Environmental Microbiology

Thermostable chitinase from Cohnella sp. A01: isolation and product optimization

Nasrin Aliabadi, Saeed Aminzadeh*, Ali Asghar Karkhane, Kamahldin Hagbeen

National Institute of Genetic Engineering and Biotechnology, Department of Industrial and Environmental Biotechnology, Bioprocess Engineering Group, Tehran, Iran

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ABSTRACT

Twelve bacterial strains isolated from shrimp farming ponds were screened for their growth activity on chitin as the sole carbon source. The highly chitinolytic bacterial strain was detected by qualitative cup plate assay and tentatively identified to be Cohnella sp. A01 based on 16S rDNA sequencing and by matching the key morphological, physiological, and biochemical characteristics. The cultivation of Cohnella sp. A01 in the suitable liquid medium resulted in the production of high levels of enzyme. The colloidal chitin, peptone, and K2HPO4 represented the best carbon, nitrogen, and phosphorus sources, respectively. Enzyme production by Cohnella sp. A01 was optimized by the Taguchi method. Our results demonstrated that inoculation amount and temperature of incubation were the most significant factors influencing chitinase production. From the tested values, the best pH/temperature was obtained at pH 5 and 70 °C, with Km and Vmax values of chitinase to be 5.6 mg/mL and 0.87 μmol/min, respectively. Ag+, Co2+, iodoacetamide, and iodoacetic acid inhibited the enzyme activity, whereas Mn2+, Cu2+, Tween (20 and 80), Triton X-100, and EDTA increased the same. In addition, the study of the morphological alteration of chitin treated by enzyme by SEM revealed cracks and pores on the chitin surface, indicating a potential application of this enzyme in several industries.

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Introduction

Chitin, a β-1,4 polymer of N-acetyl-D-glucosamine (GlcNAc), which is widely distributed among fungi, crustaceans, molluscs, coelenterates, protozoan, and green algae, is the second-most abundant biopolymer found in nature after cellulose.1,2 Several million tons of chitin is synthesized and degraded each year in the biosphere.3 This natural resource is relatively easily accessible, e.g., from sources such as shrimp, crab, and krill, which are considered as waste; chitin accounts for 20–58% of the dry weight of these wastes.4 Chitinous wastes are also produced in large amounts in industries
such as seafood processing industry, which produces prawn waste (containing 23% chitin). These wastes may pose as an environmental threat on their accumulation and due to extremely slow decomposition. Therefore, organisms that produce chitin-degrading enzymes can be useful in bioremediation and waste management as well as help release nutrients and maintain the carbon, nitrogen, and other biogeochemical cycles in the environment.\(^7,8\) Chitinases (EC 3.2.1.14) are present in a wide range of organisms, including viruses, bacteria, fungi, insects, higher plants, and animals; these enzymes are capable of catalyzing the hydrolysis of chitin.\(^9\) Chitinase participates in a variety of functions, including defense, nutrient digestion, morphogenesis, and pathogenesis.\(^3\) Most chitin-degrading prokaryotes are the gliding bacteria, pseudomonad, vibrio, enterobacteria, actinomycete, bacilli, and clostridia.\(^10\) Bacterial chitinases have a size range of 20–60 kDa.\(^11,12\) Chitinases have potential applications in various areas of biotechnology, biomedicine, agriculture, and nutrition.\(^13,14\) Microorganisms adapt to the condition in which they have to live and survive. Thermophiles synthesize proteins that are thermostable and resist denaturation and proteolysis. Due to their ecological role and growing interests of their application in biotechnology, a large number of chitin-degrading bacteria have been isolated and their respective genes have been cloned and characterized. However, only few thermostable chitinases have been reported in microorganisms.\(^15,16\) The thermostable chitinolytic enzymes can hydrolyze their substrates at high temperatures and represent important advantages against their mesophilic counterparts, for example, chemical and thermal stability, decreased viscosity, increased solubility, and significantly reduced contamination risk.\(^16\) Therefore, researches have been focused on microorganisms capable of producing such enzymes that can tolerate extreme environmental conditions.

