RESEARCH ARTICLE

Optimization of culture medium composition and fermentation conditions for chitin deacetylase production by *Lysinibacillus boronitolerans* JM-4

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Chitin was the second largest resource in nature, and chitosan obtained by deacetylation from chitin has a broad application prospect. Compared with the commonly used chemical methods, enzymatic deacetylation had the advantages of environmental protection and uniform degree of deacetylation. High quality chitosan with uniform deacetylation degree could be prepared from chitin using chitin deacetylase (CDA) to remove N-acetyl group, which is an environmentally friend process to avoid the use of strong acids and bases. The enzyme-producing conditions of the strain *Lysinibacillus boronitolerans* JM-4 (LysJM-4) were optimized to improve the CDA-producing capacity. The proportion of medium and fermentation conditions were optimized by single factor experiment. The enzyme production medium consisted of 10 g/L corn starch, 10 g/L beef extract, 5 g/L sodium chloride, and 12 g/L chitin. The optimum fermentation conditions were determined as 7.0 initial pH, 35℃ culture temperature, and 8 % (V/V) inoculation amount. Under these conditions, the CDA enzyme activity could reach 17.77 U/mL after 72 h fermentation. The results were of great significance for the industrial preparation of chitosan by enzymatic method.

Keywords: chitin deacetylase; *L. boronitolerans* JM-4; enzyme activity; fermentation; optimization.

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Introduction

Chitin is a polymer of β-1, 4 linked N-acetyl-D-glucosamine, which is widely found in the bones of crustaceans and mollusks, the shells of insects, and the cell walls of fungi [1]. Due to the existence of hydrogen bonds in chitin, chitin is difficult to decompose and insoluble in water and organic solvents, which greatly limits its application [2, 3]. Chitosan is obtained by removing acetyl group from chitin, and its solubility and biocompatibility are improved, which has a broad application prospect in food, agriculture, cosmetics, medicine, environmental protection, and many other fields due to its good biocompatibility, degradability, safety, and various biological activities. Chemical and enzyme methods are the main methods of chitosan production from chitin in industry and research [4]. A lot of strong acid and alkali are used in chemical method, which is easy to cause environmental pollution, and the uniformity of products is poor. As a metalloenzyme, chitin deacetylase (CDA, E.C.3.5.1.41) can specifically

remove N-acetylase from chitin and convert chitin into chitosan [5]. Chitosan prepared by enzymatic method has a uniform degree of deacetylation and stable molecular weight, and the use of strong acid and base can be avoided, which has become one of the most promising technologies for the industrial application of chitin [6].

In 1974, CDA was obtained by Araki *et al*. from *Mucor rouxii* for the first time [7], and then researchers successively detected CDA activity in some pathogens and bacteria. At the present stage, the microbial source of CDA is relatively narrow, including fungi such as *Mucor rouxianus* [7, 8], *Aspergillus nidulans* [9], *Absidia coerulea* [10, 11], and bacteria such as *Bacillus* and *Vibrio* [12, 13]. The CDA production capacity of these strains is low, and the obtained CDA is unstable [14], resulting in limited production and high price of CDA [15], which makes it difficult to be applied in industrialization. Screening new strains with good CDA production performance and improving the CDA production capacity of strains are of great practical significance for the industrial preparation of chitosan by enzymatic method. So far, most of the research concerning CDAs has dealt with the isolation and screening of microorganisms along with purification and characterization [7, 9, 11]. CDA production by microbial fermentation is influenced by many factors such as nutritional sources and cultivation conditions. Therefore, optimization of the nutritional suppliers and the fermentation conditions is critical to achieve high CDA yield and render the process more economical.

In this study, the enzyme-producing conditions of *Lysinibacillus boronitolerans* JM-4 (LysJM-4) isolated from naturally fermented shrimp shell were optimized, which should provide theoretical basis and technical support for the industrial production of CDA and a new direction for the green application of chitin.

