

Isolation and Optimization of Cultivation Conditions for Production of Chitinase by *Aeromonas* sp. ZD_05 from the Persian Gulf

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A newly chitinolytic *Aeromonas* strain, designated as ZD_05, was screened from marine environment of Bushehr, Persian Gulf. Optimization of cultural conditions for production of chitinase was conducted by keeping all of parameters constant except the one which was studied. Two hundred bacterial strains were isolated from different samples collected from Persian Gulf in bushehr, Iran and screened for production of chitinase enzyme. One isolate designated ZD_05 was identified as *Aeromonas* sp. based on morphological and biochemical characteristics along with 16s rRNA partial sequence analysis. This strain showed highest chitinolytic activity in media containing chitin as sole carbon source. The production of chitinase by this organism was optimized using different substrate concentrations, pH, temperature, incubation period and nitrogen sources. The maximum enzyme production by *Aeromonas* sp. ZD_05 was observed in colloidal chitin 1%, peptone 0.7%, pH 7, 30 °C and after three days of incubation. Therefore, this indigenous isolate could be considered merit as a potent chitinase producer for fishery industries and management of chitinous waste.

Key words: *Aeromonas*, Chitinase, Optimization, Screening.

Chitin, a N-acetylglucosamine (GlcNAc) polymer with glycoside β -1,4 bonds, is the second most plentiful polymer in biosphere that comes after cellulose^{1,2}. This polymer is widely distributed in cell wall of various fungi, exoskeleton of insects and also in the cyst wall of human pathogen, *Entamoeba histolytica*^{3,4}. Chitin and its related materials have a wide range of applications in food, wastewater treatment, drug delivery, wound healing

and various chemical industries⁵. Chitinases (EC 3.2.1.14), which hydrolyze chitin, are present in a broad range of organisms including bacteria, fungi, insects, higher plants and vertebrates. The roles of chitinases in these organisms are varied. In vertebrates, chitinases are generally part of the intestinal tract⁵. In insects, chitinases may take part in partial degradation of old cuticle. In plants, chitinases have protective roles to resist against fungal pathogens owing to their inducible nature and antifungal activities⁶. As for fungi, it is hypothesized to have nutritional, autolytic and morphogenetic roles. In bacteria, chitinases participate in parasitism and nutrition. In addition to the above potential applications, the chitin-derived chitooligosaccharides can be used as antibacterial agents, elicitors of lysozyme inducers,

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and immunoenhancers⁷. Moreover, Chitinases can also be used in agriculture to control plant pathogens⁵.

During the last decade, there is a much attention to chitinases due to their broader range of biotechnological applications, particularly in the biocontrol of fungal phytopathogens and wastewater treatment in the fishery industry. Therefore, the accumulation of the chitin waste in marine setting seems to be a serious problem for most of fish producing countries, including Iran. For such reason, we aimed to isolate and optimize the cultural circumstances for improved chitinase production by such potent chitinolytic bacteria, *Aeromonas* sp. ZD_05, a promising isolate from Persian Gulf, Bushehr, Iran.

MATERIALS AND METHODS

Preparation of colloidal chitin

Colloidal chitin was prepared by the modified method of Roberts⁸. Ten grams of chitin powder from the crab shell was added slowly into 80 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 500 ml of ice-cold ethanol 50% (v/v) with rapid stirring and kept at 4°C overnight. The precipitate was collected by centrifugation at 10000 g for 10 min at 4°C, and was then washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0).

Screening and Isolation of chitinolytic bacteria

Various samples from sediment, shrimp wastewater and water located in different areas of Persian Gulf, bushehr, Iran were collected. The chitinolytic bacteria were isolated by serial dilutions of water samples and plated on 1% colloidal chitin agar (CCA) medium. Medium for chitin agar plate prepared by mixing 1% colloidal chitin, 1.7% agar, 0.065% NaH₂PO₄, 0.15% KH₂PO₄, 0.0005% CaCl₂, 0.005% NH₄Cl, 0.012% MgSO₄, 0.025% NaCl, (w/v) and pH was adjusted to 6.5. The plates were incubated at 30°C for up to 10 days. Both the clear zones (CZ) and the colony size (CS) were measured and the best isolates maintained on new plates and throughout the study.

Selection of high chitinase producer

Isolates which showed a higher CZ/CS ratio in chitin agar plate in shorter time were selected and one ml of each tested bacterial

inoculums with 0.5 OD was incubated into 50 ml liquid medium. Liquid medium is similar to chitin agar medium with this different that 0.1% yeast extract was added into liquid medium. The resultant inoculated medium was cultured at 30°C for 5 days on a rotary shaker (140 rpm). A 1.5 ml sample of the culture medium was harvested every 24 h, centrifuged at 10000g for 10 min and the cell-free supernatant was assessed for chitinase activity. The cell growth was measurement by absorbance at 600nm.