Several articles have been published on the classical method of medium optimization by changing one independent parameter while fixing the others fixed. This process can be extremely time consuming, expensive, and unmanageable when involving a large number of variables as well as it cannot describe the combined effect of all the factors involved. Several factors have been reported to influence enzyme production by bacteria. Optimizing all of these affecting factors by statistical experimental designs can address these limitations. The methods of Taguchi have been used extensively in experiment designing. However, the application of Taguchi method in biological science is scarce.\(^17\) The genus Paenibacillus was originally defined in 1993 by Ash et al. after an extensive comparative analysis of 16S rRNA gene sequences of approximately 50 species of the genus Bacillus.\(^19,21\) They were reported to possess inhibitory effect on bacteria or fungi owing to their cell wall-degrading enzymes.\(^22\) Cohnella is a member of the Paenibacillaceae family.\(^23\) Earlier, we had reported the Taguchi method of chitinase production optimization from Serratia marcescens B4A\(^24\) and polygalacturonase production from Macrophomina phaseolina.\(^24\) In this study, we attempted to isolate and characterize the thermostable chitinase from the novel thermophilic strain, Cohnella sp. A01. Moreover, with the objective of obtaining accurate data and economizing the use of time and materials, we decided to use the Taguchi method for the optimization of culture medium instead of using traditional method. Only limited studies have reported statistical optimization for the production of chitinase.\(^25\) The present report is an attempt to formulate a suitable production medium by using statistical optimization that can substantially enhance chitinase production by Cohnella sp. A01.

**Materials and methods**

**Materials**

Flake crab shell chitin, 3,5-dinitrosalicylic acid (DNS), N-acetyl-d-glucosamine, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, Mo. USA). Colloidal chitin was prepared by the modified method of Roberts and Selitrennikoff. Taq DNA polymerase, 1-kb DNA ladder, standard proteins for molecular weight determination, T4 DNA ligase, IPTG, and X-Gal were purchased from Fermentas (Burlington, Canada). DNA extraction kit was purchased from Metabion (Martinsried, Germany). The High-Pure PCR Purification Kit was sourced from Roche (Indianapolis, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) and were of the highest analytical grade available.

**Isolation of microorganisms**

Samples collected from shrimp farming wastewater located in Shoebdeh-Abadan (southwestern of Iran) and used for isolation studies in our laboratory. The climate in Abadan is arid. Summers are dry and hot with temperatures of >45 °C (average 55 °C); the soaring temperatures may advance to >65 °C.

**Screening of thermophilic chitinase-producing microorganism**

For the direct screening of chitinase activity from bacterial colonies, clear zone production was monitored at 60 °C. The isolated microorganisms were cultured on agar plates containing 0.5% colloidal chitin, 0.07% K$_2$HPO$_4$, 0.03% KH$_2$PO$_4$, 0.05% MgSO$_4$·7H$_2$O, 2% agar, 0.2% NH$_4$NO$_3$, 0.1% NaCl (w/v), and 0.1% trace elements (pH 7.8). The cultures were incubated for 3 days at 60 °C. Only one chitinolytic bacterial strain (detected by a colony producing a halo around itself) was transferred into fresh chitin containing nutrient broth medium and incubated at 60 °C, following which, the strain was preserved as cell suspensions in 10% glycerol at −80 °C.

**Culture and growth conditions**

For chitinase production, the strain selected from the primary screening was cultured in a preculture medium (trace element 0.1%, tryptone 1%, yeast extract 0.5%, NaCl 0.5%, agar 0.2%, peptone 0.03%, K$_2$HPO$_4$ 0.07%, KH$_2$PO$_4$ 0.03%, CaCl$_2$·2H$_2$O 0.013%, NH$_4$NO$_3$ 0.1%, glucose 0.2%, colloid chitin 0.5%, MgSO$_4$·7H$_2$O 0.05%) for 24 h at 60 °C on a shaker incubator (180 rpm). Then, 4 mL of the preculture (3.5 × 10$^8$ cells/mL) was added to 100 mL of the production medium (preculture medium without glucose). The resultant inoculated medium...
was cultured at 60 °C for 6 days on a rotary shaker (180 rpm). The sample (4 mL) of the culture medium was removed every 24 h and centrifuged at 12,000 × g for 15 min at 4 °C. The resulting supernatant was then collected and used for subsequent chitinase activity assays.

