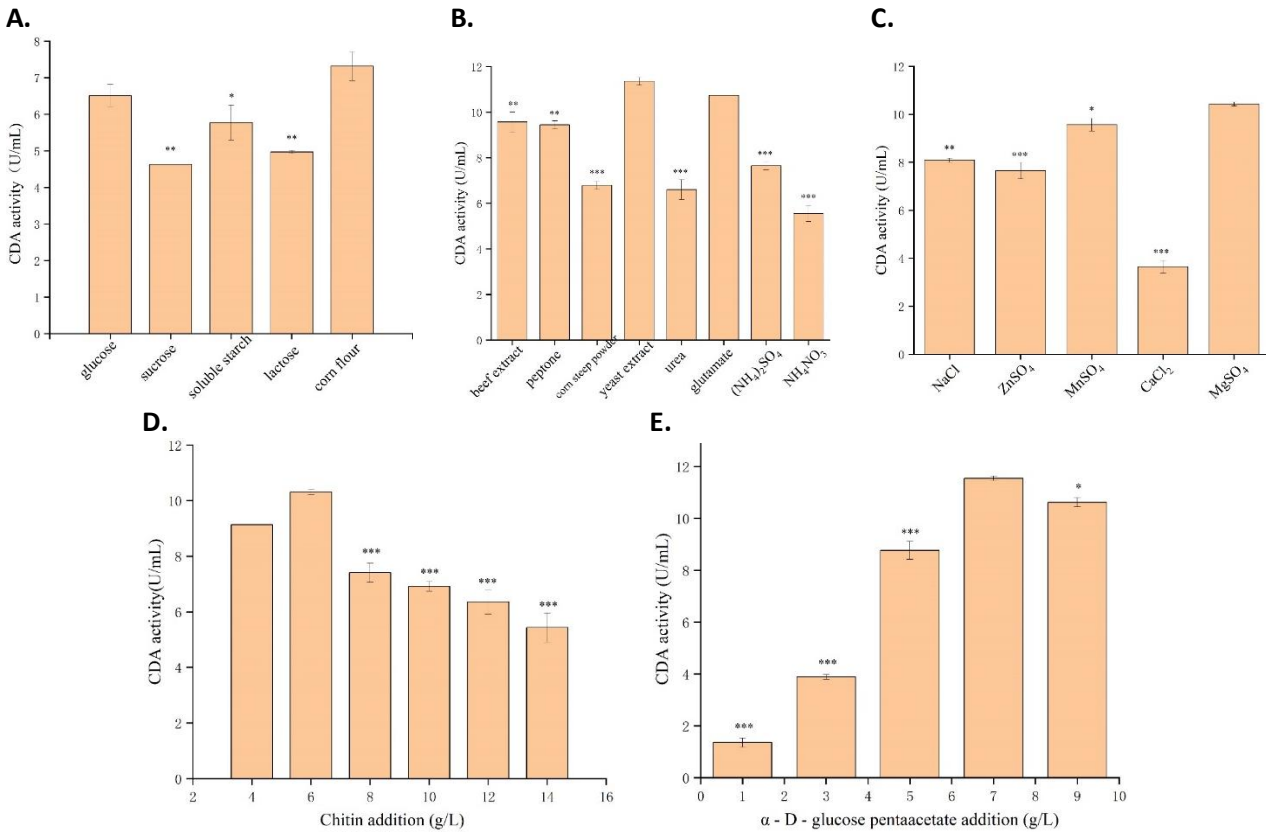

Figure 1. Optimization condition for CDA production. **A.** Carbon source. **B.** Nitrogen source. **C.** Metal ions. **D.** Chitin addition. **E.** α - D - glucose pentaacetate addition. *, **, and *** indicated significant difference $P < 0.05$, $P < 0.01$, and $P < 0.001$ within group compared to the optimal level.

Table 1. Results of Plackett-Burman design.

Run number	Factors (g/L)					CDA activity (U/mL)
	Yeast extract	Corn flour	$MgSO_4$	Chitin	αGP	
1	13	12.5	1	8	9	23.27
2	5	7.5	2.5	4	9	9.63
3	5	12.5	1	8	9	15.31
4	13	12.5	2.5	4	5	9.51
5	13	7.5	1	4	9	27.16
6	5	12.5	2.5	4	9	9.81
7	5	7.5	1	4	5	23.7
8	13	7.5	2.5	8	9	7.96
9	13	12.5	1	4	5	29.81
10	5	12.5	2.5	8	5	7.59
11	5	7.5	1	8	5	17.9
12	13	7.5	2.5	8	5	11.85

levels of yeast extract, $MgSO_4$, and chitin were significant factors ($P < 0.05$), which were selected as variables to optimize the composition of the medium with RSM.

Table 2. ANOVA analysis of Plackett–Burman design.

