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*Original paper*

# **Isolation and characterization of a thermostable alkaline chitinase-producing *Aeromonas* strain and its potential in biodegradation of shrimp shell wastes**

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## **Abstract**

Chitinases are employed to the conversion of chitin and are produced by a wide range of bacteria. The objective of this study was to isolate chitinase-producing microorganisms with high chitinolytic activity. A thermostable alkaline chitinase producing isolate strain CQNU6-2 was obtained from soil samples and showed potential in biodegradation of shrimp shell wastes. The optimal culturing conditions of isolate CQNU6-2 is at 25°C and pH 7 for 24 h. The chitinase produced by strain CQNU6-2 exhibited maximum activity at pH 6.0 and 40°C and it could tolerate the treatment of high temperature (up to 80°C) and high pH (over 10). Taxonomic study, based on biochemical and morphological analysis and phylogenetic analysis of 16S rDNA, showed that strain CQNU6-2 belongs to the genus *Aeromonas* sp. The isolate can effectively hydrolyze colloidal chitin with degradation rate of 100% and also can directly degrade the shrimp shells. Ammonium sulfate precipitation method can be used to preliminary purify the chitinase. In conclusion, strain CQNU6-2 had a promising potential for biodegradation of chitin under harsh pH or temperature conditions and could be employed to the comprehensive utilization of shrimp shell wastes.

**Keywords** *Aeromonas* sp., chitinase, thermostable, alkali-tolerant, shrimp shell wastes, biodegradation.

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## Introduction

Chitin is a linear polysaccharide with  $\beta$ -1,4-N-acetylglucosamine (GlcNAc) as the basic unit. It is widely distributed in nature and known as a major structural component in the fungal cell walls, peritrophic membrane of insect gut, exoskeletons of insects, arthropods and crustaceans (ALI *et al*, 2020, UEDA and KUROSAWA, 2015). Chitin has become the second largest biomass resource (JHA *et al*, 2016) and is treated as the potential material for yielding chitosan oligosaccharides, chitin oligosaccharides and other chitin derivatives (GUAN *et al*, 2019). In many coastal areas, biological resources including shrimp and other crustaceans are very rich, but there are large amount of shrimp and crab shells were produced. Most produced shrimp and crab shells are discarded and piled up like mountains. Unfortunately, for chitin is insoluble substrates, most of the discarded shells became wastes which visibly wasted biological resources and polluted the environment (ALI *et al*, 2020, YAN and CHEN, 2015). Additionally, in the food industry and fishery, shrimp and crab shells have become the main components of produced waste (KRITHIKA and CHELLARAM, 2016). Degrading chitin into low molecular weight products is an important way to recycle the discarded chitin in the environments (RATHORE and GUPTA, 2015). Generally, physical, chemical or biological approaches can be used to decompose chitin and among which biological method, especially for the enzymatic method with chitinase, is specially focused for the advantages of good yield, environmental friendliness, mild reaction condition and high product uniformity (JUNG and PARK, 2014, LIU *et al*, 2019).

Chitinases are capable of catalyzing the hydrolysis of chitin. Enzymatic chitin degradation is essentially implemented by chitinases, which are found in a wide range of organisms as diverse as insects, fungi, crustaceans, plants and bacteria, and are involved in nutrition digestion, energy generation, morphogenesis, and defense against chitin-containing pathogens (UEDA and KUROSAWA, 2015, LE and YANG, 2018). Up to now, chitinolytic enzymes have been commercially employed to generate chitooligosaccharides and soluble oligomers of N-acetylglucosamine (GlcNAc) as part of pharmaceutical formulations. Purified chitinase has been applied to treat chitinous waste and control malaria transmission (KIM *et al*, 2017, DAHIYA *et al*, 2006). Notably, thermostable enzymes have found lots of commercial applications in various industries (DEMIRJIAN *et al*, 2001), such as the chemical industries (BAHRAMI *et al*, 2001), the starch industry (SARIKAYA *et al*, 2000) and the food related industry (HAKI and RAKSHIT, 2003). Thermostable and alkaliphilic chitinase had major advantages over industrial catalysis for their high-hydrolytic activity at high temperature and pH, which could be useful for the chitin industry and biotechnological applications (KUZU *et al*, 2012). Especially, their application in bioconversion of marine shellfish waste to various types of high value-added products, under alkaline and high temperature conditions, became one of the key research topics (AKTUGANOV *et al*, 2018).

Numerous reported bacteria, including *Bacillus*, *Citrobacter*, *Paenibacillus*, *Sarratia*, and *Streptomyces* genera, are well known to produce chitinases (ALIABADI *et al*, 2016). Screening novel microbes and analyzing the characterization of their produced enzymes become urgent for exploring diverse sources of these enzymes. Many species of chitin-degrading microbes have been screened from various environments, and their chitinases have been studied (UEDA and KUROSAWA, 2015, BHATTACHARYA *et al*, 2007). As the important degraders of chitin in soil, bacteria play an important role in the recycling of carbon and nitrogen resources in soil ecosystems and this make soil becomes the ideal resource bank for screening chitinase-producing organisms (HAN *et al*, 2014). Most chitin-degrading prokaryotes are including the gliding bacteria, pseudomonad, vibrio, and so on. Among which, the molecular weight of bacterial chitinases usually range of 20-60 kDa (ALIABADI *et al*, 2016, HAN *et al*, 2014). Some reported bacterial chitinases also had high molecular weight ranged from 20 to 120 kDa (ALI *et al*, 2020). In bacteria, the primary role of the chitinase is thought to be the digestion and utilization of chitin as a carbon and energy source (LE and YANG, 2018, HAN *et al*, 2014). Recently, chitinases have attracted attention for their diverse functions and widely applications in many fields, especially used to generate bioactive chitooligosaccharides and GlcNAc (LE and YANG, 2018). Thus, screening and studying the properties of different originated chitinases have become hot topics of many researches. In order to isolate chitinase-producing microorganisms with high chitinolytic activity, soil samples were collected from the site with long-term accumulation of rotten leaves in the campus of Chongqing Normal University. A chitinase-producing bacteria *Aeromonas sp.* strain CQNU6-2 was obtained and the production of chitinase was optimized. Additionally, enzymatic properties and its potential in biodegradation of shrimp shell waste were also investigated.

