Increasing chitosanase production in *Bacillus cereus* by a novel mutagenesis and screen method

Chaozheng Zhang

Tianjin University of Science and Technology

Yi Li (✉ 3192281976@qq.com)

Tianjin University of Science and Technology <https://orcid.org/0000-0003-2758-6016>

Tianshuang Zhang

Tianjin University of Science and Technology

Hua Zhao (✉ zhaohua@tust.edu.cn)

Tianjin University of Science and Technology

Research

Keywords: *Bacillus cereus*, Chitosanase, Screening, Identification, Atmospheric and Room Temperature Plasma Mutagenesis

Posted Date: July 20th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-44207/v1>

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Abstract

Background

Chitosan hydrolysis by chitosanase is one of the most effective methods to produce chitosan oligosaccharides, however, the low yield of chitosanase cannot meet the current requirement. In this paper, a strain producing chitosanase was screened, and a novel mutagenesis system (1)) was selected to increase the yield of chitosanase. The hydrolyzed products from chitosan by chitosanase produced by mutant strain were also analyzed by LC-MS.

Results

A strain of *Bacillus cereus* capable of producing chitosanase was screened and identified from soil samples. A mutant strain of *Bacillus cereus* was obtained by ARTP mutagenesis and bioscreening method, and chitosanase activity was 2.49 folds that of the original bacteria. After optimized fermentation conditions, the enzyme activity of the mutant strain was 3.3 folds that of the original bacteria. The relative molecular weight of the purified chitosanase was 43 kDa. Ten chitosan oligosaccharides(2–4 oligosaccharides) were obtained by hydrolyzing chitosan with it.

Conclusions:

The results showed that the ARTP mutagenesis and bioscreening method could significantly increase the yield of chitosanase in *B. cereus*, and had little effect on the properties of the enzyme. These findings have potential applications in mutagenesis of other enzyme-producing microorganisms.

Background

Chitosan, which is insoluble in water, is a kind of biopolymer composed of β -1, 4-linked Glucosamine(GlcN or D) residues and N-acetyl Glucosamine(GlcNAc or A) residues(2). Chitosan with a degree of polymerization of 2–20 degrees, also known as Chitosan oligosaccharide (COS), are water-soluble and has a variety of biological activities. Therefore, COS has a wide range of potential applications in the fields of medicine, food, agriculture, cosmetics, and so on(3, 4). The COS has attracted more and more attention from laboratory and industry, and will become a new functional oligosaccharide. The COS can be obtained by ultrasonic irradiation (5, 6), hydrodynamic shearing, and chemical and enzymatic hydrolysis of Chitosan. Enzymatic hydrolysis of Chitosan are one of the best methods for the preparation of Chitosan oligosaccharides(7).

Chitosanases (EC 3.2.1.132) are characterized as enzymes that catalyze the hydrolysis of glycosidic bonds in chitosan to form COS. Chitosanase was first reported from soil microorganisms in 1973 (8) Although chitosanase has been studied for about 50 years (6), only certain microorganisms can produce

it. The fungi producing chitosanase are *Aspergillus*, *Penicillium*, *Fusarium*, *Gongronella*, and *Mucor*, and the molecular masses of chitosanases produced are very diverse ranging from 22.5 to 108 kDa(6, 9). Recent studies on chitosanases have been dominated by bacterial species belonging to the genera *Bacillus* (10, 11), *Pedobacter* (12), and *Streptomyces*. As COS has good biological activities and great demand, the development of new chitosanases for the preparation of COS is very important. To date, numerous chitosanases with potential industrial applications have been discovered and studied at the biochemical level (5, 13–15). However, these reported chitosanases are all induced enzymes and their production requires chitosan as the inducer (8). The yields of chitosanases from strains during fermentation are significantly low because chitosan has poor solubility in neutral pH and high viscosity aqueous solutions (16). Thus, the colloidal chitosan is introduced into the medium as an inducer. The optimization of medium composition and fermentation parameters is an important step in improving the yield of enzymes. However, the application of colloidal chitosan limits the optimization space of medium composition and fermentation parameters. To solve this problem, the use of screening strains that can produce constitutive chitosanase is one of the ways to enhance the yield of chitosanase. A constitutive chitosanase was screened in our laboratory. The enzyme was obtained from *Bacillus cereus* screened from soil. However, the disadvantage of this strain is that the yield of chitosanase is not high, so it is necessary to increase the yield by mutagenesis.

