Optimization of Low-Temperature Lipase Production Conditions and Study on Enzymatic Properties of *Aspergillus Niger*

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ABSTRACT: *In order to obtain the optimum fermentation medium and conditions for extracellular lipase production by Aspergillus Niger, the fermentation conditions of Aspergillus Niger were optimized by single factor and response surface design, the enzymatic properties of the crude enzyme were also studied. The results showed that the optimum fermentation medium was soluble starch 4%, $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, peptone 3%, olive oil 1.05%, initial pH 7. The optimal fermentation conditions were 30℃, the sample size was 26 mL/250 mL and the shaking speed was 213 r/min. The optimized lipase activity was 1.55 U/mL, which was 7.75 times of the pre-optimized lipase. It was found that when the pH value of lipase was 7.0, the activity of lipase reached its maximum value of 79.3±6.82%. When the pH value was between 6.0 and 8.0, the activity of lipase could be kept above 60% and the stability was good. At the same time, through the study of the temperature stability of lipase, found that the lipase was stable at 25℃- 35℃, its activity could reach more than 70%. When the enzyme activity reaches the maximum (107.6±9.57%), the temperature was 30℃.*

KEYWORDS: *Low-temperature lipase; Conditions of enzyme production; Enzymological properties; Orthogonal experiment; Response surface analysis.*

INTRODUCTION

Lipase is a kind of glyceryl hydrolase, which can hydrolyze triacylglycerols into glycerol and free fatty acids, and is widely found in mammals, plants and microorganisms [1-5]. Lipase produces a lot of energy when it breaks down fat, which can be used for the body's growth and metabolism [6]. Lipase can also be regulated

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by externally characterized effectors to form "liposomes" to control lipid and energy homeostasis [7]. Lipase not only has good performance and function under extreme conditions, high catalytic activity, mild reaction conditions, environment-friendly but also has broad industrial application prospects [8], it is the preferred enzyme for potential applications in textiles, cosmetics, and paper making, mainly in food, detergents and pH pharmaceuticals [9]. Analysis of the existing literature shows that microbial lipase is one of the most productive enzymes [1], it is also an important biocatalyst in industry, especially microbial lipase [10]. Compared with other lipases, microbial lipases have higher stability, substrate specificity [3], higher yield, production costs are lower and no seasonal fluctuation [11,12], and are more stable in organic solvents [13]. At the same time, microbial lipases have attracted much attention due to their various biochemical activities and the simplicity of isolation and production [1,14]. In view of its advantages and its current and potential applications, lipases are considered to be an important and promising industrial enzyme [5,15].

Fungi, bacteria, and yeasts are currently the main sources of microbial lipases [8], the fungi are mainly *Aspergillus*, *Mucor*, *Rhizopus*, *Geotrichum*, *Rhizopus*, *Pythium*, *Candida*, and *Penicillium* [1,16,17]. It has been found that low-temperature lipase has good effects on wastewater treatment, organic pollutant degradation, and in situ bioremediation in a low-temperature environment [1]. Cold-adapted lipase has been one of the hot topics in recent years due to its unique evolutionary mechanism, low cost, simple use, and high commercial availability [18]. It is widely used in detergents, food processing, pH pharmaceutical synthesis, and fine chemical industries [1,17,19]. Although the low-temperature fermentation conditions and low enzyme yield required by the bacteria have brought great difficulties to large-scale industrial production, the researchers have solved this problem to a certain extent by cloning the enzyme-producing gene into normal-temperature bacteria [20].

Fungal lipases are known to be one of the major sources of microbial lipases, fungal lipases have received extensive attention in the industrial field for their selectivity, stability, low production cost, wide substrate specificity, and simple genetic manipulation [7,21]. At present, filamentous fungi are considered the best producer of lipase and the preferred source of lipase production. The main reason is that filamentous fungi mostly produce extracellular lipase, which is convenient to extract in a medium [22]. *Aspergillus.* is a group of widespread filamentous fungi that evolved over 200 million years [3], it can be used as an effective source of lipase-producing microorganisms and can produce potential lipases [5]. Its lipase is one of the important industrial catalysts and has high stability and selectivity [3,23], the application in industrial production has also been further studied [24]. In this study, a high lipase-producing strain, *Aspergillus* was selected from a laboratory-preserved strain. The fermentation conditions and enzymological properties of lipase were studied in order to obtain the optimal culture conditions and enzymological characteristics of lipase activity, and to provide a theoretical basis and technical guidance for its application.