Materials and methods

Optimization of CDA production condition

(1) Determination of medium composition

The effects of medium composition and culture conditions on CDA yield were investigated by single factor experiment. The basic medium for CDA production was prepared at 7.0 initial pH with 10 g/L of carbon source, 10 g/L of nitrogen source, 5 g/L of mineral salt, and 10 g/L of chitin (Shanghai McLean Bio-Chemical Co., Ltd., Shanghai, China). 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. Glucose, sucrose, maltose, lactose, and corn starch were selected as carbon source, ammonium sulfate, sodium nitrate, beef extract, peptone, and corn pulp were used as nitrogen source, NaCl, $MgSO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$, and $ZnCl_2$ were selected as mineral salt, respectively.

(2) Determination of initial pH and chitin amount

Appropriate amounts of chitin were added into the optimized fermentation medium at the final concentrations of 6, 8, 10, 12, 14 g/L to investigate the induction effect of chitin on the production of enzymes. 200 mL of culture medium was filled into a 500 mL conical flask for sterilization, then the initial pH of culture medium was adjusted to 4, 5, 6, 7, 8, respectively, to explore the effect of initial pH on CDA production.

(3) Determination of inoculation quantity and cultural temperature

After inoculated 10% ($V_{seed\ liquid}$:V_{medium}) of logarithmic growth stage seed liquid, *L. boronitolerans* JM-4 (LysJM-4) isolated from naturally fermented shrimp shell was fermented at 28℃ for 72 hours in a MQD-B3R oscillating incubator (Shanghai Minquan Instrument Co., Ltd., Shanghai, China). On the premise of the above culture conditions and keeping other conditions unchanged, 2, 4, 6, 8, and 10% (V/V) inoculated amount were tested to determine the suitable amount of seed liquid, and the culture temperature was set at 28, 30, 35, and 40℃, respectively, to explore the influence of

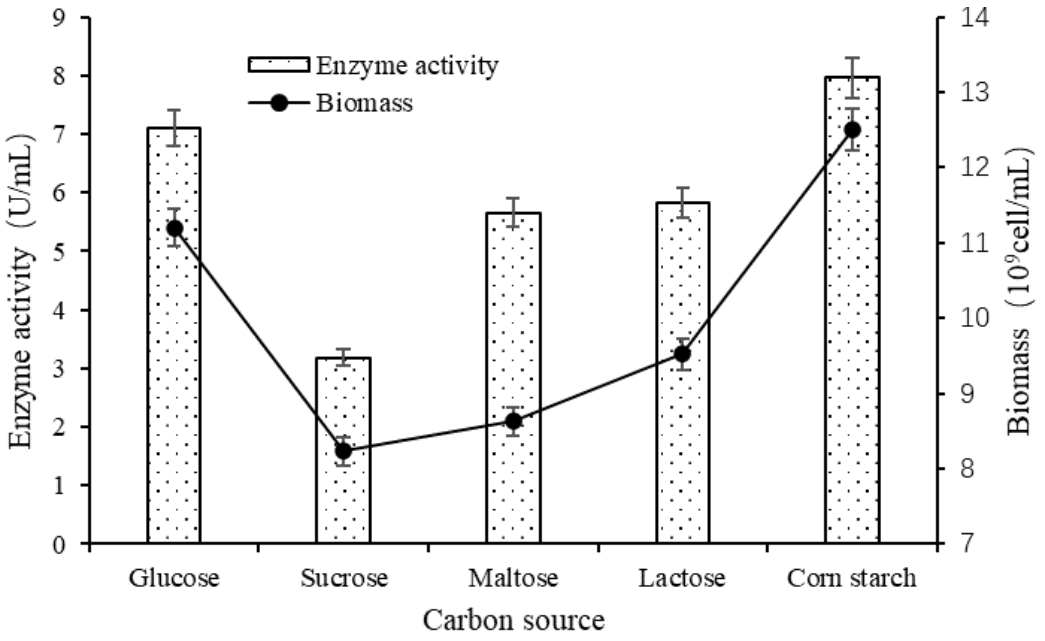

Figure 1. Effect of carbon source on CDA production of LysJM-4.

temperature on CDA production. The fermented liquor samples were taken every 24 h and centrifuged at 8,000 rpm for 15 minutes at room temperature. The supernatant was considered as crude enzyme and used to determine CDA activity, while the sediment was used to determine the microbial biomass.