Atmospheric and Room Temperature Plasma (ARTP) is a kind of plasma source of non-equilibrium discharge at atmospheric pressure (17, 18). With ARTP treatment, DNA can be destroyed by chemical active substances rather than high temperatures, ultraviolet rays, charged particles, or strong electric fields. ARTP is a new type of physical mutagenesis technology, which has the characteristics of a higher mutation rate, diversity, and no pollution than traditional mutagenesis (1, 18, 19). Therefore, it is more and more widely used in microbial mutation (20–22).

In this study, a strain of *Bacillus cereus* capable of producing chitosanase was screened and identified from soil samples. Whereafter, the ARTP mutagenesis method was used to increase the yield of chitosanase produced by *B. cereus*. The culture conditions of chitosanase production were studied, and the optimum culture conditions were determined, which increased the yield and activity of chitosanase. A new type of chitosanase without chitosan induction was proposed, and its potential application in COS production was studied.

Results And Discussion

Isolation of strain producing constitutive chitosanase

In the current work, chitosan was used as the sole carbon source to separate strain from the diluents of enrichment culture. Many bacterial colonies and molds were observed in agar plates. A total of 33 colonies with good growth and transparent circles was selected and numbered successively for further purification. To investigate the enzyme production capacity, the strains 01, 06, 10, and 12 with relatively large D/d values were selected for rescreening. Four strains were inoculated into both rescreening media

A and B and chitosanase activity was determined after incubation for 36 h. Chitosanase activities in the rescreening medium A for four strains positively correlated with the size of D/d. Strain 01, was the only inoculum found to have chitosanase activity in re-screening medium B broth; therefore considered a consisted-enzyme. At present, chitosanase from most microbial sources belongs to the inducible enzyme type and gene expression was mostly controlled by repressor and inducible substance (2, 10, 12) . Chitosan was generally used as the inducible substance and the by-product was the repressor. However, the consisted-chitosanase produced by strain 01 could exactly overcome this problem.

Identification of strain 01

The colony and cell forms of strain 01 have been shown in Fig. 1. The colony was round, with the rough and waxy surface, opaque, white, and looked like frosted glass. Gram staining, identified the bacteria as gram-positive. Cells were rod-shaped, with obtuse ends and formed short chains with an approximate size of $0.4\text{-}0.6 \times 2\text{-}3 \mu\text{m}$ under SEM, as shown in Fig. 1.

Biolog Microstation system showed that strain 01 can fully utilize 42 of the 95 carbon resources, and it was confidently identified as *Bacillus cereus*. Sequencing with 16S rDNA was blasted into the public database (Gene Bank) and revealed that the strain was *Bacillus cereus* at 100% homology. The strain 01 was preserved in Tianjin University of Science and Technology's Center of Culture Collection (TCCC) and numbered TCCC 10028.

ARTP mutagenesis and screening of mutant

The enzyme activity of the strain TCCC 10028 was only 3.81 U/mL, which could not meet the production requirements. Therefore, ARTP mutagenesis has been used to improve the enzyme activity of the strain. The lethality curve of *Bacillus cereus* mutagenized by ARTP has been shown in Fig. 2. When the irradiation time was between 20s and 50s, the lethality fluctuated within a constant range (62.7-71.9%). It was inferred that DNA repair machinery of the bacteria played positive role, which machinery repaired the damaged DNA in bacteria caused by ARTP. Because the cell contained many proteins and specific DNA repair machinery, it maintained its correct structure (23) . When DNA was severely damaged and/or its synthesis was blocked, the DNA repair machinery was induced and it repaired itself. However the induction of the repair machinery took time, it was impossible to repair the damaged parts all the time. When the irradiation time was the 60s, the lethality rate of the strain was more than 90% and the DNA repair machinery was no longer working. Then the 60s was selected for the irradiation time of ARTP. The bacteria after ARTP mutagenesis according to method were transferred to a bioscreen system. In the case of a 90% fatality rate, only one mutated cell can be assigned to each micropore in bioscreen system. This may reduce the tedious work required for subsequent screening.