EXPERIMENTAL SECTION

Research materials

The low-temperature lipase strain is *Aspergillus Niger*, which is stored in a refrigerator at 4 °C.

Seed culture medium: 2% glucose, 0.50% $(NH_4)_2SO_4$, 0.10% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 2.50% peptone, 1% olive oil.

Fermentation medium: 0.05% sucrose, 0.10% $(NH_4)_2SO_4$, 0.10% K_2HPO_4, 0.05% MgSO4·7 H_2O, 2% peptone, 1% olive oil.

Determination of seed culture process and low-temperature lipase activity

Aspergillus Niger colony was inoculated in seed medium and incubated at 28 °C for 12 h in a constant temperature incubator at 150 r/min. The culture medium was inoculated with 2% inoculum for 24 hours under the same condition and the supernatant were retained.

4 mL P-Nitrophenol palmitate (0.09 mg/mL) was preheated at 37 °C for 5 minutes, then 0.1 mL supernatant was added to react for 10 minutes, then added 5 mL of 0.5 mol/L trichloroacetic acids, the reaction was stopped after 5 minutes, the pH value was adjusted by 0.5 mol/L NaOH to match the pH value before adding acid, then the absorbance was measured at 410 nm. The measured absorbance is taken as the standard curve of P-Nitrophenol, the concentration of P-Nitrophenol is calculated, and the lipase activity is calculated according to the lipase activity formula.

The formula of lipase activity is $X= CV_1/TV_2$.

In which: *X:* lipase activity, U/mL. *C:* p-Nitrophenol

concentration, mol/L. V_1: final volume of solution, mL. *T:* reaction time, min. V_2: the amount of enzyme solution added in the solution, mL.

Effects of different carbon sources on the characteristics of fermentation enzyme

The fermentation medium is used as the basic medium, using glucose, soluble starch, sucrose, lactose, and maltose as the carbon source respectively, the activity of low-temperature lipase produced by fermentation with different carbon sources was determined by the method of enzyme activity, to select the most suitable carbon source. Then, the optimum concentration of carbon source was used as the independent variable, and 1, 2, 3, 4, and 5% of carbon source were selected respectively. In the same way, the activity of low-temperature lipase produced by fermentation of different concentrations of carbon source was determined by the method of enzyme activity determination, and the optimum concentration of carbon source was obtained.

Effects of different nitrogen sources on the characteristics of the fermentative enzyme

The fermentation medium serves as the basic medium, using urea, peptone, ammonium sulfate, ammonium chloride, and sodium nitrate as nitrogen sources respectively, the low-temperature lipase activity produced by fermentation with different nitrogen sources was determined by the method of enzyme activity, to select the most suitable carbon source. Then the optimum nitrogen source concentration was taken as the independent variable, and 1, 2, 3, 4, 5% were weighed respectively. In order to obtain the optimum concentration of nitrogen source, the activity of low-temperature lipase produced by fermentation of nitrogen source with different concentrations was determined by the same method.

Effects of different initial pH values on the characteristics of the fermentative enzyme

The fermentation medium was used as the basic medium and the initial pH (5, 6, 7, 8, 9) was used as the variable to determine the low-temperature lipase activity produced by fermentation in different pH, so as to select the optimum pH. At the same time, the enzyme solution was placed in different pH values and kept at the same temperature for 1 h, then the remaining enzyme activity without denaturation was determined according to the same method, and the effect of pH on the stability of lipase was studied.

Effect of different temperatures on the characteristics of fermentation enzyme

Taking the fermentation medium as the basic medium and the initial temperature of the medium (20, 25, 30, 35 °C) as the variable, the low-temperature lipase activity produced after fermentation at different temperatures was determined by the method of enzyme activity determination, so as to select the optimum temperature. Under the same pH condition, the enzyme solution was incubated at different temperatures for 1h, then the activity of the remaining enzyme without denaturation was determined by enzyme activity assay. Further study on the effect of temperature on the stability of lipase.

Orthogonal test

According to the results of the single-factor experiment, 3-factor-2-level experiments of soluble starch (3, 4%), peptone (3, 4%), and culture pH (7, 8) were designed. The enzyme production conditions were optimized by Box-Behnken Design analysis, and the enzyme activity was used as the response value. The contents of olive oil (1, 2, 3%), 250 mL liquid (10, 20, 30 mL), and the rotational speed of the flask were independent variables (150, 200, 250 r/min), design 3-factor 3-level experiment.