**PCR amplification and study of 16S rRNA gene**

A partial DNA sequence for the 16S rRNA gene (ca. 1.4-kbp fragment) was amplified by PCR with the primers f16s: AGAAGGAGGTATCCAGGCGC and r16s: TCTTTGAGACTTTGATCCT. Isolation of genomic DNA and PCR amplification were conducted as suggested by. The amplification was performed by using the Techne® TC-512 Gradient Thermal Cycler (UK) with the following cycling parameters: 94 °C for 1 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 51 °C, and 1.5 min at 72 °C, with a final extension of 5 min. The PCR product was purified by using the High Pure PCR Purification Kit (Roche, Germany). The purified PCR fragment was then ligated into the pTZ75R (Fermentas, Canada) by the T/A cloning procedure, and the construct was transformed into Escherichia coli DH5α. The amplified construct harboring the 16S rDNA was then extracted, purified, and sequenced by SeqLab (Germany) with appropriate primers. The nucleotide sequences were analyzed by using the DNASIS program (version 3.2; Hitachi Software Engineering Co. Ltd.); the obtained sequences were compiled and compared with the sequences in the databases (http://www.ncbi.nlm.nih.gov) by using the BLAST program. Other morphological and physiological characteristics for the selected strain were analyzed according to the Bergey’s Manual of Systematic Bacteriology.

**Selection of carbon, nitrogen, and phosphorus sources**

For the selection of the best source of carbon, nitrogen, and phosphorus for chitinase production, several carbon, nitrogen, and phosphorus sources were used in the production liquid medium. To select the carbon sources in the production medium without chitin, 1% (w/v) glucose, galactose, mannose, fructose, arabinose (as monosaccharide); lactose, sucrose, maltose (as disaccharide); and starch, polygalacturonic acid, cellulose, chitin plus glucose, and chitin powder (as polysaccharide) were added to each basic medium separately. To select the nitrogen sources, 1% (w/v) peptone, NH₄HCO₃, (NH₄)₂SO₄, NH₄NO₃, NH₄Cl, Ca(NO₃)₂, 4H₂O, CaH₂NO₃, (NH₄)₂HPO₄, and triptone NH₄H₂PO₄ were added to the basic medium with optional carbon source. Similarly, 1% (w/v) Na₂HPO₄, NaH₂PO₄, K₂HPO₄, KH₂PO₄, and NH₄H₂PO₄ were added to the medium to determine the optimum phosphorus sources. The carbon:nitrogen (C:N) ratio are the same in all the media.

**Experimental design and analysis**

We first determined the various factors to be optimized in the culture medium that can have critical effect on the chitinase yield. Factors and their related levels were selected based on the consensus among the design engineers, scientists, and technicians with relevant experience involved in this experiment. Based on the obtained experimental data, eight factors were considered to significantly influence the chitinase yield. Table 1 displays the eight control factors and their levels employed in the Taguchi’s robust experimental design. A standard orthogonal array L27 was used to examine this eight-factor (six chemical parameters, two physical parameters) system. A run involved the corresponding combination of levels to which the factors in the experiment were set. Thus, the Taguchi’s method can calculate the condition of least variability from the SN ratios and the condition of the best reaction performed by maximizing the overall desirability.

**Extraction and partial purification of chitinase**

All experimental steps were conducted at 4 °C in a cold room. The resulting supernatant (100 mL) of the production medium was fractionated by 20–80% solid ammonium sulfate precipitation and then centrifuged at 12,000 × g for 15 min at 4 °C; the precipitated proteins were suspended in 20 mM phosphate buffer (pH 7.8) and dialyzed against the same buffer for 24 h at 4 °C.

**Enzymatic activity**

The chitinase activity was determined by measuring the reducing end-group N-acetylglucosamine (GlcNAc) (as a final product) produced from colloidal chitin as a substrate. Colloidal chitin was prepared from chitin, as described previously. The standard reaction mixture containing 0.1 mL of 1% (w/v) colloidal chitin and 0.1 mL of the partially purified enzyme was incubated at 60 °C for 60 min. The reaction was stopped by the addition of 0.2 mL of DNS, followed by heating at 100 °C for 5 min. Then, the samples were cooled to room temperature and centrifuged at 6000 × g for 15 min. The concentration of the reducing sugar was determined by the modified DNS method. The absorption of the test sample was measured at 540 nm by a UV spectrophotometer (Beckman DU530, USA) along with reaction blanks. One unit (U) of the chitinase activity was defined as the amount of enzyme required to liberate 1 mmol of the reducing sugar per minute under the described conditions.