According to the value of OD_{600} obtained from the bioscreen system, 30 wells with a larger increase in OD_{600} value than others were selected, and the enzyme activities were determined according to the after mentioned method. The yield of chitosanase by the strain was closely related to the concentration of the

strain, so several holes with high OD₆₀₀ increase were selected to determine the enzyme activity, and the ratio of enzyme activity to OD₆₀₀ was calculated and arranged in descending order, as shown in Table. 1.

Table. 1 The screening of mutant strains

Well	OD ₆₀₀	Activity [U/mL]	Activity/OD ₆₀₀	Well	OD ₆₀₀	Activity [U/mL]	Activity/OD ₆₀₀
202	3.509	3.728676	1.0626	142	3.5	3.320143	0.9486
129	3.515	3.716221	1.0572	196	3.514	3.330107	0.9477
115	3.524	3.591668	1.0192	189	3.506	3.311424	0.9445
221	3.509	3.548074	1.0111	109	3.498	3.105912	0.8879
113	3.505	3.535619	1.0087	182	3.558	3.037408	0.8537
188	3.517	3.492026	0.9929	116	3.523	2.975131	0.8445
126	3.515	3.485798	0.9917	295	3.675	3.093456	0.8418
121	3.538	3.485798	0.9852	284	3.516	2.956448	0.8409
207	3.505	3.435977	0.9803	275	3.599	2.975131	0.8267
131	3.505	3.429749	0.9785	122	3.504	2.881717	0.8224
242	3.557	3.45466	0.9712	233	3.547	2.906627	0.8195
138	3.503	3.392383	0.9684	246	3.531	2.887944	0.8179
276	3.531	3.398611	0.9625	245	3.526	2.869261	0.8137
212	3.5	3.342562	0.955	104	3.503	2.844351	0.812
117	3.505	3.342562	0.9537	193	3.51	2.638838	0.7518

The first six Wells in the ratio of enzyme activity to OD₆₀₀ were selected for coating to obtain the single colonies. The twenty-one mutants with different colony shape were isolated and tested enzyme activity in the shaking bottle. The results have been listed in Table 2. The enzyme activity of 15 of the 21 mutant strains was higher than that of the original strain. The highest enzyme activity of the mutant was 9.46(mutant 202(1)), 2.49 folds that of the original strain. ARTP mutagenesis has been widely used in the screening of yeast and filamentous fungi, and the desired results have been obtained(24, 25). There were some studies that use ARTP mutagenesis to screen of Bacillus, which was 1.2-3.3 folds higher than the original strain(26, 27). The yield of mutant starin in this paper reached to 2.49 fold that of the original strain, which was consistent with the results reported.

Table. 2 The mutants strains in shake flask screening

Number	113(1)	113(2)	113(3)	113(4)	113(5)	113(6)	115(1)
Activity(U/mL)	5.2745	6.6940	4.3249	7.2521	5.9011	5.0200	3.0326
Number	115(2)	129(1)	129(2)	129(3)	129(4)	202(1)	202(2)
Activity(U/mL)	4.2368	3.0228	4.3640	3.3655	4.1878	9.4606	3.2578
Number	202(3)	202(4)	188(1)	188(2)	221(1)	221(2)	221(3)
Activity(U/mL)	5.8913	3.2578	6.7626	6.7528	4.0997	2.1417	5.0591

Stability of enzyme production

In order to investigate the stability of the mutated strain, continuous transfer experiment was carried out. A mutant displaying high chitosanase activity after the ARTP mutagenic treatments was selected, and its stability of the enzyme production was investigated for six generations by successive inoculations of the strain on chitosanase production medium. The changes of enzyme activity in fermentation broth were very small when the strains were cultured for six generations. By comparing the changes of chitosanase activity in each generation, the stability of enzyme production in the mutant strain was determined.