Statistical analysis

SPSS 17.0 was used to analyze the experimental data, and the data were drawn in Origin 8.0. The 3D graph of response surface and contour curve was completed in Design-Expert 8.0.

RESULTS AND DISCUSSION

Optimization of lipase production conditions

Single-factor test

Low-temperature lipase activity was measured with 5 different carbon sources (Fig. 2A) in this study. When the carbon source was glucose, sucrose, lactose, and maltose, the lipase activity was about 0.2 U/mL. When the carbon source was soluble starch, the enzyme activity reached 1.10 U/mL. This result is the same as that of *Alkan HÜseyin et al.* [25-30], the best carbon source is starch, which is produced by *Bacillus Coagulans* by solid-state fermentation. When the carbon source concentration in the fermentation medium was used

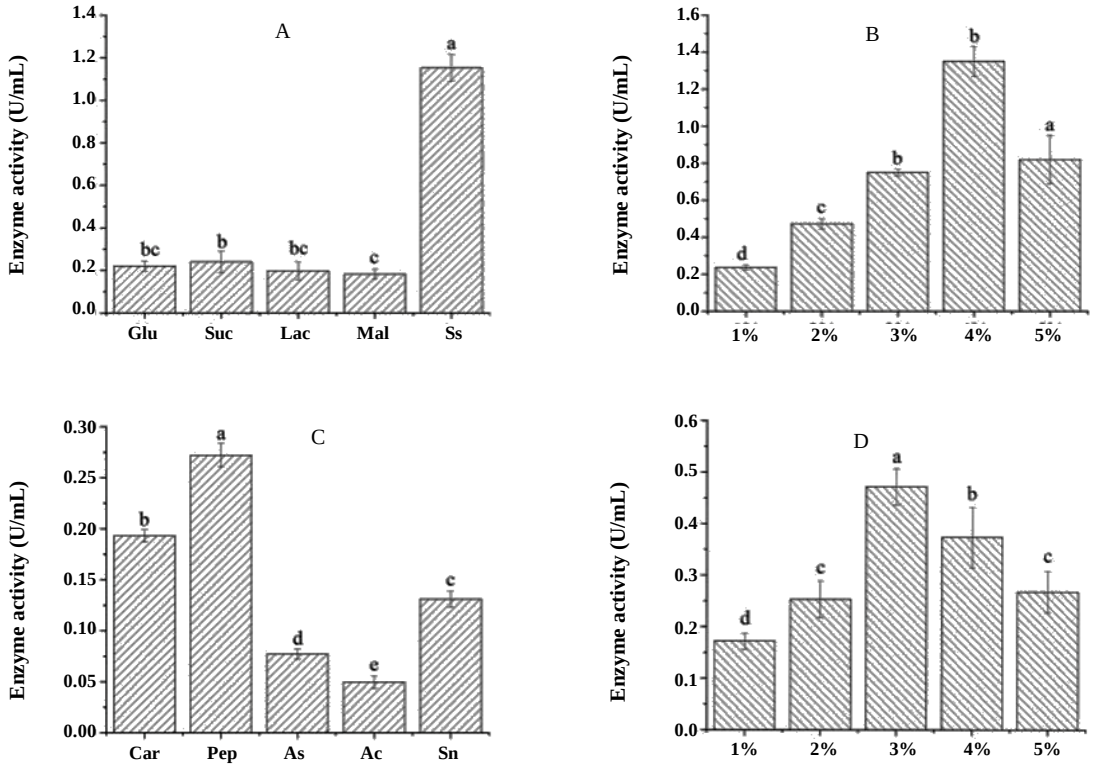


Fig. 1: Optimization of enzyme production conditions.
Note: A: determination of the best carbon source. B: determination of the optimum carbon source concentration. C: determination of the best nitrogen source. D: determination of optimum nitrogen source concentration. Glu is glucose, Suc is sucrose, Lac is lactose, Mal is maltose and Ss is soluble starch, Car is urea, Pep is peptone, as is ammonium sulfate, Ac is ammonium chloride, Sn is sodium nitrate.

as an independent variable to determine the activity of low-temperature lipase (Fig. 2B), it was found that the activity of lipase increased first and then decreased with the increase of the soluble starch concentration when the concentration was 4%, the highest enzyme activity was 1.401 U/mL. When the concentration increased to 5%, the enzyme activity decreased to 0.795 U/mL, so the optimum concentration of soluble starch was 4%.