CDA activity assay

The CDA activity was determined according to the p-nitroaniline method reported by Yang *et al.* [16]. Briefly, 1 mL crude enzyme solution was mixed with 1 mL 0.2 g/L 4-nitroacetanilide (Shanghai Yuanye Bio-Co., Ltd., Shanghai, China) and 3 mL 0.2 M phosphate buffer (pH 7.0) before incubated at 50℃ for 15 min. The reaction was stopped by heating in boiling water for 20 min, and replenished volume with deionized water to 10 mL. The generation of 4-nitroaniline during the enzymatic reaction was detected at 400 nm using different concentrations of 4-nitroaniline as standard. The distilled water was set as blank control. A unit of CDA activity was defined as the amount of enzyme required to produce 1 μg 4-nitroaniline per hour under the above reaction conditions.

Assay of microbial biomass

The microbial biomass in sediment portion was determined by turbidimetric method [14]. The sediment was washed with deionized water and dried overnight at 100℃. The OD was determined at 400 nm when the dried powder was re-suspended in normal saline and diluted to an appropriate concentration.

Statistical analyses

All tests were repeated three times, and the data was expressed as mean ± standard error. SPSS software (IBM, Armonk, New York, USA) was used to conduct one-way ANOVA and multiple comparison through Tukey-Kramer test.

Results and discussion

Optimization of medium composition

(1) Effect of carbon source on CDA production

Carbon source was an essential nutrient component in medium. The effect of carbon source on CDA production was shown in Figure 1. When corn starch was used as carbon source, the growth of strain LysJM-4 was the most vigorous

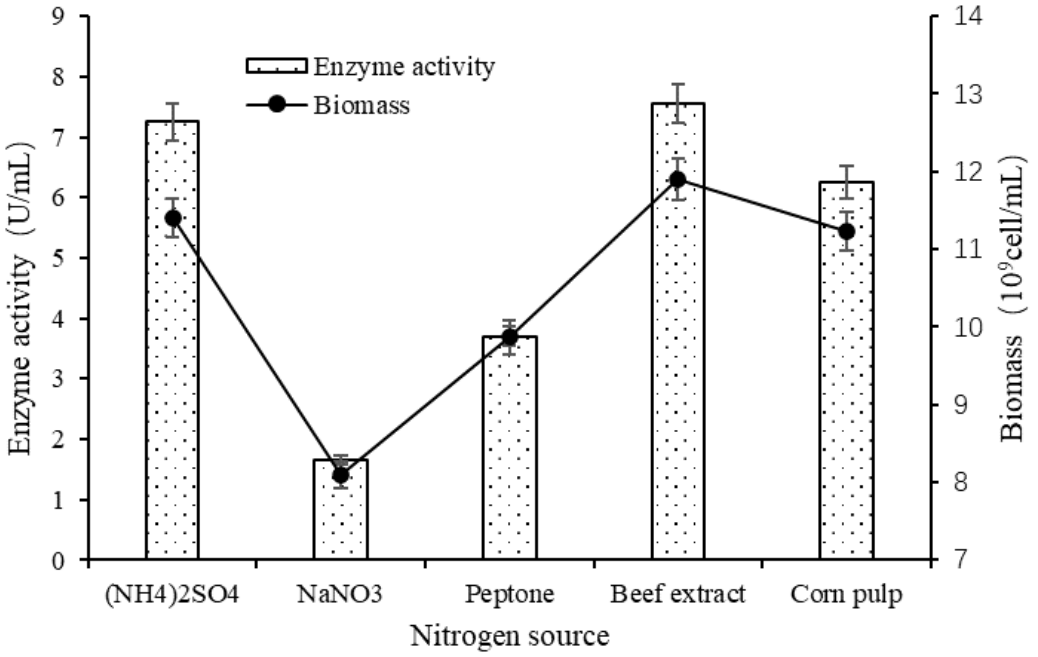

Figure 2. Effect of nitrogen source on CDA production of LysJM-4.

reaching 1.25 × 10^{10} cells/mL, and the activity of CDA was the highest of 7.96 U/mL. When glucose was used as carbon source, the CDA activity was slightly lower than that of corn starch as 7.10 U/mL. The CDA activities of the other three carbon sources experimental groups were much lower. Therefore, corn starch was selected for the following experiments.