## Materials and Methods

### *Samples, culture, and isolation of chitinase-producing bacterial strains*

Putrefying soil samples were collected from the sites with long-term accumulation of rotten leaves in the campus of Chongqing Normal University, China. Chitinase-producing bacterial strains were isolated from these samples following classical method. Briefly, 2 g of the sample was resuspended before settled in 200 mL deionized water, and the upper suspension fluid was serially diluted, 200  $\mu$ L of 100 times dilution were subcultured on a chitinase isolation medium (CIM) consisting of 3 g tryptone, 0.7 g  $K_2HPO_4$ , 0.5 g  $MgSO_4 \cdot 7H_2O$ , 0.3 g  $KH_2PO_4$ , 0.02 g  $FeSO_4 \cdot 7H_2O$ , 0.01 g  $ZnSO_4 \cdot 7H_2O$ , 100 mL colloidal chitin, 15 g Agar powder in 1000 mL ultrapure water (pH 7.2-7.4). Cultures were incubated at 30°C under aerobic conditions for 2-5 days until clear colonies appeared on the plate. The strains with transparent circles around the colonies were selected and the size of the transparent circles were measured. The strain with the largest ratio of the diameter of the transparent circle to the diameter

of the colony was selected and then purified by plate streaking on the chitinase isolation medium (CIM).

### **Preparation of colloidal chitin**

Colloidal chitin was prepared by the optimized method refer to Hsu and Lockwood (1975). Briefly, 10 g chitin (Sigma-Aldrich, St. Louis, Mo., U.S.A.) were added into 100 mL 85% Phosphoric acid and left at 30°C for 36 h. Subsequently, distilled water was added to precipitate snowflake-like colloidal chitin. The precipitate was collected by centrifugation at 10000 g for 10 min at 4°C. The colloidal chitin was washed several times with sterile distilled water till pH 7.0. It was freeze-dried to powder and stored at 4°C. A total of 5% colloidal chitin was used for the chitinase activity experiments.

### **Determination of chitinase activity**

One unit of chitinase activity was defined as the amount of enzyme that released 1  $\mu$ mol of GlcNAc per minute at 40°C. The determination method is as follows: the fermentation broth is centrifuged at 10000 g for 10 min, and 1 mL fermentation supernatant plus 1 mL substrate reaction solution were added to 1 mL phosphate buffer (pH 6.0). The reaction was maintained at 40°C for 30 min. Then, 1 mL supernatant was obtained after centrifugation at 4000 g for 2 min and mixed with 2 mL 3, 5-dinitrosalicylic acid (DNS). Subsequently, the mixture was heated in boiling water bath for 10 min and then made up to 10 mL after cooling down. An absorbance at 540 nm exhibited the amount of released reducing sugar in the reaction mixture. The standard curve was generated from known concentrations of GlcNAc (0 to 1.6 mg). The enzyme activity was calculated according to the DNS method for the standard curve of GlcNAc.

### **Morphological and phenotypic characterizations**

The selected strain was streaked on the isolation plate and cultivated for 24 h at 30°C. Then, colony characteristics, including colony color, gloss, size, texture, shape, surface condition, pigment production status, etc, were recorded. For morphological characterization, a single colony was picking into the fermentation medium (FM) consisting of 3 g tryptone, 1.5 g yeast extract, 0.7 g  $K_2HPO_4$ , 0.5 g  $MgSO_4 \cdot 7H_2O$ , 0.3 g  $KH_2PO_4$ , 0.02 g  $FeSO_4 \cdot 7H_2O$ , 0.01 g  $ZnSO_4 \cdot 7H_2O$ , 100 mL colloidal chitin in 1000 mL ultrapure water (pH 7.2-7.4) and cultivated for 24 h at 30°C. Subsequently, the bacteria were collected by centrifugation at 12000 g for 2 min. The samples were washed and postfixed in 2.5% glutaraldehyde fixative solution for 5 h at room temperature. Following postfixation, the sample was centrifuged at 12000 g for 2 min and wash with PBS buffer (PH7.4) for three times. After 1% osmium acid was added and allowed to stand for 1 h, it was washed again with PBS buffer (PH7.4) for three times. The samples were dehydrated in a graded ethanol series (30%, 50%, 60%, 70%, 80%, 90%, and 100% with each change for 15 min), and subsequently treated with 50%, 75% and 100% tert-butanol with each change for 10 minutes. After drying, the grains were attached to Scanning Electron Microscope stubs using doublesided conductive tape and sputter coated with gold. The samples were examined using Hitachi S-3000N Scanning Electron Microscope

(Hitachi High-Technologies Corp., Japan) with an acceleration tension of 15 kV.

### **Taxonomic Study**

Biochemical and morphological analysis of the strain were performed following Bergey's Manual (BRENNER et al, 2005, MA et al, 2015). Total genomic DNA was extracted according to the method by Huber et al. (2002). The 16S rRNA gene was polymerase chain reaction (PCR)-amplified using the universal primers, 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') in an Eppendorf Mastercycler nexus gradient (Hamburg, Germany). The PCR mixture was consisted of 1.0  $\mu$ L of template DNA, 2.0  $\mu$ L of 2.5 mM dNTPs, 2.0  $\mu$ L of 2.5 mM  $MgCl_2$ , 2.5  $\mu$ L of 10 $\times$ PCR buffer ( $Mg^{2+}$  free), 1.0  $\mu$ L of each primer (10 mM), 0.25  $\mu$ L of rTaq DNA polymerase (2.5 U/ $\mu$ L) (TaKaRa, Dalian), in a final volume of 25  $\mu$ L. Samples were preheated for 10 min at 94°C and then amplified for 35 cycles at 94°C for 20 s, 55°C for 30 s, and 72°C for 80 s; 72°C for 10 min. Subsequent to PCR amplification, 2  $\mu$ L of each reaction was run on a 0.8% agarose gel, and the DNA was visualized by UV illumination at BIO-RAD Gel Doc 2000 (Hercules, California, USA) followed with ethidium bromide staining. Then, the 16S rDNA gene was cloned into pMD19-T vector. Nucleotide sequence of 16S rDNA was sequenced and identified through the online BLAST program. Multiple alignment was completed with the CLUSTAL W software and a neighbor-joining phylogenetic tree was reconstructed using the MEGA 7 with performing 1000 replicates and marked into branching points (RASTOGI et al, 2009, HAN et al, 2014, MA et al, 2015).