The lipase activity at low temperature was measured by adding 5 different nitrogen sources to the fermentation medium (Fig. 2C). When the nitrogen source was ammonium sulfate and ammonium chloride, the lipase activity was low, about 0.05 U/mL. When peptone was used as a nitrogen source, the enzyme activity was the highest (0.272±0.01 U/mL). The results were slightly different from those of *Khadija Ouaissa et al.* [31]. When *Bacillus Subtilis* strain and rapeseed cake were used as a substrate for lipase production, the optimum inorganic nitrogen source was NH_4N0_3. When the concentration of nitrogen source in fermentation medium was used as an independent variable to determine the activity of low-temperature lipase, it was found that with the increase of the concentration of nitrogen source, the activity of lipase first increased and then decreased. When the concentration of peptone was 3%, the activity of lipase was highest, 0.469±0.04 U/mL. When the concentration of peptone increased to 5%, the enzyme activity decreased to 0.268±0.04 U/mL, so the optimum concentration of peptone was 3%.

Optimization of lipase production conditions by orthogonal test

With peptone as nitrogen source (A), soluble starch as carbon source (B), and initial pH value (C), three factors and two levels of the orthogonal test were designed (Table 1).

Table 1: Orthogonal Design Table L8 (2^3).

Experiment number	Peptone (A)	Soluble starch (B)	pH (C)	Enzyme activity (U/mL)
1	1 (3%)	1 (4%)	1 (7.0)	1.648
2	1 (3%)	1 (4%)	2 (8.0)	1.402
3	1 (3%)	2 (3%)	1 (7.0)	1.237
4	1 (3%)	2 (3%)	2 (8.0)	0.935
5	2 (4%)	1 (4%)	1 (7.0)	1.504
6	2 (4%)	1 (4%)	2 (8.0)	1.037
7	2 (4%)	2 (3%)	1 (7.0)	1.143
8	2 (4%)	2 (3%)	2 (8.0)	0.735
K1	5.222	5.591	5.532	
K2	4.419	4.050	4.109	
K1	1.306	1.398	1.383	
K2	1.105	1.013	1.027	
R	0.201	0.385	0.356	

The results of range analysis showed that carbon source had the most effect on enzyme yield, followed by initial pH value, and nitrogen source had the least effect on enzyme yield. The optimum scheme was $A_1B_1C_1$, when the soluble starch was 4%, the peptone was 3% and the initial pH of the fermentation medium was 7, the enzyme activity was the highest.

Optimization of lipase production conditions by response surface methodology

The enzyme activity was taken as the response value, the amount of olive oil, the amount of liquid in 250 mL shake flask, and the rotation speed of shake flask as independent variables, the experiment with three-factor and three-level was designed (Table 2).

The regression analysis of lipase activity was carried out after the data were processed by Design-Expert 8.0 software (Table 3). The functional relationship between the response value Y and each factor (A, B, C) can be obtained from the regression coefficient column in the table.

$$Y = 1.54+0.082A-0.044B+0.023C+0.098AB+0.050AC-0.065BC-0.036A^2-0.13B^2-0.077C^2$$

According to the regression equation, in order to further intuitively see the influence of various factors on lipase activity, the response surface and high-level curve 3D graph were made by using Design Expert 8.0 software (Fig. 2). The order of enzyme activity influenced by various factors is A (olive oil addition) > C (rotational speed) > B (liquid volume). Among them, the amount of olive oil has the most significant effect. By solving the regression equation, the optimal experimental scheme is as follows: the amount of olive oil is 1.05%, the liquid volume in 250 mL shake flask is 26 mL, and the rotation speed of shake flask is 213 r/min. Under this experimental scheme, the maximum enzyme activity can be obtained, which is 1.55U/mL. According to the experimental verification, the actual enzyme activity is 1.49 U/mL, and the error between theory and practice is 3.87%.