(2) Effect of nitrogen source on CDA production

The effects of nitrogen source on CDA production of LysJM-4 showed that, when beef extract was used as nitrogen source, the growth of strain Lys-X4 was the fastest with its biomass reaching 1.19 × 10^{10} cells/mL and the CDA activity reaching the highest 7.56 U/mL. When sodium nitrate was used as the nitrogen source, the enzyme activity was the lowest at 1.75 U/mL, and the biomass was only 8.10 × 10^{9} cells/mL (Figure 2). This was because organic nitrogen could provide other nutrients such as growth factors for microorganisms. Among the three organic nitrogen sources, beef extract showed the most significant effect on bacterial growth and CDA production. Therefore, beef extract was selected as the nitrogen source for the next experiments.

(3) Effect of mineral salt on CDA production

Inorganic salt can not only maintain enzyme activity, but also regulate osmotic pressure during microbial growth and metabolism. In this study, six mineral salts were selected to study the effects of mineral salt on LysJM-4 production CDA (Figure 3). The results showed that NaCl had the strongest effect on the growth and CDA production of LysJM-4, the biomass and CDA activity could reach 1.49 × 10^{10} cells/mL and 10.93 U/mL, respectively. LysJM-4 was isolated from fermented shrimp shell, salt environment should be more conducive to the growth and CDA production of LysJM-4. The effects of $CuSO_4$ and $FeSO_4$ were slightly weaker than that of NaCl, which suggested that Cu^{2+} and Fe^{2+} might be the activators of CDA produced by LysJM-4.

Optimization of CDA production conditions

(1) Effect of pH on CDA production

The charge of cytomembrane will change with pH to a certain extent, which will affect the growth and metabolism of microorganisms. LysJM-4 could produce CDA when pH was between 4 - 8, and the enzyme-producing capacity increased gradually with the increase of pH. The maximum

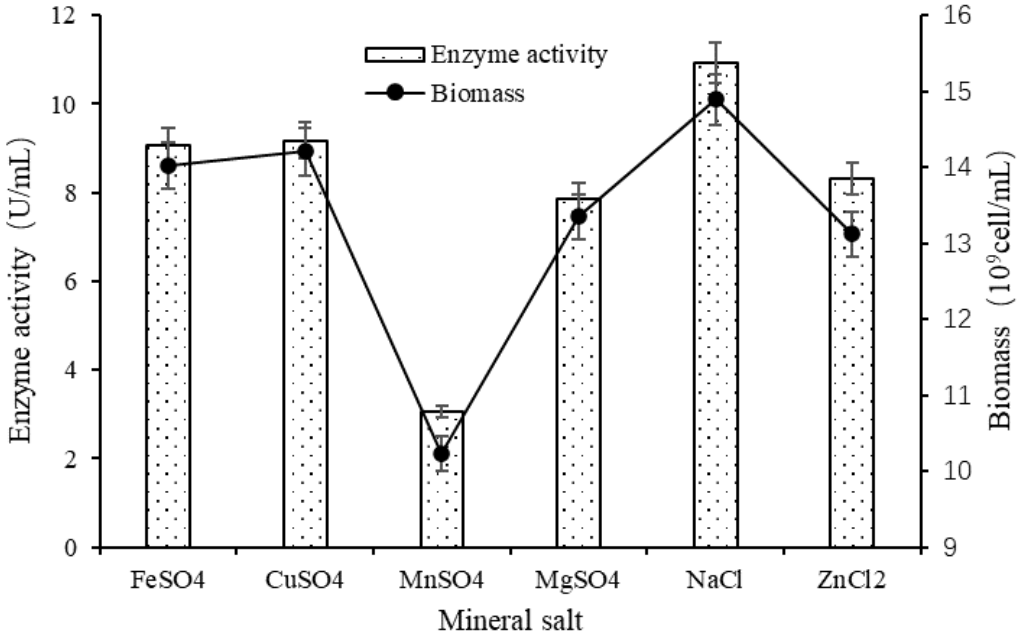

Figure 3. Effect of mineral salt on CDA production of LysJM-4.