### **Effect of media type on strain growth and chitinase production**

Six different media were used to study their effect on the growth of strain and chitinase production. The basic medium (BM) was 3 g tryptone, 0.7 g  $K_2HPO_4$ , 0.5 g  $MgSO_4 \cdot 7H_2O$ , 0.3 g  $KH_2PO_4$ , 0.02 g  $FeSO_4 \cdot 7H_2O$ , 0.01 g  $ZnSO_4 \cdot 7H_2O$ , 15 g Agar powder in 1000 mL ultrapure water (pH 7.2-7.4). The tested media was basic medium containing 0.5% colloidal chitin (CBM), Glucose basic medium containing 0.5% Glucose (GBM), Shrimp basic medium containing 0.5% shrimp shell powder (SBM), Luria-Bertani (LB) broth and Shrimp Luria-Bertani medium (SLB) with 0.5% shrimp shell powder. The strain was then streaked to the above mediums and cultivated for 48 h at 30°C. In order to evaluate the growth of bacteria more clearly, the cells of CQNU6-2 were gradient diluted and then the same quantity bacterium was coated on the above mediums. Three repeated plates were performed for each cultural medium. The colony growth status and transparent circle were observed.

### **Optimization of culturing conditions for enzyme activity**

The bacterial cultures were inoculated into the fermentation medium (FM) with 2% inoculation volume. Under the cultural condition of 180 r/min in constant temperature incubator, the effects of different initial pH (5, 6, 7, 9 and 11), different culture temperature (20°C, 25°C, 30°C, 37°C and 42°C) and different culture time

(12 h, 24 h, 36 h and 48 h) on chitinase activity were explored. At the end of the growth period, chitinase activity assay was performed by the stated protocol above. All experiments were performed independently in triplicate.

#### **Effect of reaction conditions and stability for enzyme activity**

To investigate the effect of temperature on chitinase activity, a standard assay was performed at various temperatures (10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C). Thermal stability was determined by incubating the crude enzyme at various temperature (from 20-90°C) for 3 h under pH 7.0 and then measured the chitinase activity under standard assay conditions (KUZU *et al*, 2012). The effect of pH on chitinase activity were assessed over the range of pH 3-10 under standard assay conditions with different buffers as follows: citrate buffer (50 mM, pH 3, 4 and 5), phosphate buffer (50 mM, pH 6, 7, and 8), and borate buffer (50 mM, pH 9 and 10) (UEDA and KUROSAWA, 2015). For pH stability assay, the enzyme solution was pre-incubated in different buffers of pH range from 3-10 and then the residual activity was determined under standard conditions (GUO *et al*, 2017). Additionally, Effect of ions and additives on enzyme activity were explored by adding Na<sup>+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, Ba<sup>2+</sup>, Urea, EDTA at high concentration of 0.1 mM and low concentration of 0.01 mM. Chitinase activity was measured according to the standard procedure. The relative activity was calculated with the reaction, which performed in the absence of any additive, as the control. All experiments were repeated independently for three times.

#### **Growth curve and degradation experiment of colloidal chitin**

The cell growth was monitored at 600 nm using an UV-spectrophotometer (Lingguang 722S UV spectrophotometer, Lingguang Ltd, Shanghai, China) at different times from 3-36 h. The strain was cultured at 30°C in liquid chitinase isolation medium (LCIM) with 180 r/min in constant temperature incubator. In order to explore the degradation rate of colloidal chitin, six experimental groups are set up for different culture times (0 h, 8 h, 12 h, 24 h, 30 h and 48 h). The bacterial cultures were inoculated into the LCIM with 2% inoculation volume and cultivated at 30°C with 180 r/min in constant temperature shaking incubator. The fermentation broth was collected after fermentation, centrifuged at 3000 r/min for 2 min to collect and weigh the

undegraded chitin to calculate the degradation rate of chitin. All experiments were repeated independently for three times.

#### **Degradation experiment of Shrimp shells**

Shrimp shells were collect from the shrimp shell waste and were washed with deionized water. The shells were heated in boiling water bath for 5 min and soaked for 24 h. Subsequently, an electric thermostatic blast drying oven was employed to remove the water for 30 min. Then, 0.2 g shrimp shells were added into 30 mL liquid basic medium (LBM) consisted of 3 g tryptone, 0.7 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 g KH<sub>2</sub>PO<sub>4</sub>, 0.02 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g ZnSO<sub>4</sub>·7H<sub>2</sub>O in 1000 mL ultrapure water (pH 7.2-7.4). All experiments were performed independently in triplicate. To study the effect degradation rate, six experimental groups are set up for different culture times (1 d, 2 d, 3 d, 4 d, 5 d and 6 d). The bacterial cultures were inoculated into the LBM with 2% inoculation volume and cultivated at 30°C with 180 r/min in constant temperature shaking incubator. After incubating for corresponding period, the remaining shrimp shells in the triangular flask were took out and put into electric thermostatic blast drying oven for 30 min to remove the water. The weight of the sample was obtained and used for the calculation of degradation rate and construction of the shrimp shell degradation rate curve.

## **Results**

#### **Isolation of chitinase-producing bacterial strains**

A total of 6 morphologically different chitinolytic bacterial colonies were screened from 6 collected soil samples. On the basis of diameter of transparent circle (> 0.2 cm) on a chitinase isolation medium (CIM), 1 colonies named CQNU6-2 were selected for secondary screening and purification (Fig. 1A), because that the ratio of diameter of transparent circle to colony diameter reached 3.05. Three parallel assessments were conducted for purifying the colonies and the testing of enzyme activity (Fig. 1B). The selected strain CQNU6-2 exhibited the maximum chitinase activity on CIM with 0.5% (w/v) colloidal chitin, showing clear transparent circle around colonies. The strain CQNU-6-2 grew fast and the colonies were looser and milky white with smooth surface. The colonies were slightly protruding with neat edges. Scanning electron microscopic investigations revealed the morphology of the isolated strain (Fig. 1C). The results showed that the cells were rod-shaped with raised at both ends. Both ends of the cell are blunt and the size is (0.79 to 1.37 μm) × (0.44 to 0.49 μm).



**Figure 1.** Screening of and re-screening of chitinase producing strains (A), purification of CQNU6-2 (B) and Electron microscopic observation on the morphology of strain CQNU6-2 strain (C).