**Effect of temperature and pH on the chitinase activity and stability**

The best pH value of partially purified chitinase was determined by using 50-mM mixed buffer (glycine, NaHCO₃, NaCl, MgCl₂, and KCl) and incubating at 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C, respectively, for 30 min at pH 4.5 to 9.5. The enzyme activity was determined by measuring the reducing end-group N-acetylglucosamine (GlcNAc) produced from colloidal chitin as a substrate. The enzyme solution (0.1 mL) was incubated at 4 °C with an equal volume of substrate solution (1 mL, 0.05 g/mL prepared in distilled water) in a reaction mixture containing 0.1 M MgCl₂ and 0.05 M KCl. The reaction was stopped by the addition of 0.2 mL of DNS, followed by heating for 5 min at 100 °C. The concentration of the reducing sugar was determined by the modified DNS method.

<table>
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<tr>
<th>Serial number</th>
<th>Factors</th>
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<th>Level 2</th>
<th>Level 3</th>
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<td>65</td>
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<td>7.5</td>
<td>8.5</td>
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<tr>
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<td>NaCl (w/v)</td>
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<td>0.07</td>
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<td>Chitin (w/v)</td>
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<td>0.5</td>
<td>1.5</td>
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<td>7</td>
<td>Trace element (w/v)</td>
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<td>0.2</td>
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<tr>
<td>8</td>
<td>Inoculation (w/v)</td>
<td>2</td>
<td>4</td>
<td>8</td>
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</tbody>
</table>

NaH2PO4 (pHs 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0) by performing the standard assay. The best temperature value of the chitinase activity was tested at 10, 20, 30, 40, 50, 60, 70, 80, and 90 °C in 50-mM phosphate buffer (pH 7.8). After that, the residual activity of the chitinase was measured by using the standard assay.

To determine the temperature stability, chitinase was initially preincubated at different temperatures (10–90 °C). Every 10 and 20 min (up to 90 min), the samples were placed on an ice bath for 30 min and then the colloidal chitin was added and the standard enzyme assay was performed at 60 °C. To obtain the pH stability, the enzyme was added to buffers with different pHs (3–12) for 90 min at 25 °C, followed by the standard enzyme assay at the best pH value.

**Effect of metal ions and detergents on chitinase activity**

To study the detrimental effects of metal ions and detergents, the chitinase activity was determined by the standard assay method in the presence of Ag⁺, Al³⁺, Ba²⁺, Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Fe³⁺, K⁺, Ni²⁺, Mg²⁺, Mn²⁺ (1, 5, 10, and 15 mM) and ethylenediaminetetra acetic acid (EDTA) iodoacetamide, iodoacetic acid, Tween 20, Tween 80, Triton X-100, and SDS at 0.1% (w/v). The relative activity was calculated with respect to the control, where the reaction was performed in the absence of any additive.

**Michaelis and rate constant determination**

The Michaelis constant (Kₘ) and the maximum velocity (Vₘₐₓ) of the enzyme (U/mL) were evaluated by using a substrate concentration of 0–10 mg/mL. The reaction mixture was incubated at 60 °C for 1 h. Analysis of the Michaelis–Menten curve was conducted by using the GraphPad Prism 6 software to determine the enzyme’s Kₘ and Vₘₐₓ.

**Scanning electron microscopy (SEM)**

In order to observe the morphological features of the microorganisms and the effect of chitinase on the morphological changes of chitin, the bacteria and chitin in the absence and presence of enzyme were provided and photographed under an SEM (LEO, 1455 VP; Germany) as described in previous articles. 3,33,34

**Statistical analyses**

All data are presented as means ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed by using the Student’s t-test. p < 0.05 was considered as statistically significant.

**Software**

Qualitek-4 software (Nutek Inc., MI) was used for automatic designing of experiments by using the Taguchi approach. Qualitek-4 software is provided to use L-4 to L-64 arrays along with a selection of 2–63 factors with two, three, and four levels to each factor. The automatic layout option permits Qualitek-4 to select the array used and to assign factors to suitable columns.