Studies on the enzymological properties of cold-adapted lipase strains

Fig. 3A shows the effect of different pH on enzyme activity. When pH is 4.0, the lipase activity is 21.20±2.88% and the enzyme activity is the lowest. When pH is between 4.0 and 7.0, the enzyme activity increases with the increase of pH, when pH is 6.0, the enzyme activity reached 79.51±6.55%, and reached the maximum. When pH value continued to increase, the enzyme activity decreased gradually, and when pH increased to 10, the enzyme activity decreased to the lowest (35.66±4.25%). Therefore, the optimum pH value of the enzyme is 6, which belongs to acid lipase.

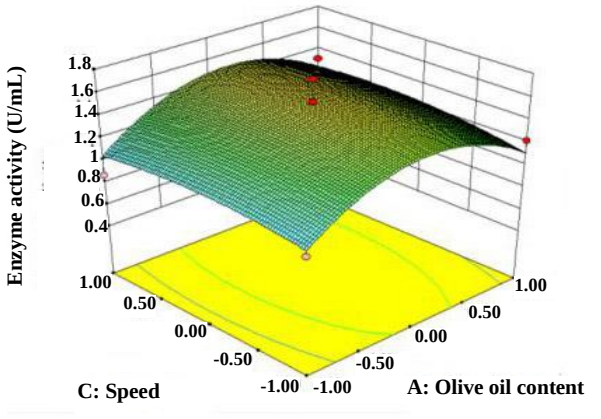
Table 2: BBD Experimental Design For Response Surface Analysis.

Experiment number	Olive oil content (A)	Liquid loading (B)	Speed (C)	Enzyme activity (U/mL)
1	0 (1.0%)	-1 (10)	-1 (150)	1.224
2	1 (1.5%)	1 (50)	0 (200)	1.061
3	-1 (0.5%)	0 (30)	1 (250)	0.875
4	0	0	0	1.733
5	-1	0	-1	0.985
6	0	1	-1	1.339
7	0	0	0	1.428
8	0	0	0	1.539
9	-1	-1	0	1.234
10	0	0	0	1.476
11	1	0	1	1.312
12	0	-1	1	1.456
13	-1	1	1	0.876
14	0	1	1	1.312
15	1	0	-1	1.224
16	0	0	0	1.508
17	1	-1	0	1.025

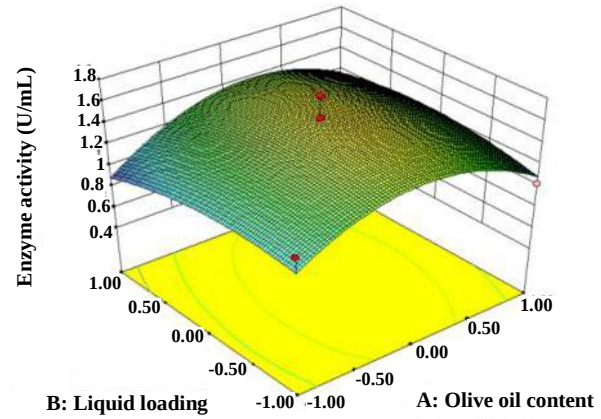
Table 3: Analysis of variance of response surface quadratic model.

Source of error	Master model					
	Sum of Squares	Degree of freedom	Mean Square	F Value	P-value Prob>F	Significant
A	0.034	1	0.034	1.53	0.2554	
B	0.00679	1	0.00679	0.31	0.5952	
C	0.00950	1	0.00950	0.43	0.5315	
AB	0.017	1	0.017	0.78	0.407	
AC	0.00322	1	0.00322	0.15	0.7132	
BC	0.00778	1	0.00778	0.35	0.5701	
A^2	0.48	1	0.48	21.83	0.0023	**
B^2	0.052	1	0.052	2.37	0.1674	
C^2	0.017	1	0.017	0.75	0.4139	
Model	0.8	9	0.089	4.06	0.039	*
Residual	0.15	7	0.022			
Lack of Fit	0.099	3	0.033	2.4	0.2087	
Pure Error	0.055	4	0.014			
Cor Total	0.96	16				

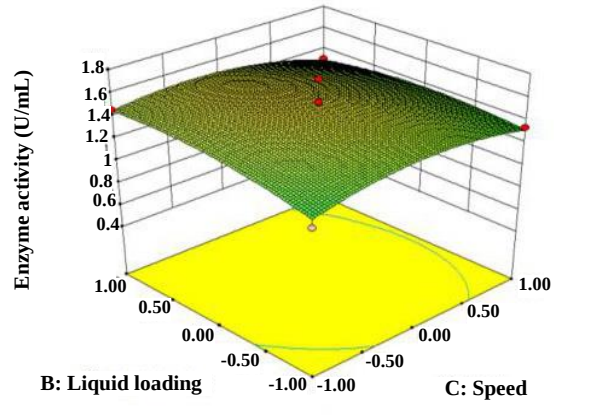
*Note: p<0.01 is extremely significant, which is indicated by **. P<0.05 is significant, which is indicated by *. P>0.05 is not significant.*



A



B



C

Fig. 2: Response surface analysis.