To test the chitinolytic activity, CQNU6-2 strain was cultured in LCIM medium and the cell-free supernatant was collected as crude enzyme for the activity assay. Results showed that the activity of chitinase was 0.415 U/mL. These activities were stronger than those of *Pseudomonas* (LIU et al, 2019) and *Bacillus licheniformis* (SLIMENE et al, 2015). Strain CQNU6-2 was stored at the Chongqing Key Laboratory of Animal Biology, Chongqing Normal University, Chongqing, China.

#### Identification of the selected bacterial isolate CQNU6-2

After Gram staining, the isolates CQNU6-2 appeared

under the light microscope as Gram-negative bacterium. The cells were rod-shaped (0.44-0.49 by 0.79-1.37  $\mu\text{m}$ ) and were facultative anaerobic bacteria. CQNU6-2 could utilize glucose and fructose as the carbon source. The contact enzymatic reaction exhibited a negative result. Other biochemical properties were listed in Table 1. All the properties were in accordance with the standard identification of *Aeromonas* in the Bergey's Manual of systematic Bacteriology (BUCHANAN and GIBBONS, 1975), the CQNU6-2 was classified as a bacteria belongs to the genus *Aeromonas*.

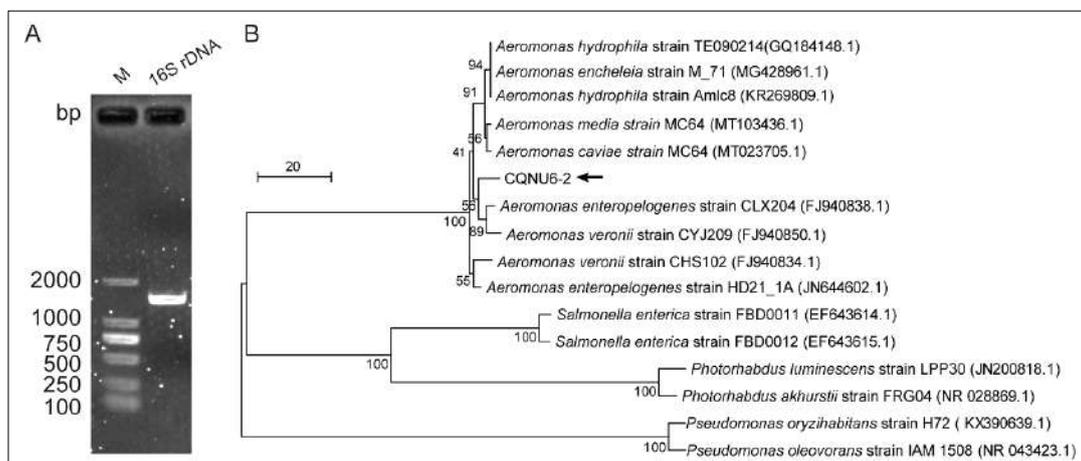
**Table 1.** Biochemical and physiological tests of strain CQNU6-2

Test items	Result	Test items	Result
Gram stain	-	Glycerol hydrolysis	+
Glucose	+	Sorbose	-
Sucrose	-	Xylose	-
Fructose	+	Contact enzyme reaction	-
Maltose	+	Acetyl methyl methanol (V.P.)	+
Trehalose	+	Esculin test	+
Starch hydrolysis	+	Methyl red	+

+ Positive, - Negative

PCR was used to obtain the 16S rDNA gene fragment (Fig. 2A) and partial 16S rDNA sequence of strain CQNU6-2 was determined (GenBank No. MT463495) and the BLAST results showed that its share of more than 99% identifies it with those of *Aeromonas* at nucleic acid level. Based on multiple alignments of all related 16S rDNA

sequences comprised of sixteen sequences derived from ten *Aeromonas* and six elements from other species, the NJ phylogenetic tree was constructed for further identification. Strain CQNU6-2 is the member of clade comprised of various *Aeromonas* strains (Fig. 2B). This result confirmed the strain as being *Aeromonas sp.*



**Figure 2.** Analysis of 16S rDNA fragment (A) and Phylogenetic analysis based on 16S rDNA sequences (B) showing the position of strains CQNU6-2. The tree was constructed using the neighbor-joining method. Numbers at nodes represent the bootstrap percentage (based on 1000 replicates). The GenBank accession number is provided following the species name.

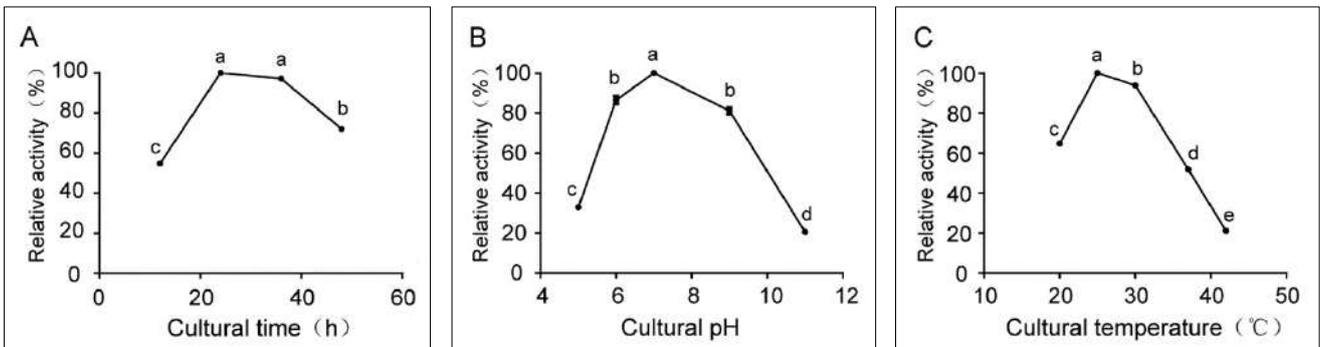
#### Optimization of culturing conditions of isolate CQNU6-2

To obtain an suitable cultural time for producing chitinase, four different cultural time (12 h, 24 h, 36 h and 48 h) were explored (Fig. 3A). It was observed that the enzyme activity was higher when the cultural time was 24 to 36 h. And the enzyme activity significantly decreased after 48 h of culture. Therefore, the fermentation time of CQNU6-2 strain should be controlled within 24-36 h.