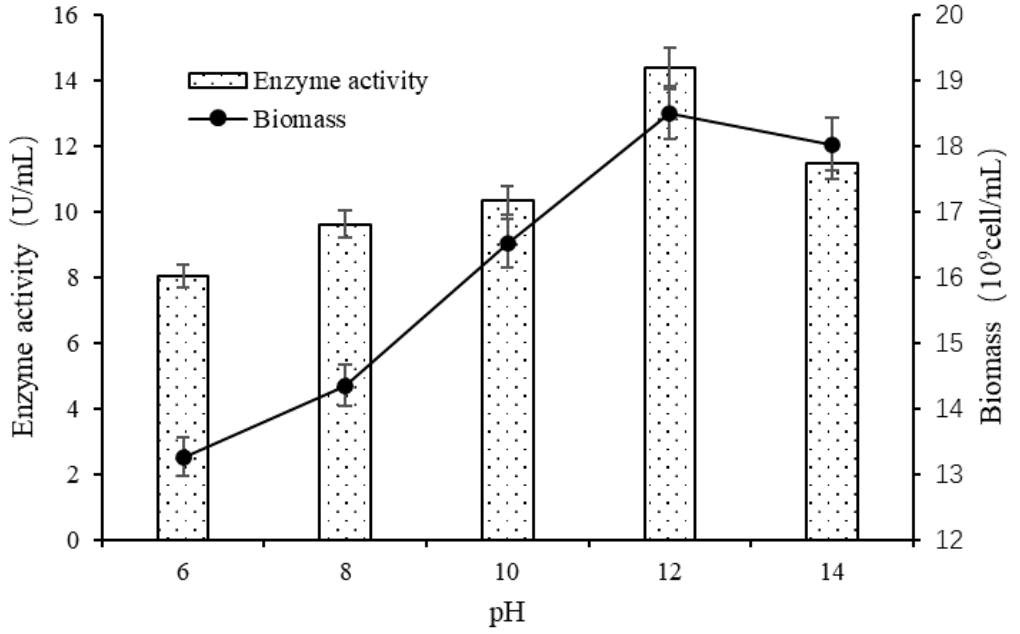

Figure 4. Effect of pH on CDA production of LysJM-4.

CDA activity reached 14.38 U/mL at pH 7, then both enzyme activity and biomass showed a downward trend when pH exceeded 7. The results demonstrated that the optimum environment for CDA production was neutral (pH 7.0) (Figure 4).

(2) Effect of culture temperature on CDA production

The growth and synthesis of metabolites of microorganisms are closely related to the culture temperature. Low temperature may cause slow growth of bacteria and low enzyme activity, and