Optimization of culture conditions

To obtain more chitosanase, the growth conditions of strains have been optimized (Fig. 3). The optimal temperature was 32°C (Fig. 3a) and the pH of the basic fermentation medium is adjusted to 6.0 with 2 mol L⁻¹ NaOH (Fig. 3b). B. Ceres mutant was suitable for growing in a weakly acidic environment. The initial pH of the medium did not change after mutagenicity, but the optimal culture temperature was increased from 30°C to 32°C. Under the optimal conditions, the chitosanase activity reached to 12.77 U/mL, 3.3folds that of the original strain. It could be seen that ARTP mutagenesis had little influence on the culture conditions of the strain.

Application and purification of chitosanase

After centrifugation, the fermentation broth was precipitated with ammonium sulfate, and then the dialyzed, and finally the chitosan powder was obtained by freeze drying. The chitosanase produced by two strains before and after ARTP mutagenesis were analyzed by SDS-PAGE gel electrophoresis. Comparing the standard protein makers with the obtained chitosanase, it was found that the relative molecular weight of the two enzymes were both about 43KDa. It showed that the relative molecular weight of chitosanase had no obvious change before and after mutagenesis. It was reported in the literature that the relative molecular weights of chitosase was between 22.5 kDa and 108 kDa. The results of this study were consistent with those reported.

The application of chitosanase can be divided into several kinds. An alkalophilic extracellular chitosanase (pH 10 medium) was obtained from *B. cereus* and the corresponding COS were prepared (28). The Exo- and endo-chitosanase were obtained from *Gongronella butleri* (9) and *B.*

amyloliquefaciens. A cold-adapted chitosanase was used for preparing the COS under the normal temperature process (29). The chitosanase obtained in this paper can hydrolyze chitosan at 60°C to produce COS. The hydrolysis of chitosan by chitosanase were analyzed by LC-MS. Ten COSs have been identified from the hydrolyzed products, including chitobiose, chitotriose, tetrasacchside and their related acetyl derivatives.

Conclusions

In this study, a chitosanase producing strain was successfully isolated from marine soil rich in shrimp shells in Tianjin. It was identified as *B. cereus* by morphology, biology test, and 16S rDNA sequence analysis. *B. cereus* was mutagenized by ARTP. The results showed that the lethality rate was more than 90% after mutagenesis with ARTP for the 60 s, and a mutant strain with 2.49 fold enzyme activity was obtained. The optimal culture conditions of fermentation medium were 30 °C and the initial pH 6.0. The activity of chitosanase increased by 3.36 fold to 12.77 U mL⁻¹ under optimal fermentation conditions. Although there were many studies on chitosanase production by microorganisms, there has been few reports that ARTP mutagenesis can be used to improve chitosanase production by *Bacillus*. The properties of chitosanase did not change after ARTP mutagenesis. A total of ten COSs were isolated and identified by LC-MS from chitosan hydrolysate. The further application of this chitosase laid the foundation.

Materials And Methods

Matrix of the isolation and cultivation media

The matrix of isolation was sampled from a fishpond near the Bohai Sea(Tianjin, China). The enriched medium contained (in g L⁻¹) the following: Chitosan powder, 10 g; yeast powder, 2.5 g; sodium chloride, 5 g. The pH was not artificially regulated. Sterilization was executed at 121 °C for 20 min.

The seed medium contains peptone 10g, yeast extract 5g, NaCl 5g L⁻¹ and glucose 10g. The pH was adjusted to 6.5. Sterilization was executed at 121 °C for 20 min.