Moreover, five different cultural pH (5, 6, 7, 9 and 11) has been selected to study the enzyme production by strain CQNU6-2 (Fig. 3B). The Maximum activity was showed in the pH 7 and > 50% of its maximum activity was observed in the pH range of 6 to 9. The chitinase can be stable and maintained under both neutral and alkaline culture conditions. Additionally, it was exhibited that the chitinase produced by strain CQNU6-2 had maximum

chitinase activity under the cultural temperature at 25°C, the chitinase activity declined markedly at temperature of 42°C, when only 21% of its initial activity retained (Fig. 3C). This result indicated that the chitinase can be

produced at a lower temperature than other bacterial isolates, such as *Salinivibrio* (LE and YANG, 2018), *Cohnella* (ALIABADI *et al*, 2016), *Paenibacillus* (HAN *et al*, 2014), and so on.



**Figure 3.** Effect of fermentation conditions on enzyme activity of CQNU6-2 strain. (A) Cultural time; (B) Cultural pH; (C) Cultural temperature.

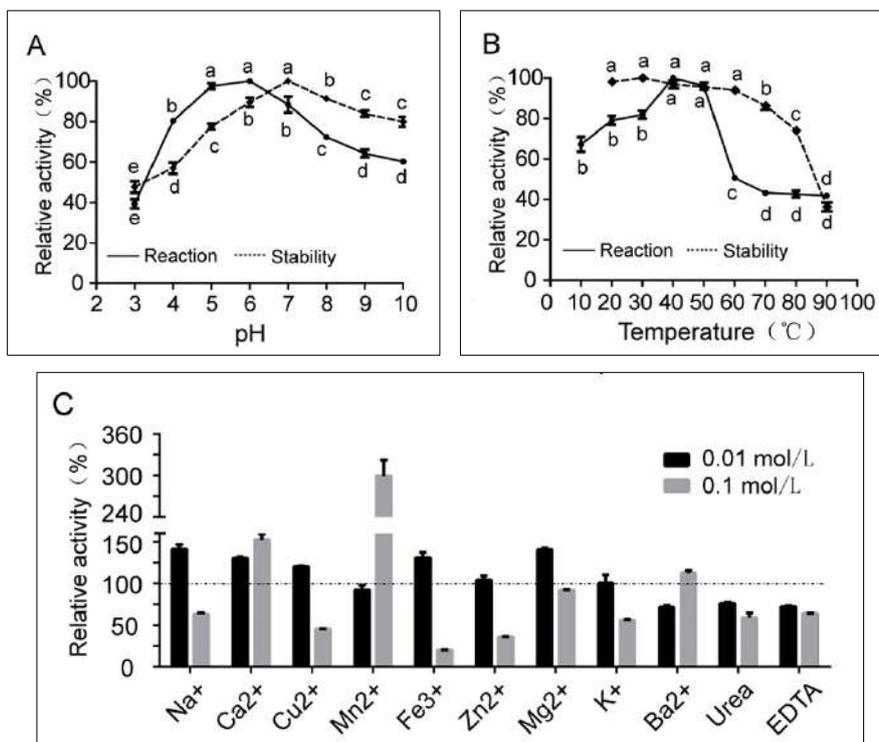
**Enzymatic characterization of chitinase produced by strain CQNU6-2**

After optimizing the cultural conditions of enzyme production, the highest enzyme activity of the crude enzyme of strain CQNU6-2 was 0.415 U/mL, which was 71.3% higher than the strain before optimization. The chitinase activity was as high as chitinases produced by other reported *Aeromonas* (GUO *et al*, 2004, AL-AHMADI *et al*, 2008).

In order to determine the optimum pH of the chitinase, the chitinase activity was measured under different reaction pH (from 3 to 10). The optimal reaction pH of this enzyme

is 6 and > 50% of its maximum activity was observed in the pH range from 4 to 10. It also showed 60.1% activity when the reaction pH at 10. With regard to pH stability, the enzyme was stable over a broad pH range from 3 to 10. The residual enzyme activity was 79.8% at pH 10. However, the activity was much lower at an acidic pH, in particular, approximately 39.3% of its activity was observed (Fig. 4A).

The optimum temperature for the chitinase were determined by varying the reaction temperature. Among all the tested temperature, the chitinase activity began at 10°C and achieved its maximum level at 40-50°C (Fig. 4B).



**Figure 4.** Effect of pH (A), temperature (B) and additives (C) on enzyme activity of CQNU6-2 strain. Chitinase activity was measured according to the standard procedure. The relative activity was calculated with the reaction, which performed in the absence of any additive, as the control.

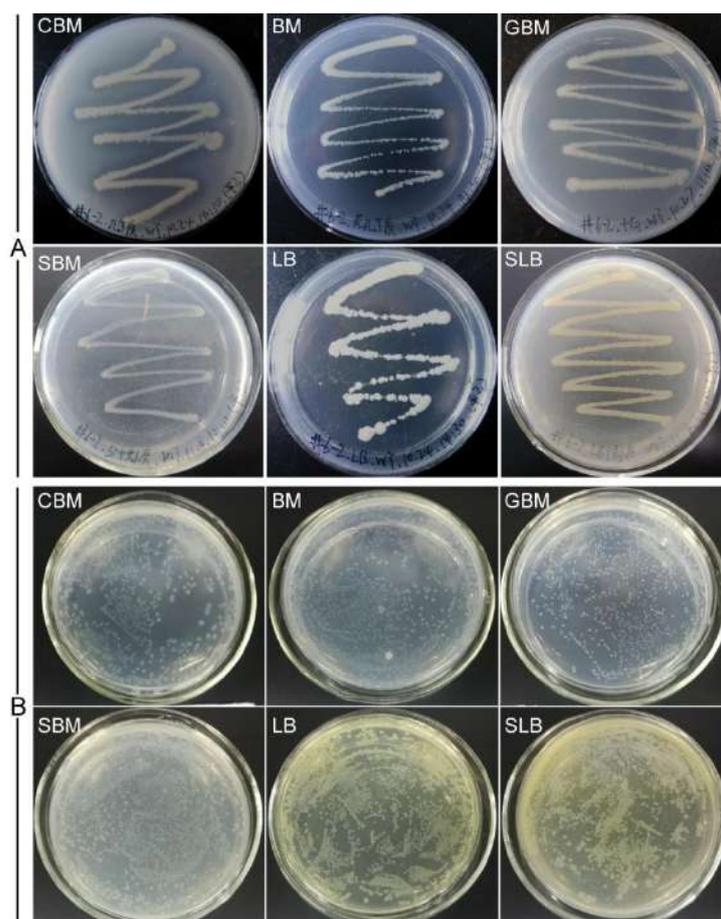
Strain CQNU6-2 also exhibited 67.2% of the optimized activity at low temperature of 10°C. Chitinase activity increased from 10 to 50°C and then declined at temperature of 60 to 90°C. Notably, the chitinase also could effectively hydrolyze chitin (> 40%) when the reaction temperature high to 90°C. It was stable at temperatures up to 80°C with 73.9% of the optimized enzyme activity. It showed a significantly decline when the temperature up to 90°C and the residual enzyme activity reduced to 36.2%. In contrast, most previously reported bacteria did not functionally work under the wide range of temperature and pH (STUMPF et al, 2019, DAS et al, 2019). The chitinase produced by the strain CQNU6-2 had a wide temperature range of catalytic activity and exhibited extremely strong heat-resistance, so it may have potential application under high-temperature production conditions.