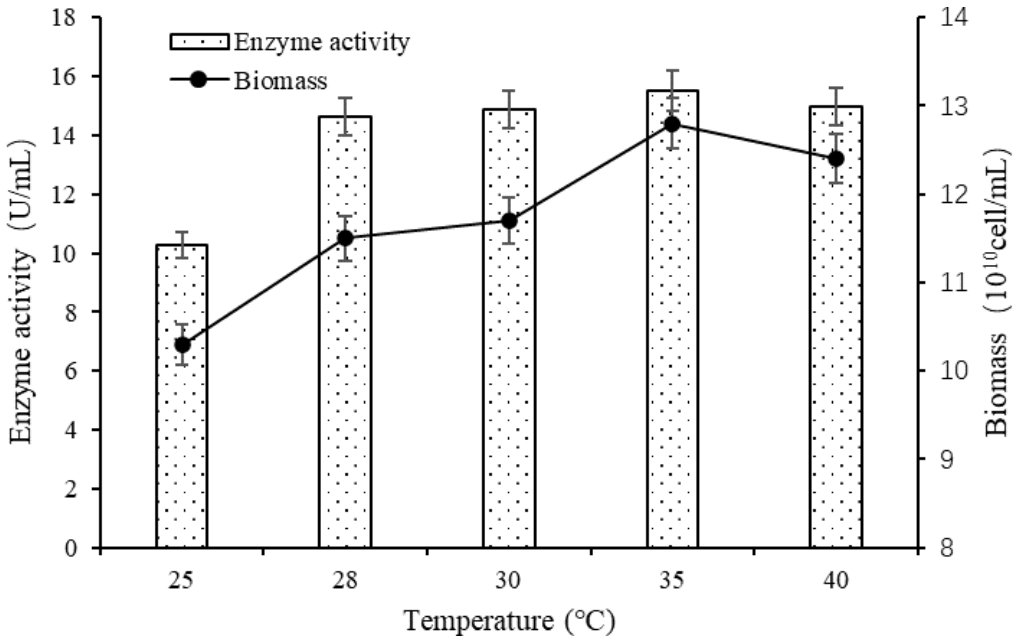

Figure 5. Effect of culture temperature on CDA production of LysJM-4.

excessive temperature can lead to intolerance of bacteria, as well as enzyme inactivation. The effect of culture temperature on CDA production of LysJM-4 was shown in Figure 5. The CDA activity and biomass of LysJM-4 increased with the increase of temperature and reached the maximum value at 35℃. When the temperature exceeded 35℃, the enzyme activity decreased slightly, and the biomass decreased significantly, which indicated that the growth of the strain was inhibited at high temperature that also affected the synthesis of CDA. As a result, 35℃ was selected as the fermentation temperature of CDA production.

(3) Effect of culture time on CDA production

With the extension of fermentation time, the biomass of LysJM-4 and CDA enzyme activity increased gradually, and the maximum CDA activity reached 16.93 U/mL at 72 h (Figure 6). Then the CDA activity tended to be stable and slightly decreased with the extension of fermentation time. With the extension of fermentation time, the pH of the culture system decreased slowly, and nutrients were also gradually consumed, leading to a decline in enzyme production.

(4) Effect of inoculation quantity on CDA production

The CDA activity increased with the increase of the inoculation quantity, and the maximum CDA activity reached 16.48 U/mL when the inoculation quantity was 8% (V/V), and then the CDA activity began to decline with the increasing of inoculation quantity (Figure 7). Most nutrients in the medium were mainly used for the growth of bacteria when the inoculation quantity was too high, resulting in less CDA synthesis. When the inoculation amount was too small, the bacterial concentration was too low, resulting in less enzyme production.

Effect of crystalline chitin on CDA production

Previous studies have shown that both *Absidia coerulea* and *Aspergillusni dulans* showed an increase in CDA activity when soluble chitosan was used as carbon source [11, 17]. Chitin was the substrate of CDA, and appropriate addition of chitin might have a certain conducive effect on the CDA production by LysJM-4. Because most CDAs are difficult to act on crystalline chitin, colloidal chitin is generally used as the substrate of CDA in the studies [18]. In this research, crystalline chitin (CC) was used as the substrate