Fig. 3B shows the effect of pH value on the remaining enzyme activity without denaturation. When pH value is between 4.0 and 7.0, the enzyme activity increases with the increase of pH value. When pH value is 7.0, the enzyme activity is 79.3±6.82%, reaching the maximum value. When pH increased, the enzyme activity decreased gradually, but when pH value was between 6.0 and 8.0, the enzyme activity remained above 60%. The results were consistent with the optimal pH value of lipase produced by solid-state fermentation of *Bacillus Coagulans.* When the enzyme solution was pretreated in the pH range of 6.0 - 8.0 for 1 h, the lipase activity remained above 60% and kept the best activity. This showed that the lipase was stable in the pH range of 6.0 - 8.0, this is close to the optimal pH of the lipase produced by the reported *Halobacillus SP.* AP-MSU 8 strain [17] and *Xanthomonas oryzae pv. oryzae* YB103 strain [32].

Fig. 3C shows the effect of temperature on lipase activity. When the temperature was between 20 °C and 30 °C, the lipase activity increased with the increase of temperature. When the temperature reached 30 °C, the lipase activity reached its maximum value (107.6 ± 9.57%). When the temperature reached 45 °C, the lipase activity decreased gradually and reached the lowest (49.6 ± 4.55%).

Fig. 3D shows the effect of different temperatures on the activity of undenatured residual lipase. When the temperature is 20-35 °C, the thermal stability of the lipase is good, and the activity of the lipase can be maintained above 70%, when the temperature reached 50 °C, the residual enzyme activity was only 11.5 ± 3.72%.

Lipases come from a wide variety of sources, and different lipases have different effects. The majority of lipases and their preparations used in general factories are produced by microorganisms [12]. Because of the variety, rapid propagation, and high mutation rate of microorganisms, it is possible to better screen out the kinds of lipases needed for practical production, and even to mutate according to the requirements of factories in order to meet the production requirements, a high the lipase-producing strain was screened out [5].

In this study, five different carbon sources and nitrogen sources were selected to determine their low-temperature lipase activity. It was found that the strain had the best utilization effect on soluble starch and reached the highest value. When the carbon source is glucose, the enzyme