The crude chitinase activity was analyzed under the optimum pH and temperature by adding various metal ions and chemical compounds (Fig. 4C). Metal ions such as  $\text{Na}^+$ ,  $\text{Fe}^{3+}$  and  $\text{Mg}^{2+}$  increased the enzyme activity up to 40% at 0.01 mM, but when the concentration reached 0.1 mM,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  inhibited the enzyme activity up to 50%.  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  could increase the activity at 0.01 mM up to 50%. Urea and EDTA always decreased the activity at both 0.01 mM and 0.1 mM. This effect of EDTA was

not identical to the previous study of *Cohnella* sp. (ALIABADI et al, 2016).

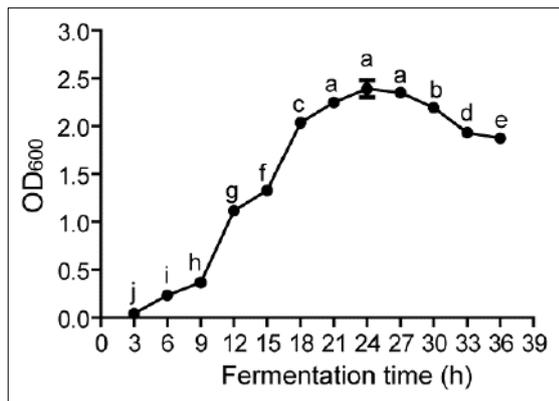
#### ***Analysis of chitin utilization and degradation rate of strain CQNU6-2***

In order to investigate the growth of strains on different nutrient types of media, the experiment of strain CQNU6-2 chitin utilization assay was shown in Fig. 5. The results suggested that CBM containing 0.5% colloidal chitin is most suitable for CQNU6-2 growth, showing the largest (Fig. 5B) and closely arranged colonies (Fig. 5A). Meanwhile, a clear transparent area could be observed around the colonies, which indicated the chitinase was secreted and hydrolyzed the colloidal chitin. CQNU 6-2 also could form the close colonies and weak chitin degradation area (Fig. 5A), which suggested the strains could have a better growth status and more colonies on SBM and SLB than BM and LB. Notably, the colonies on GBM with 0.5% glucose were more and larger than the medium without chitin (BM) (Fig. 5B). Although the results showed that strain CQNU6-2 could grow in the above 6 types of medium, it showed better status on the medium containing chitin, shrimp shell powder. Thus, colloidal chitin and shrimp shell powder could be suggested as the ideal source of nutrients for this strain.



**Figure 5.** Analysis of chitin utilization of strain CQNU6-2. The tested media was basic medium (BM), Chitin basic medium containing 0.5% colloidal chitin (CBM), Glucose basic medium containing 0.5% Glucose (GBM), Shrimp basic medium containing 0.5% shrimp shell powder (SBM), Luria-Bertani (LB) broth and Shrimp Luria-Bertani medium with 0.5% shrimp shell powder (SLB). The strain was then streaked (A) or coated (B) to the above media and cultivated for 48 h at 30°C.

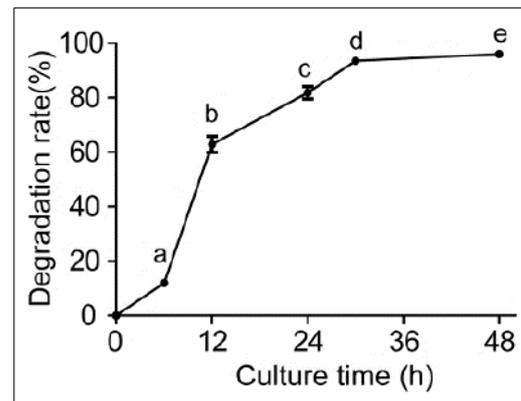
Since strain CQNU6-2 can grow well on plates containing colloidal chitin and shrimp shell powder, the degradation rate of colloidal chitin was further explored. Firstly, the growth situation of CQNU6-2 in a liquid medium containing colloidal chitin (LCIM) was observed, and the growth curve was drawn according to the OD<sub>600</sub> of the bacteria (Fig. 6). The result demonstrated that the cells, which cultured under at 30°C with shaking culture at 180 r/min, reached the maximum growth rate at 12 h post inoculation (hpi), and the cell concentration raised up to the maximum and entered the stationary phase at 21 hpi.



**Figure 6.** Determination of growth curve of strain CQNU6-2.

From 30 hpi, the cell concentration was gradually decreased and the cells entered the decline phase.

Subsequently, the strain CQNU6-2 was cultured at 30°C with 180 r/min rotation speed and the degradation rate of the substrate colloidal chitin at different culture times were analyzed (Fig. 7). The results showed that the chitin degradation rate of the strain reached 50% after 12 hours culture, up to 100% after 30 hours culture. It indicated that the strain has a strong degradation ability to colloidal chitin substrate.