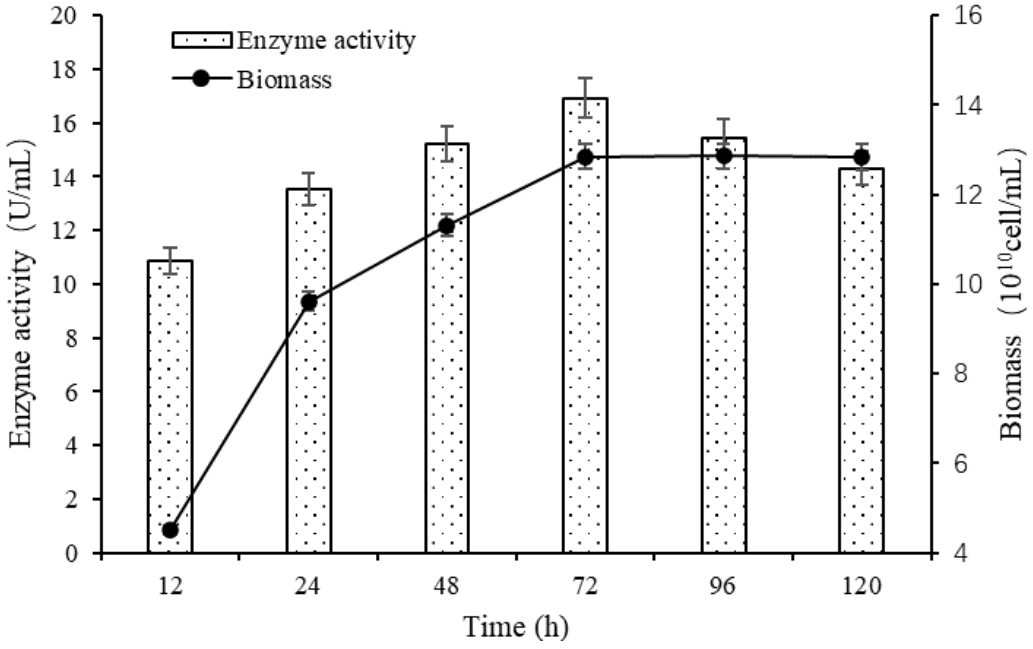

Figure 6. Effect of culture time on CDA production of LysJM-4.

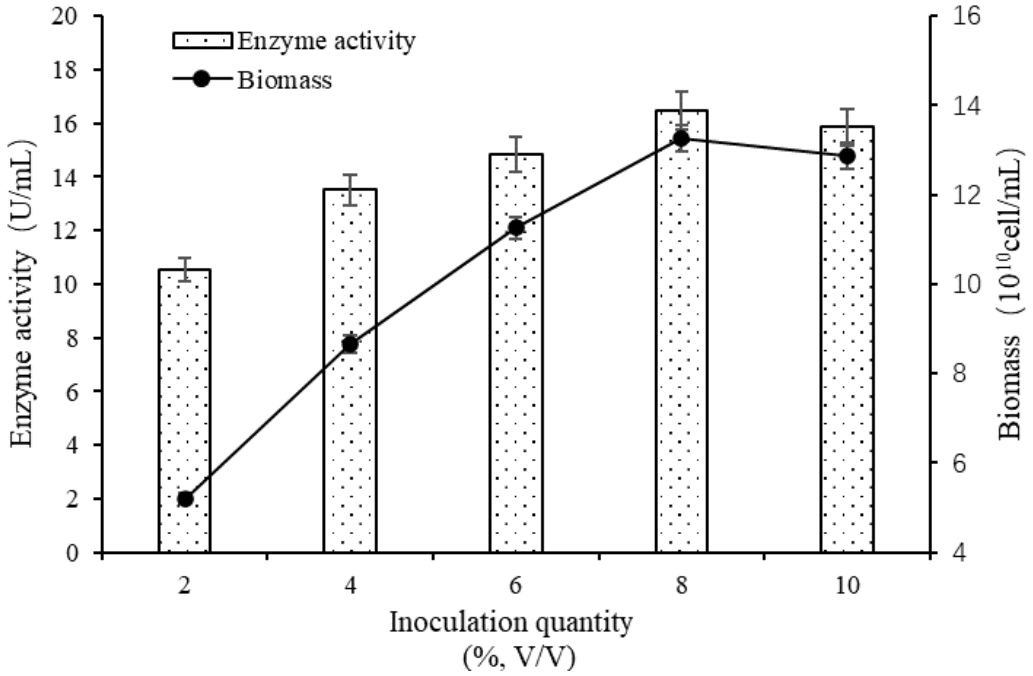

Figure 7. Effect of inoculation quantity on CDA production of LysJM-4.

to induce the production of CDA with the different concentrations in the medium. The results showed that the addition of chitin could promote CDA production to a certain extent, and the CDA activity gradually increased with the increase of chitin dosage. When the addition of chitin was 12 g/L, the enzyme activity reached a maximum of 17.23 U/mL, and the biomass was