GraphPad Prism 6 (San Diego, CA, USA) was used to determine the enzyme’s Michaelis–Menten kinetic parameters, Kₘ (substrate concentration that yields a half-maximal velocity), and Vₘₐₓ (maximum velocity) values by nonlinear regression analysis.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Morphological, physiological and biochemical characteristic of Cohnella sp. A01.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characters</td>
<td>Results</td>
</tr>
<tr>
<td>Form</td>
<td>Rod</td>
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<tr>
<td>Gram stain</td>
<td>Positive</td>
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<tr>
<td>Spore</td>
<td>Spore central and oval</td>
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<tr>
<td>Motility</td>
<td>Positive</td>
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<tr>
<td>Catalase</td>
<td>Positive</td>
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<tr>
<td>Oxidase</td>
<td>Negative</td>
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<tr>
<td>Utilization of</td>
<td></td>
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<td>Glucose, maltose</td>
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<tr>
<td>Arabinose, xylose, mannitol, lactose, fructose</td>
<td>Negative</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
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<tr>
<td>Hydrolysis of gelatin</td>
<td>Negative</td>
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<tr>
<td>Indol formation</td>
<td>Negative</td>
</tr>
<tr>
<td>Growth on NaCl</td>
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<tr>
<td>Growth temperature</td>
<td>40–60 °C</td>
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</tbody>
</table>

**Results**

**Isolation and identification of chitinolytic microorganisms**

We isolated 12 strains from the water and waste water of Abadan shrimp farming ponds in southwestern Iran. In the initial screening, qualitative plate assay was performed for chitinase production, indicating one isolate as the most active one. 3 Biochemical and microbiological analyses were performed to characterize the screened strain (Table 2). In accordance with the Bergey’s Manual of Systematic Bacteriology, the A01 strain was classified as a bacteria belonging to the genus Cohnella. Subsequent sequence analysis of the 16S rDNA gene confirmed the isolate as being Cohnella sp.

**Nucleotide sequence accession number**

The nucleotide sequence of 16S rDNA from Cohnella sp. A01 would be made available in the GenBank nucleotide sequence databases (accession number: JN208862).

**Effects of incubation time, carbon, nitrogen, and phosphorus sources on chitinase production**

Chitinase was produced in the media containing 0.5% of colloidal chitin. During the early period of the incubation, minimal enzyme activity was detected in the culture; however, after 72 h of incubation, the enzyme activity was significantly increased (Fig. 1A). The growth of Cohnella sp. and the production of enzyme by this bacterium were studied by using different carbon...
sources. Chitinase production was induced by chitin and inhibited in the presence of easily metabolized monosaccharides, such as glucose, galactose, mannose, arabinose, and fructose (Fig. 1B). These compounds inhibited enzyme biosynthesis and presented with low chitinase activities when these monosaccharides were used as carbon sources. Lactose, sucrose, and maltose detracted chitinase production (approximately 70–80%). Polygalacturonic acid, starch, and cellulose decreased enzyme production by approximately 50–60%; nevertheless, chitin gave the highest enzyme activity (100%). The medium containing NH₄NO₃ (Fig. 1C) and KH₂PO₄ (Fig. 1D) showed the highest enzyme activity as compared to the others.

**Results of Taguchi design analysis**

We used different media with different inducers and under different conditions to analyze the possible fractions of chitinases synthesized by Cohnella sp. The main effects of the eight control factors at their three levels on crude enzyme biosynthesis are shown in Fig. 2. The contribution of each factor is presented in Table 3. According to these data, inoculation amount and temperature were the most significant factors influencing the production of chitinase in addition to trace elements and NaCl constitution.

**Effect of temperature and pH on enzyme activity and stability**

The effect of temperature on the crude enzyme activity was studied at several temperatures. The best temperature value was obtained at 70 °C (Fig. 3A) and the best pH value at 5 (Fig. 3B). The temperature stability in 10- and 20-min incubation of enzyme in different temperature conditions showed that the enzyme possessed >50% activity until at 80 °C temperature (Fig. 3C). The enzyme displayed activity over a relatively wide range of pH (4.0–11.0), i.e., >75% (Fig. 3D).

**Effect of metal ions, chelators, and some chemicals on chitinase activity**

The crude chitinase activity was measured at the optimum pH and temperature in the presence of various metal ions and chemical compounds. Metal ions such as Ag⁺ and Co²⁺ inhibited the enzyme activity up to 40% at 15 mM concentration. On the other hand, Mn²⁺ and Cu²⁺ increased the enzyme activity up to 25% at 5 mM concentration (Table 4). Iodoacetamide and idoacetic acid (1% w/v) inhibited the enzyme activity by approximately 55%. Tweens (20 and 80) and Triton X-100 slightly increased the enzyme activity (110, 115, and
Table 3 – The analysis of variance (ANOVA) table.

<table>
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<tr>
<th>Serial number</th>
<th>Factors</th>
<th>Degree of freedom (DOF)</th>
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<th>F-ratio</th>
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Table 4 – Effect of some metal ions on the enzyme activity.