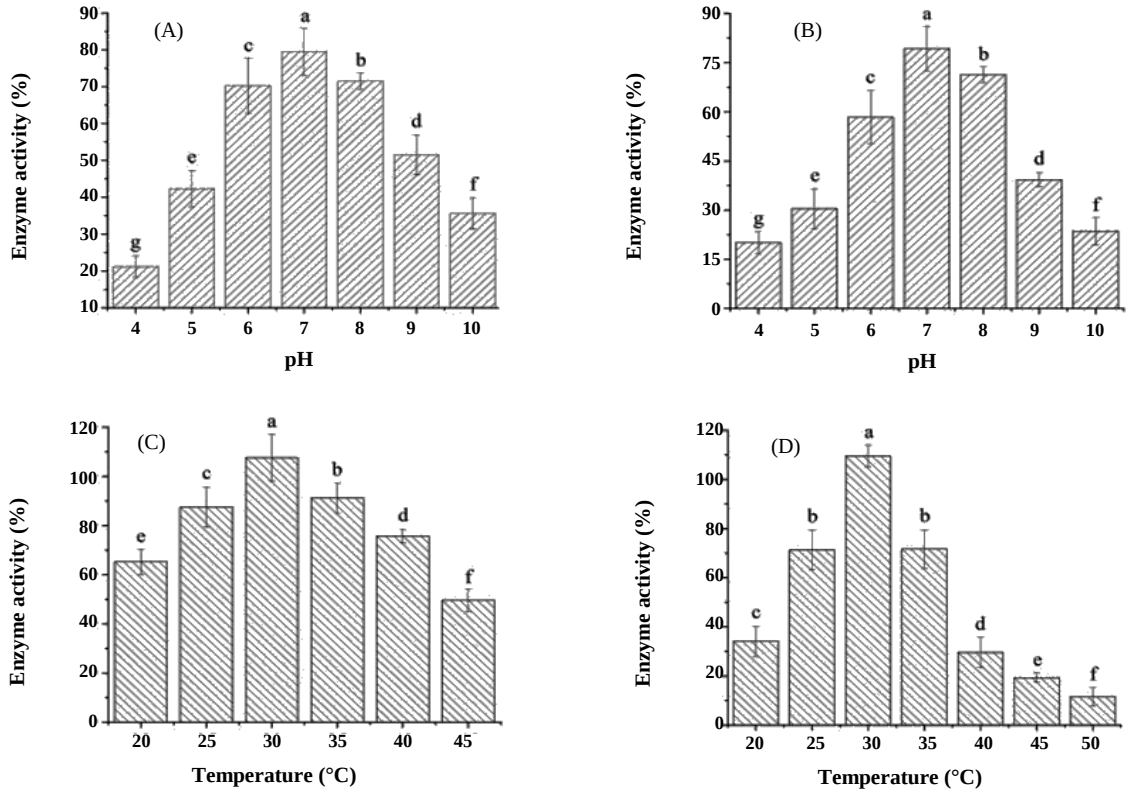


Fig. 3: Enzymatic properties. Note: A: the effect of pH on lipase activity. B: the effect of pH on enzyme stability. C: the effect of temperature on lipase activity. D: Effect of temperature on enzyme stability.

activity is lower, which is consistent with the conclusion that glucose can inhibit the production of lipase reported by *Dalmau et al.,* [33]. When the nitrogen source is peptone, the enzyme activity is the highest, probably because peptone is the organic nitrogen source, in addition to rich protein, peptide, free amino acid, but also contains a small amount of sugar, fat, and growth factor [24]. Because organic nitrogen source is rich in nutrients, microorganisms often show the characteristics of vigorous growth and rapid growth of cell concentration in the medium [22]. However, too low or too high a concentration of nitrogen source is not conducive to the growth of the mycelium, if the concentration is too low, the energy supply for the normal growth of the mycelium is insufficient, too high will hinder the normal metabolic process of the mycelium, and only when the concentration of nitrogen is moderate, the mycelium will grow well [6].

It was found that the lipase activity increased first and then decreased with the increase of reaction temperature. When the reaction temperature was 30 °C, the lipase activity was the highest. This result is the same as the optimum temperature of lipase produced by *Burkholderia sp* [34] and *Pseudomonas aeruginosa* LX1 strain [35,38]. When the pH of the enzyme solution was maintained at 7.0, the enzyme activity was maintained at about 110% after pretreatment at 30 °C for 1 h. After being pretreated at 25 °C and 35 °C for 1 h, the enzyme activity remained at about 70%, after being treated at 20 °C and 30 °C for 1 h, the enzyme activity remained at about 30%, and after being treated at 45 °C for 1 h, the lipase activity decreased to about 10%. It can be seen that temperature has a great effect on the lipase, this is consistent with the results of *Liu Wenshan et al.,* [23]. Therefore, if the enzyme is used in practical production, some additives should be added to improve its thermal stability [39-53].

CONCLUSIONS

The optimal fermentation medium of this low temperature lipase strain was soluble starch 4%, $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, peptone 3%, olive oil 1.05%, and the initial pH value is 7. The optimal fermentation conditions were 30 °C, sample

loading 26mL/250mL, and shaking flask rotation speed 213 r/min. The lipase activity of the strain reached 1.55 U/mL after medium optimization. It was found that the lipase was stable at pH 6.0-8.0 and could keep over 60% of the enzyme activity. When pH value was 7.0, the enzyme activity reached the maximum, which was 79.3±6.82%. At the same time, it was found that when the temperature reached 30 °C, the lipase activity reached the maximum of 107.6±9.57%, the lipase was stable between 25 °C and 35 °C, and the lipase activity could reach more than 70%. However, the thermal stability of the low-temperature lipase was poor over 35 °C.

Based on the results of a single-factor test, the optimum scheme was determined by orthogonal test and Design Expert 8.0 software. The experimental results showed that the actual enzyme activity is 1.49 U/mL, and the error between theory and practical is 3.87%. Therefore, this experiment can better predict the actual fermentation of low-temperature lipase.

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