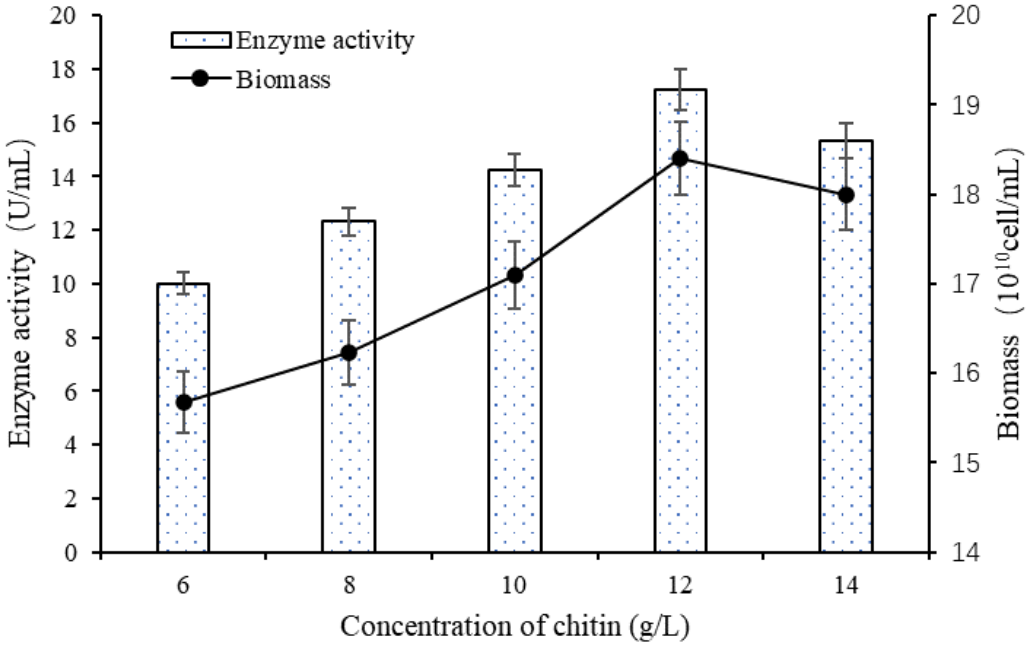

Figure 8. Effect of chitin addition on CDA production of LysJM-4.

1.84×10^{11} cells/mL. When the addition of chitin increased to 14 g/L, the CDA activity began to decline, and the biomass also decreased slightly to 1.80×10^{11} cells/mL (Figure 8). The reason might be that the higher concentration of substrate might reduce the fermentation environment to be acidic (pH 5.16) [12], thus inhibiting the growth of bacteria, and resulted in a decrease in enzyme production. According to the preliminary optimized experimental conditions, 8 % (V/V) seed liquid of LysJM-4 was inoculated in the medium (pH 7.0) consisted with 10 g/L corn meal, 10 g/L beef extract, 5 g/L sodium chloride, and 12 g/L chitin. After culturing at 35℃ for 72 h, 17.77 ± 0.28 U/mL CDA activity was obtained.

Many studies were carried out to improve the production of CDA by microorganisms. The maximum CDA yields were obtained as 547.38 ± 12.06 U/L by *Rhizopus japonicus* M193 [19], 40.6 U/μg by *Alcaligenes faecalis Alca* F2018 [20], 2.24 U/ml by *Paenibacillus sp*. Hu1 [21], and 1.05 U/mg by *Colletotrichum gloeosporioides* [14], respectively. Currently, there are few microbes having high CDA production capacity in the process of cultivation, and the substrate of CDA was generally deacetylated chitin or chitosan [22]. In contrast, CDA produced by LysJM-4 showed higher enzyme activity with crystalline chitin as substrate, which suggested that this enzyme could decompose crystal chitin directly. Therefore, how to improve the ability of LysJM-4 to produce CDA is still one of the important topics to solve the industrial application of CDA.

Conclusion

The ability of the microorganism to produce CDA is of great significance for the industrial preparation of chitosan by enzymatic method. The culture conditions of LysJM-4 were optimized to improve the CDA production capacity. The enzyme production medium consisted of 10 g/L corn meal, 10 g/L beef extract, 5 g/L sodium chloride, and 12 g/L chitin. After adjusting the culture medium pH to 7.0, 8% (V/V) LysJM-4 seed solution was inoculated and cultured at 35℃. Under these conditions, the CDA activity could reach 17.77 U/mL after 72 h fermentation. This study provided theoretical

basis and technical support for industrial production of chitin deacetylase.

Acknowledgements

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