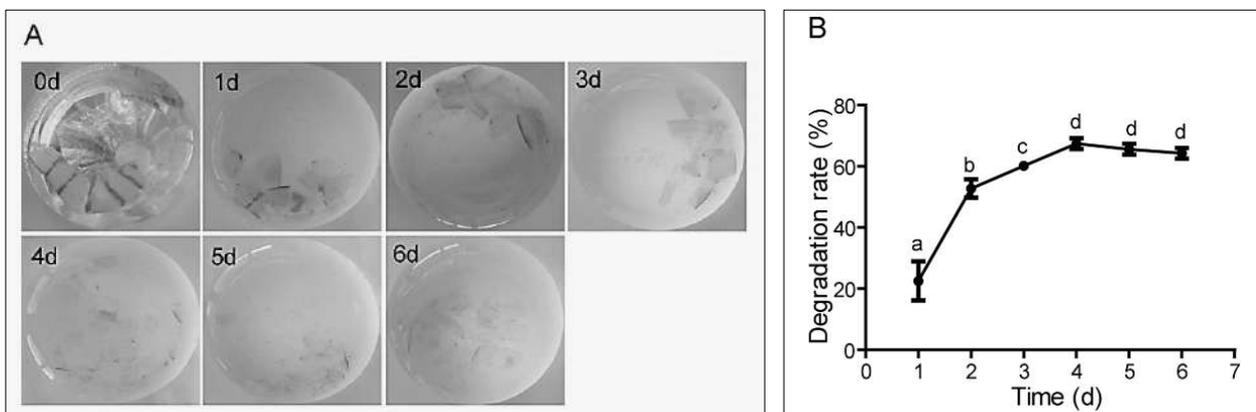


**Figure 7.** Analysis of colloidal chitin degradation curve of strain CQNU6-2.

**Degradation of shrimp shell waste of strain CQNU6-2**

Strain CQNU6-2 exhibited a high degradation rate of colloidal chitin and could express a well growth status on SBM and SLB with shrimp shell powder added. For further exploring its potential application in biodegradation of shrimp shells in food waste, the degradation rate of integral shrimp shells were carried out. According to the procedure mentioned above, shrimp shells were added to the liquid basic medium (LBM). After inoculation with CQNU6-2, fermentation culture was carried out at 30°C and 180 r/min. With the extension of the cultivation time, the concentration of bacteria in the erlenmeyer flask gradually increased

and after 1 day post inoculation (dpi), the shrimp shells were degraded from intact to shrimp shell fragments. From 2 dpi to 6 dpi, the shrimp shell fragments were gradually degraded into debris (Fig. 8A). Then, the degradation rate curve was drawn (Fig. 8B) and it showed that the degradation speed was up to the maximum after 2 days post inoculation. Until 4 dpi, the degradation rate reached the maximum, about 65%. From 4 dpi to 6 dpi, the degradation curve was flat and the quality of residual shrimp shells tended to be stable. The remaining ingredients may be other substance of the shrimp shells, such as proteins and glucans (KRITHIKA and CHELLARAM, 2016).

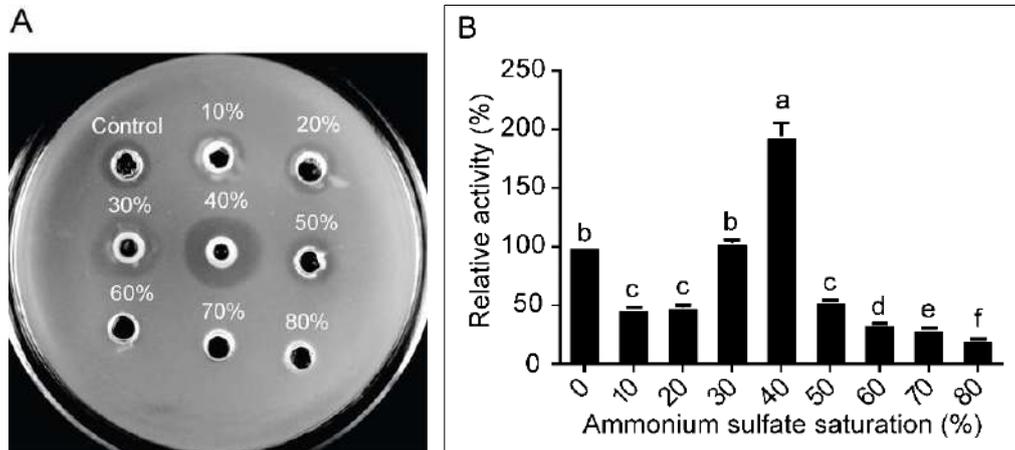


**Figure 8.** Shrimp shells degradation experiment (A) and analysis of shrimp shells degradation curve (B) of strain CQNU6-2.

### Preliminary isolation of crude chitinase by ammonium sulfate precipitation

In order to further isolate the chitinase from the fermentation supernatant of CQNU6-2, ammonium sulfate precipitation method was employed and the chitinase activity was determined by active plate detection and DNS

method (Fig. 9A). The result indicated that the optimal fractionation range of chitinase is 30%-50% of ammonium sulfate saturation. The activity of chitinase isolated reached to the maximum when the saturation of ammonium sulfate was 40% (Fig. 9B). This assay would lay a solid experimental foundation for the subsequent purification of the chitinase produced by strain CQNU6-2.



**Figure 9.** Ammonium sulfate precipitation of chitinase from strain CQNU6-2.

**A.** Enzyme activity detection of chitinase on screening plate;

**B.** Enzyme activity of chitinase measured by DNS method.

## Discussion

Chitin exists naturally and becomes the second most abundant carbohydrate polymer around the world. Biomass with high proportions of chitin, including shrimp, crab and krill, has already been widely used for generating active substances in agriculture, medicine and food industries (LE and YANG, 2019). However, degradation and application of chitin were so difficult that large amount of shrimp waste generated from shellfish industry. Meanwhile, the utilization of chitin resources became extremely essential for proper carbon-nitrogen balance (BHATTACHARYA et al, 2016). Comparing to physical and chemical methods, biological approaches can perform more advantages to decompose chitin, including good yield, environmental friendliness and so on (LIU et al, 2019). More and more studies focused on the screening and utilization of novel chitinase-producing organisms (STUMPF et al, 2019, KUMAR et al, 2017). In this study, a thermostable alkaline chitinase-producing strain was isolated from the oil samples collected from the site with long-term accumulation of rotten leaves and confirmed to be *Aeromonas* sp. CQNU6-2 based on the taxonomic study including biochemical and morphological analysis, and the phylogenetic analysis of 16S rDNA. Chitinase have been found in many organisms (LIU et al, 2019) and Chitinase genes have been cloned from diverse bacterial groups (HAN et al, 2014). Several species of *Aeromonas*, including *Aeromonas caviae* (CARDOZO et

al, 2017, CARDOZO et al, 2019), *Aeromonas hydrophila* (ZHANG et al, 2017, STUMPF et al, 2019) and *Aeromonas veronii* (KANG et al, 2016), were reported to produce chitinase and have great potential application in the bio-synthesis of pullulanase and chitinase, the bioconversion of  $\alpha$ -chitin into N-acetyl-glucosamine, the degradation of chitin within fungal mycelium.