Source	Sum of squares	df	Mean square	F value	*P* value	Prob > F
Model	659.09	5	131.82	17.2	0.0017	significant
Yeast extract	54.70	1	54.70	7.14	0.0369	
Corn flour	0.6912	1	0.6912	0.0902	0.7740	
$MgSO_4$	544.05	1	544.05	71.01	0.00020	
Chitin	55.30	1	55.30	7.22	0.0362	
αGP	4.34	1	4.34	0.5607	0.4800	
Residual	45.97	6	7.66			
Cor Total	705.06	11				
R^2			0.9348			
R^2_{adj}			0.8805			
Pred R^2			0.7392			
Adeq Precisior			11.2555			

Table 3. Results of Steepest ascent design.

Variable	Yeast extract (g/L)	Corn flour (g/L)	$MgSO_4$ (g/L)	Chitin (g/L)	αGP (g/L)	CDA activity (U/mL)
0	5	7.5	2.6	8	5	17.78
0 + 1Δ	7	7.5	2.2	7	5	22.59
0 + 2Δ	9	7.5	1.8	6	5	24.07
0 + 3Δ	11	7.5	1.4	5	5	25.93
0 + 4Δ	13	7.5	1.0	4	5	28.64
0 + 5Δ	15	7.5	0.6	3	5	27.65
0 + 6Δ	17	7.5	0.2	2	5	25.19

Note: Δ was a unit of change. It represented yeast extract 2 g/L, $MgSO_4$ 0.4 g/L, chitin 1 g/L.

Table 4. Results of Box–Behnken design.

Run number	Factors (g/L)			CDA activity (U/mL)
	Yeast extract (A)	$MgSO_4$ (B)	Chitin (C)	
1	15	0.6	4	24.38
2	15	1.0	5	24.44
3	13	1.0	4	26.48
4	11	0.6	4	23.15
5	13	1.0	4	26.67
6	13	1.0	4	26.56
7	15	1.4	4	21.67
8	11	1.0	3	24.26
9	15	1.0	3	27.04
10	11	1.0	5	24.63
11	13	1.0	4	26.11
12	13	0.6	3	25.59
13	11	1.4	4	18.24
14	13	0.6	5	21.79
15	13	1.4	5	19.91
16	13	1.0	4	26.66
17	13	1.4	3	20.18

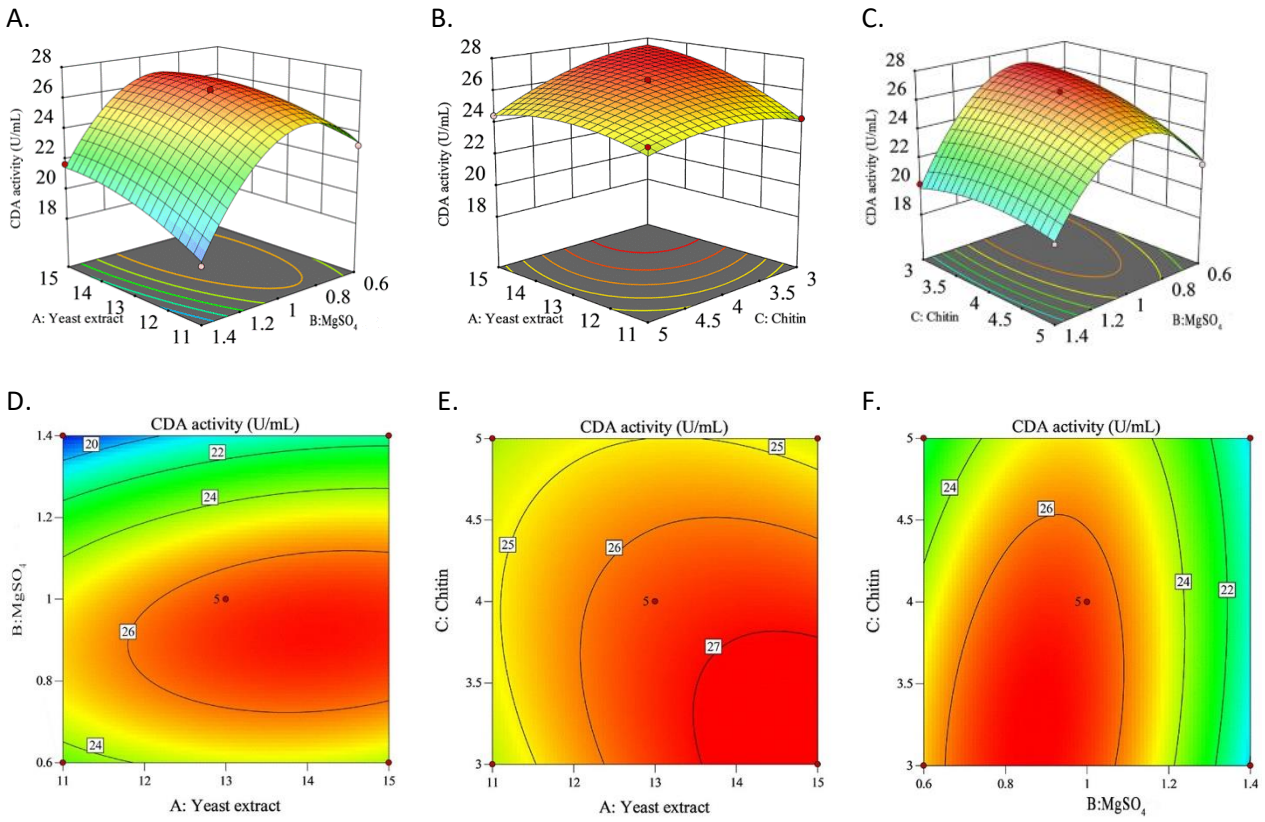

Figure 2. Response surface plots (A, B, and C) and contour plots (D, E, and F) presented the influence of yeast extract (A), $MgSO_4$ (B), chitin (C) on the effect for CDA activity.