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<th>Residual activity at 15 mM</th>
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<th>Residual activity at 5 mM</th>
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<td>ZnSO4</td>
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</table>

Fig. 2 – The main effect plot (data means) for means on chitin production. For more details, please refer to the “Materials and methods” section.

108%, respectively, while EDTA stimulated the activity up to 120% (Table 5).

**Determination of Michaelis and rate constant**

The Michaelis constant (Km) and the maximum rate (Vmax) of the enzyme (U/mL) were assayed at chitin concentration of 0.0–10 mg/mL. Analysis of the Michaelis–Menten curve was accomplished with GraphPad Prism 6. In enzyme kinetics, Vmax illustrated the maximum velocity received by the system at saturating substrate concentrations. The substrate concentration at which the reaction velocity was half of Vmax value represented the Michaelis–Menten constant (Km). The Km and Vmax values of the enzyme were 5.7 mg mL⁻¹ and 0.87 units (μmol min⁻¹) in the presence of colloidal chitin, respectively (Fig. 4).

Table 5 – Effect of some chemical compounds and chelators on the enzyme activity.

<table>
<thead>
<tr>
<th>Chemical compounds and chelators (%)</th>
<th>Enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Citric acid</td>
<td>96.9</td>
</tr>
<tr>
<td>EDTA</td>
<td>120</td>
</tr>
<tr>
<td>SDS</td>
<td>87.4</td>
</tr>
<tr>
<td>PMSF</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>45.7</td>
</tr>
<tr>
<td>Iodoacetate acid</td>
<td>45.5</td>
</tr>
<tr>
<td>Tween 20</td>
<td>110</td>
</tr>
<tr>
<td>Tween 80</td>
<td>115</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>108</td>
</tr>
</tbody>
</table>
The SEM image illustrated in Fig. 5A shows Cohnella sp. A01 cells as long, straight, rod-shaped bacilli. To investigate the morphological changes in chitin after chitinase treatment, we performed SEM trials. In the absence of enzyme, chitin illustrated more or less a smooth outside layer (Fig. 5B). After chitinase treatment (Fig. 5C and D), the SEM image revealed cracks and several small holes on the chitin surface, with the amount of pores remarkably increasing with the period of exposure (incubation).

**Discussion**

The increase of enzyme products often depends on screening a large number of microorganisms for an enzyme activity. In this study, a chitinolytic strain was isolated from the shrimp farming wastewater located in Choebdeh-Abadan and confirmed to be Cohnella sp. A01 based on its morphology, physiological and biochemical tests, and the 16S rRNA sequence analysis reports. The 16S rRNA gene sequences are largely used for the identification of prokaryotes.

This paper present product optimization of chitinase by Cohnella A01 for the first time. Other studies have reported similar studies on Pseudomonas sp. TKU015, Aeromonas schuberti, and Bacillus sp. 13.26. In the A. hydrophila HS4, maximum chitinase activity was detected to occur after 24 h of incubation, and remained invariant up to 48 h, while A. punctata HS6 produced maximum enzyme after 48 h of incubation. The primary reasons for the reduction in chitinase production may be due to the lack of nutrients in the culture medium or the production of toxic chemicals in the medium, eventuating in the inactivation of secretary machinery of the enzymes or breakup of enzyme by proteases.

In the first stage of the medium ingredients optimization for maximum enzyme production by Cohnella sp. A01, the optimum carbon, nitrogen, and phosphorus sources were selected by the one factor-at-a-time procedure. The study on
the effect of carbon sources showed that monosaccharides inhibited chitinase biosynthesis and its synthesis in small levels with low activity. Inhibition of the enzyme efficiency in the presence of simple sugars may be due to catabolite repression. These outcomes are in agreement with that for chitinase production by *Streptomyces viridiflancus* and *Trichoderma harzianum*, respectively. The results of the present survey demonstrates that the addition of monosaccharides to the culture medium reduces the chitinase activity of *Cohnella* sp. A01, which was similar to chitinase production by *Streptomyces* sp. and *Serratia marcescens* B4A.

The efficacy of nitrogen sources on enzyme production demonstrated that NH₄NO₃ presumably is the most optimal nitrogen source for enzyme production. It may be due to ammonium (in ammonium nitrate) is the inorganic nitrogenous form of simpler assimilation and also because of lower energetic costs of metabolism.