The preliminary screening medium consisted of solution A and B. Solution A contained 1% (w/w) colloidal Chitosan and Solution B contained (in g L⁻¹) 5 g peptone, 5 g (NH₄)₂SO₄, 1.4 g K₂HPO₄, 0.6 g KH₂PO₄, 5 g NaCl, 1 g MgSO₄·7H₂O, and 30 g agar power 30. The pH was adjusted to 6.5. Solutions A and B were sterilized at 115 °C for 30 min. Upon cooling to 60 °C, Solutions A and B were mixed with a ratio of 1:1. Colloidal Chitosan (1%) was prepared according to by adding 1 g of Chitosan powder (deacetylation above 90%) in a 250 ml beaker containing 20 ml purified water for 2 min followed by the addition of 30 ml 0.2 Mol L⁻¹ acetic acid. The solution was constantly stirred until the Chitosan presented as a transparent gel. The pH of colloid Chitosan was adjusted to 5.5 with 1% sodium hydroxide solution and the capacity to 100 ml with distilled water.

The re-screening medium A contained (g L^{-1}) 5 g Chitosan powder, 5 g peptone, 5 g $(\text{NH}_4)_2\text{SO}_4$, 1.4 g K_2HPO_4 , 0.6 g KH_2PO_4 , 5 g NaCl, and 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH of the media was adjusted to 6.5 with 2 mol L^{-1} NaOH and was sterilized at 121 °C for 20 min.

The re-screening medium B contained glucose instead of Chitosan powder from the re-screening medium A. Meanwhile, the seed culture medium contained (g L^{-1}) 10 g peptone, 10 g glucose, 5 g yeast extract powder, and 10 g NaCl. The pH of the medium was adjusted to 6.5 with 2 mol L^{-1} NaOH and then sterilized at 121 °C for 20 minutes.

Basic fermentation medium contained (g L^{-1}) glucose 5 g, yeast extract 5 g, K_2HPO_4 1.4 g, KH_2PO_4 0.6 g, NaCl 5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g, and Tween-80 1 mL. The pH of the medium was adjusted to 6.0 with 2 mol L^{-1} NaOH and was then sterilized at 121 °C for 20 minutes.

Enrichment and screening of chitosanase-producing strain

The collected soil sample was used as the inoculums to enrich chitosanase-producing bacteria in a 250 ml conical flask containing 100 ml of the enrichment medium at 30°C for 48 h, with constant shaking at 160 rpm. Using a sterile saline solution, 1 ml bacterial solution in the enrichment medium was successively diluted from 10^{-1} to 10^{-6} . Approximately 0.1 ml of each diluent was plated on the preliminary screening medium plate and cultured at 30 °C for 3 d. Each sample was done with three replications. Single colonies with good growth and transparent circle were selected for further purification. These colonies were inoculated by a sterile toothpick on preliminary screening medium plates and incubated at 30 °C for 3 d. The diameter (d) of the colony and size of the transparent circle around each colony (D) was measured. The five largest strains on the specific value of D/d were selected for further fermentation screening. The inoculated seed medium was incubated at 30 °C for about 8 h, under constant shaking at 160 rpm. The inoculation volume of the seed broth was 2% when the concentration of bacteria in seed broth was 1.0 as determined by OD_{600} . The fermentation process was executed in 30 °C for 40 h under 160 rpm conditions. Then crude enzyme liquid was obtained by subjecting the fermentation broth to centrifugation, after which enzyme activity was measured. The strain with the highest enzyme activity was used in the next step.

Inductivity of chitosanase produced by strains

The strain was inoculated into the re-screening media A and B and cultivated for 40 h at 30 °C, with constant agitation at 160 rpm. The inductivity of the enzyme was confirmed based on its activity in different fermentation broths.

Identification of strain

The cellular morphologies of strains from the agar plate were observed under the scanning electron microscope (SEM, FEI Apreo HIVac, Czech). Pure culture preserved in a test tube was initially identified by the Biolog test (Biolog ML3420, USA) and by 16S-rDNA sequencing.