Steepest ascent design

The results demonstrated that the enzyme activity of CDA increased from 0 to 0 + 4Δ and decreased from 0 + 4Δ to 0 + 6Δ (Table 3). Therefore, 0 + 4Δ was taken as the central point of the Box–Behnken response surface methodology experiments.

Box–Behnken response surface methodology experiments

Since the CDA activity was greatly affected by yeast extract, $MgSO_4$, and chitin, a three-factor, three-level experiment was designed using Box-Behnken with the above three factors as independent variables and the CDA activity as the response. The experimental levels and results were shown in Table 4. The model equation was obtained to describe the response surface as follows.

$$Y = 26.50 + 0.9063A - 1.86B - 0.7875C + 0.55AB - 0.7425AC + 0.8825BC - 0.7055A_2 - 3.93B_2 - 0.698C_2$$

where Y was the predicted response (CDA activity). A, B, C were the concentrations of yeast extract, $MgSO_4$, and chitin, respectively. The results of response surface methodology showed the effect of interaction among the yeast extract, $MgSO_4$, and chitin on CDA activity (Figure 2). When the concentrations of yeast extract, chitin, and $MgSO_4$ were at their optimal values, the interaction of the other two factors and the relationship between CDA activity directly reflected the relationship between each factor and the response value. The transition from blue to red in the response surface diagram indicated a shift from lower to higher values of the response with a more pronounced and curved surface indicating a stronger interaction between the experimental factors. With the increase of

Table 5. ANOVA analysis of Box–Behnken design.

Source	Sum of squares	df	Mean square	F value	*P* value	Prob > F
Model	118.28	9	13.14	77.77	<0.0001	significant
A-Yeast extract	6.57	1	6.57	38.88	0.0004	
B-$MgSO_4$	27.79	1	27.79	164.44	<0.0001	
C-Chitin	4.96	1	4.96	29.36	0.0010	
AB	1.21	1	1.21	7.16	0.0317	
AC	2.21	1	2.21	13.05	0.0086	
BC	3.12	1	3.12	18.43	0.0036	
A^2	2.10	1	2.10	12.40	0.0097	
B^2	65.05	1	65.05	384.92	<0.0001	
C^2	2.05	1	2.05	12.14	0.0102	
Lack of Fit	0.9724	3	0.3241	6.16	0.0557	Not significant
Pure Error	0.2105	4	0.0526			
C.V.%			1.71			
R^2			0.9901			
R^2_{adj}			0.9774			
Pred R^2			0.8670			
Adeq Precisior			28.5096			

the dosage of primary yeast extract and $MgSO_4$, the CDA yield first increased and then decreased, while, with the increase of chitin addition, the CDA yield showed a downward trend (Figure 2A-2C). In addition, the contour plot (Figures 2D to 2F) presented an oval shape, further indicating that the interaction between the corresponding variables was significant. The results of the variance analysis of response surface methodology were shown in Table 5. The results, Prob > F < 0.0001, indicated that the model was significant for the response. The correlation coefficient R^2 = 0.9901 and the correction coefficient R^2_{adj} = 0.9774 indicated that the model had a good fit and could explain 97.74% of the response variation. From the value of F, the results demonstrated that the order of influencing factors on the activity of CDA was $MgSO_4$ > yeast extract > chitin, and the optimal levels were 13.324 g/L for yeast extract, 0.937 g/L for MgSO4, and 3.374 g/L for chitin. The maximum CDA activity could reach 27.04 U/mL under the above conditions. To being operated easily, the levels of each factor were adjusted to 13.4 g/L for yeast extract, 0.94 g/L for $MgSO_4$, and 3.4 g/L for chitin. The CDA activity could reach 27.22 ± 0.37 U/mL under this condition, which was close to the theoretical value and met the prediction. Compared with the optimized fermentation conditions reported by Zhang *et al*. [28] and Pareek *et al*. [29] for the production of CDA, the CDA activity obtained in this study was obviously higher than the previous reports.

Conclusions

In this study, the Plackett-Burman design was used to screen out the factors that significantly influenced the CDA production of *B. subtilis* H9 in the medium. The optimal medium composition was determined by the Box-Behnken design as yeast extract of 13.4 g/L, corn flour of 7.5 g/L, $MgSO_4$ of 0.94 g/L, chitin of 3.4 g/L, and α - D - glucose pentaacetate of 5 g/L. Under the above conditions, the CDA activity reached 27.04 U/mL, which was 3.91 times higher than that 6.91 U/mL of basic fermentation medium without optimization. This study provided the basis for the subsequent research to improve the level of enzyme production.

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