The rapid and ecofriendly chitinase production by microorganisms shows great promise of possible applications in many industries. However, several deficiencies of chitinase, including critical reaction conditions, low enzyme activity and complex purification procedures of crude enzyme, hindered their application in industries (LE and YANG, 2019). Excellent applicability of chitinase with activity under wide reaction range of pH and temperature would perform high potential in process of bioconversion. The pH and temperature effect on the crude chitinase activity of *Aeromonas* sp. CQNU6-2 were determined and the enzyme was active at broad range of pH from 3 to 10 and the activity reach to the maximum at pH 6. The result suggested that this chitinase could exhibit high activity under more alkaline conditions and alkaline chitinase was reported to have a certain bacteriostatic effect *in vitro*. Thus, the reported chitinase has the potential to be developed as an inhibitor of pathogenic bacteria and used in the biological control of pests and diseases (AJAYI et al, 2016). Additionally, enzyme can hydrolyze chitin (> 40%) at reaction temperature of 10 to 90°C and 67.2% of the optimized activity at low temperature of 10°C was observed. Even the temperature

was high to 90°C, it had shown 41.9% retain activity. The analysis of stability of enzyme also showed the crude chitinase was able to tolerate wide range of pH (3-10) and temperature at 20 to 80°C. Therefore, CQNU6-2 chitinase could be named as a thermostable enzyme. A comparable feature has been known by *Cohnella* sp. (ALIABADI et al, 2016), *Paenibacillus thermoaerophilus* (UEDA and KUROSAWA, 2015), *Bacillus pumilus* (BHATTACHARYA et al, 2016), *Micrococcus* sp. (ANNAMALAI et al, 2010). The strain CQNU6-2 could be considered as the producer of alkalitolerant and thermostable chitinase, which have the possibility to the bioconversion of chitin-containing materials under harsh pH or temperature conditions.

Metals ions and chemical compounds can interfere the activity of chitinase of the strain CQNU6-2 at both 0.01 mM and 0.1 mM concentration. Under the low concentration, most of the metals ions could increase the enzyme activity, such as Na<sup>+</sup>, Ca<sup>2+</sup>, Fe<sup>3+</sup>, Cu<sup>2+</sup> and Mg<sup>2+</sup>. But when the concentration of Cu<sup>2+</sup> and Fe<sup>3+</sup> reached up to 0.1 mM, the enzyme activity was inhibited up to 50%. Whether under high concentration or low concentration, Urea and EDTA have played the same effect on chitinase. Other chitinases produced by *Micrococcus* sp. (ANNAMALAI et al, 2010), *Paenibacillus pasadenensis* (GUO et al, 2017) and *Bacillus pumilus* (BHATTACHARYA et al, 2016) were also inhibited by Fe<sup>3+</sup> and Cu<sup>2+</sup>. Chitinase from *Bacillus pumilus* (BHATTACHARYA et al, 2016) was activated by Ca<sup>2+</sup>. EDTA also interfered the chitinases from *Paenibacillus pasadenensis* (GUO et al, 2017) and *Paenibacillus elgii* (KIM et al, 2017), but acted as an activator to *Cohnella* sp. (ALIABADI et al, 2016).

Traditionally, shrimp shells and crab shells were used as the resources to generate commercialized chitin (DOAN et al, 2019). In addition, making effective use of these resources also can solve the problem of environmental pollution induced by shrimp shells waste. Colloidal chitin and shrimp shell powder were confirmed to be the ideal source of nutrients for strain CQNU6-2. And the strain can effectively hydrolyze colloid chitin and the degradation rate was up to 100% after 30 hours culture. Meanwhile, shrimp shells could be directly degraded by CQNU6-2 and the degradation rate reached the maximum of 65% after 4 days post inoculation. It indicated that this strain have the possibility to be developed to decompose the shrimp shells waste to bioactive substances and realize the environmental recycling of disposable chitin waste (ALI et al, 2020).

Chitinase was considered as one of the important enzymes applied in industrial and commercial fields (GANGWAR et al, 2016). Although the crude chitinolytic enzymes from bacterium could be commercially used to chitin degradation (ALI et al, 2020), high purity and high activity of the enzyme were urgently required. The purified enzyme had wider application space and chitinase purified from *Serratia marcescens* (WANG et al, 2014), *Salinivibrio* sp. (LE and YANG, 2018), *Aeromonas hydrophila* (STUMPF et al, 2019) were purified and suggested to have

potential application in biocontrol of aflatoxin, antifungal, producing chitobiose, degradation of chitin. The crude chitinase from strain CQNU6-2 were systematically studied here and was testified to have strong ability to decompose colloidal chitin and shrimp shells. Thus, it would be necessary to explore the purification of produced chitinase and the ammonium sulfate precipitation was employed to isolate chitinase from CQNU6-2. The results showed that the activity of chitinase were maximum when the saturation of ammonium sulfate was 40%. This would provide a solid foundation for subsequent enzyme purification. Streptactin Affinity Chromatography (STUMPF et al, 2019), DEAE-cellulose chromatography (ALI et al, 2020), ultrafiltration and chitin sorption (AKTUGANOV et al, 2018) would be used as the main purification method to obtain the purified chitinase from strain CQNU6-2.

## Conclusions

In conclusion, the *Aeromonas* sp. strain CQNU6-2 proved as a thermostable alkaline chitinase-producing organism. The characters of its highly stable enzyme system, which could be active under wide range of temperature and pH, suggested a potential application in the industries of biomass utilization or biocontrol. Meanwhile, colloidal chitin and shrimp shell powder could be used as carbon and nitrogen source for the strain growth and it can effectively hydrolyze colloidal chitin. Shrimp shells could be directly degraded by strain CQNU6-2, which performed its potential in biodegradation of shrimp shell waste to produce useful-bioactive substances.

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## Conflict of Interest

The authors have no conflict of interest to declare.

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