Chitosanase production

Chitosanase production was performed in 250 ml flasks containing 100 ml fermentation medium. According to 2% (v/v) seed inoculum, the broths were incubated at 30 °C, 160 rpm for 30 h in a rotary shaking incubator. After fermentation, the inoculum pellets were separated from the broth by subjecting to a centrifuge at 10000 g for 20 min and the supernatant was treated as the crude chitosanase for enzyme activity measurements.

Detection of chitosanase activity

Chitosanase activity was determined as the rate at which reducing sugar molecules were generated. Enzyme activity was detected by the dinitrosalicylic acid (DNS) method according to a previous study (30) with some modifications. The enzyme activity was calculated by measuring optical density (OD) at a wavelength of 540nm, which was related to the content of reducing sugar in the solution using D-glucosamine as standard(29). In this experiment, an enzyme activity unit (U) is defined as the amount of enzyme required to produce 1 μmol of glucosamine per minute, 1 $\mu\text{mol}/\text{min}$. The enzyme activity unit per milliliter of fermentation broth (or per milligram of enzyme protein) is U/ml (or U/mg).

ARTP mutagenesis

The bacteria in the logarithmic phase were collected by centrifuging the fermentation broth and washed twice with sterilized saline solution. Then bacterial suspensions of about 2×10^5 CFU/ml were obtained through diluting them with sterilized saline solution. The slide of ARTP was coated evenly by 10 μL of suspension and was placed in a hole on the rotary table in the ARTP system, then the mutagenic procedure was executed. The operation parameters in the mutagenic procedure were as follows: irradiation distance was 2 mm; gas flow rate was 10 L min^{-1} ; mutagenic power was 100 W; the bacterial strains were treated with 0 s, 10 s, 20 s, 30 s, 40 s, 50 s, 60 s, and 70 s, respectively. Three parallel slides were treated under each mutagenic time. In this experiment, the mortality rate of the bacteria was calculated through a colony count and the mortality curve was plotted. The treated time with a lethality rate of 90% for mutagenesis operation was selected as the operation time of mutagenesis experiments.

Screening of mutagenic strains

According to the lethality curve, the optimal irradiation time was selected for the experiment, and the slide coated mutant was shaken with hand for 1 min in the 5mL fermentation medium. Then the mutated bacterial solution was inoculated in two 100-well microwell plates with 200 μL in each well. The microwell plates were placed in the bioscreen system (OY Growth Curves Biocscreen C, Finland) and cultured for 48 hours. The micropores with high increase in OD_{600} were selected for measuring the activity of chitosanase. And the hole with larger value of enzyme activity / OD_{600} was obtained. The remaining bacterial liquids in the micropores with high enzyme activity were evenly spread on the plate of seed medium and cultured for 24 hours, then some single colonies were selected and preserved according to the shape and size of colonies on the plate, and then the selected strains were tested for

enzymatic activity verification again. The strains with high enzyme activity were obtained by culturing at 30 °C for 72 hours. The obtained strains were stored at 4 °C.

Optimization of culture conditions

The optimal temperature of fermentation was evaluated by producing chitosanase under different temperatures, including 28°C, 30°C, 32°C, 35°C and 37 °C with initial pH 6.0. The optimal initial pH of fermentation medium has been investigated by producing chitosanase in the presence of different pH at 5, 5.5, 6, 6.5, and 7, respectively, with 30°C. Chitosanase activities in fermentation broth were analyzed at the end of fermentation.

Declarations

Acknowledgments

The authors would like to thank Key Laboratory of Ministry of Education Industrial Fermentation Microbiology.

Funding

This work has been supported by the Foundation (No. ZXKF20180304) of the Tianjin Engineering Research Center of Microbial Metabolism and Fermentation Process Control.

Authors contributions

Competing interests

The authors declare that they have no competing interests.

Consent for publication

No applicable.

Ethics approval and consent to participate

This paper is in compliance with ethical standards.

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Figures

Figure 1

The forms of strain 01(a- cell form, b- colony from)

Figure 2

The lethality curve of ARTP mutagenesis

Figure 3

The effect of culture conditions on enzyme activity a-fermentation tempreature; b- the initial pH of medium