K₂HPO₄ was recognized as the best phosphorus source for the enzyme production by *Cohnella* sp. A01, in concordance with that reported for chitinase biosynthesis by *Paenibacillus* sp. D1 and *Vibrio alginolyticus* JN863235.

Cultural conditions are significant factors that affect the product efficiency and cell growth. In this research, the effects of various parameters, including incubation temperature, initial pH, NaCl, K₂HPO₄, NH₄NO₃, chitin levels, trace elements, and inoculation percentage on the efficiency of chitinase production were evaluated and optimized by the Taguchi L27 array method. The data obtained from this method suggested that the temperature and NaCl as well as inoculation amount were more effective than others on the enzyme production. The temperature of incubation and inoculation percentage were the most important factors influencing the change in the production level (Fig. 2).

The temperature and pH of enzyme are usually distinctive parameters that determine whether an enzyme is appropriate for biotechnological applications. The chitinase of *Cohnella* sp. A01 was active at 80 °C after 20 min of temperature treatment and showed the best activity at 70 °C (Fig. 3A and C). Therefore, *Cohnella* sp. A01 chitinase can be called as a thermostable enzyme. A comparable thermostability has been reported for alkaline chitinase by *Bacillus thuringiensis* subsp. *Kurstaki* strain HBK-51, *Bacillus thuringiensis* Mexican isolates, *Brevibacillus formosus* BISR-1, and *Rhodothermus marinus*. The highest enzyme activity was found at pH 5 (Fig. 3B), although activity was detected over a wide pH range of 4–11 (Fig. 3D). A comparable pH optimum has been reported for Bbchit1 of *Beauveria bassiana* NCIM 1216. This enzyme has it is the best pH in the acidic range and can possibly be used for the control of plant fungal pathogens.

The studied enzyme did not show requirement of any specific metal ions for its activity, although Mn²⁺ and Cu²⁺ had a stimulatory effect on chitinase activity. Idoacetate acid and

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**Fig. 5** – Scanning electronic microscopy photographs of *Cohnella* A01 (A); untreated chitin (B); treated chitin after 1 h (C); and 2 h (D). For more details, please refer to the “Materials and methods” section.
iodoacetamide inhibited the enzyme activity at 1 mM concentration by approximately 55%, indicating that several cysteine residues form a part of the catalytic site of chitinase. Twenos 20, 80, and Triton X-100 stimulated the chitinase activity by up to 15%. As the surface-active reagents may have enhanced the turnover number of enzyme due to the increased contact frequency between the substrate and active site of the enzyme with disrupting surface tension of the aqueous medium.\(^{33,43}\)

In the test of substrate concentration against chitinase activity, the activity of enzyme was found to increase with increase in the substrate concentration to about 7 mg/mL of colloidal chitin, but no effect with further increase. The enzyme showed a \(K_m\) value of 5.6 mg/mL against colloidal chitin, which is relatively lower than some other reported \(K_m\) values, such as 12 mg/mL for chitinase from Bacillus sp. BG-11\(^{59}\) and 8.3 mg/mL for chitinase from Serratia marcescens B4A\(^{35}\), the chitinase of Cohnella sp. A01 revealed a high affinity for colloidal chitin and possibly greater potential in the industry.

Study of the morphological alterations of chitin treated by enzyme via SEM experiments exhibited cracks and pores in the substrate surface. The same morphological changes have been reported for Serratia marcescens B4A chitinase\(^{55}\) and chitinase from penicillium sp. LYGO704.\(^{54}\) This direct evidence implies that the isolated chitinase was quite effective on chitin degradation.

**Conclusions**

The isolation study revealed that shrimp farming ponds are an appropriate environment for screening of chitinolytic enzyme-producing bacteria. Our study affirmed the efficiency of the Taguchi experimental design for specifying the most appropriate medium components to obtain maximum production of enzyme by Cohnella sp. A01. This work revealed that the production of chitinase is feasible from a newly isolated Cohnella sp. A01. Chitinase produced from Cohnella sp. A01 is active over a wide range of temperature and pH, with good tolerance to high temperatures. The growth of enzyme products mostly relies on screening of extensive number of organisms for their enzyme activity with a specific set of biochemical characteristics that suits the targeted population. Thus, merging enzyme screening with protein engineering, directed evolution, and metagenome studies, novel enzymes with improved efficiency under specific applications and conditions can be built.

**Conflicts of interest**

The authors declare no conflicts of interest.

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