Chitinase activity assay

The extra cellular chitinase activity was determined by incubating 1 ml of culture supernatant with 1 ml of colloidal chitin in phosphate buffer, pH 7. After incubation at 50°C for 1h, the reaction mixture was subjected centrifugation at 10000g for 10 min, the amount of N-acetyl-D-glucosamine release in the supernatant was determined using DNS method⁹. The absorption of the appropriately diluted test sample was measured at 540 nm. One unit of chitinase activity was defined as the amount of enzyme that produces 1 μmol reducing sugar per hour under the reaction conditions.

Identification of chitinase producer bacteria

The morphological, physiological and biochemical traits of chitinolytic bacteria were performed according to Bergey's Manual of Systematic Bacteriology¹⁰. The identification of selected bacterium at the gene level, was conducted using the partial 16s rRNA gene of the selected strain was amplified by PCR. Isolation of genomic DNA and PCR amplification were adopted from Ghasemi *et al.*,¹¹. The nucleotide sequence 16s rRNA of the tested isolate was compiled and compared with sequences in NCBI using a BLAST program. Finally, the nucleotide sequences of 16s rRNA genes were deposited to GenBank under the accession numbers JQ435774.

Optimization of chitinase production

In present study, the effects of cultural conditions and media composition on chitinase production by *Aeromonas* sp. ZD_05 were performed by using one-factor-at-a-time method, in which holding all the factors steady except the one which was studied. The investigated factors include several nitrogen and carbon source with their various concentrations, time period of incubation, initial pH, various fermentation

temperatures and chitin concentration. For each of factor, three sets of experiment were conducted and the mean of the values was reported.

RESULTS AND DISCUSSION

Isolation and identification

A total of 200 different chitinolytic bacteria were isolated from various marine environment of Persian Gulf which capable of using colloidal chitin as a sole carbon source. Thirty isolates which produced large and clear zones in shorter time were selected and transferred into liquid medium. Chitinase activity was measured by DNS method. Among the potent chitinase producers, strain ZD_05 showed the highest chitinolytic activity (8.7u/ml) at 72 h after incubation. Therefore, strain ZD_05 was opted for future study and identification through 16s rRNA sequence analysis and phenotypic characterization. The taxonomic analysis of isolated ZD_05 was identified to the genus *Aeromonas* on the basis of Bergey's Manual of Systematic Bacteriology. The phenotypic tests showed this bacterium is gram negative, rod shaped, catalase and oxidase positive (Table 1). Further 16s rRNA sequence analysis also confirmed that this bacterium is belong to a member of *Aeromonas* sp with more than 98% nucleotide sequence homology.

Effect of incubation period time on chitinase production

The influence of the course of time on chitinase production by the ZD_05 strain is depicted in Fig. 1. Chitinase production was primarily found after 24 h of incubation and reached maximum levels after 72 h of cultivation. The cell growth in this strain showed that the enzyme increase linearly with growth up to 72 h and then declined as the incubation period further expanded. Zarei *et al.*,¹² reported the maximum production of extra-cellular chitinase by *Serratia marcescens* B4A after 72 h of incubation. In similar study, Wang *et al.*,¹³ provided that the production of chitinase by *Pseudomonas* sp. TKU008 was high after 3 days of incubation. In contrast, Kamil *et al.*,³ found that maximum yield of chitinase by *Bacillus licheniformis* after 96 h of incubation. To our best knowledge, there is no report concerning on chitinase production shorter than 3 days. Therefore, this isolate could be considered as a

potent chitinase producer.

Effect of chitin concentration on chitinase production

The production of chitinase by *Aeromonas* sp. ZD_05 was investigated in different concentrations of colloidal chitin (0.5 to 2.5%). The results revealed that culture medium supplemented with 1% chitin, produce maximum enzyme activity (Fig. 2). These outcomes were in confirmity with the finding of Nandakumar *et al.*,¹⁴ who reported that the production of the chitinase from *Pseudomonas fluorescens* was at highest rate with 1% colloidal chitin concentration. In another study, Al-Ahmadi *et al.*,¹⁵ reported that the maximum of chitinase production by *Aeromonas* sp. JK1 was in 0.75% of colloidal chitin. According to Brzezinska and Donderski^{16,5,17,18,2}, *Aeromonas salmonicidai* produced maximum level chitinase in 1.5% of chitin amended medium after 8 days of incubation at 30°C.

Impact of pH and Temperature on chitinase production

The effect of pH and temperature on chitinolytic enzyme production by *Aeromonas* sp. ZD_05 was studied by growing culture at different temperature (25, 30, 37, 42°C) and initial pH between 5 and 9. A high level of chitinase activity was

Table 1. Biochemical properties of *Aeromonas* sp. ZD_05 strain

Biochemical properties	Result
Catalase	+
Oxidase	+
Motility	-
Urease	-
MR	+
VP	-
Indole production	-
Hydrolysis of Pectin, Xylane, Starch	-
Utilization of	
Lactose	-
Sucrose	-
Mannitol	+
Glucose	+
Utilization of citrate	+
Nitrate reduction	-
Growth in NaCl	
0-4 %	+
5-7 %	-
5-9 %	-

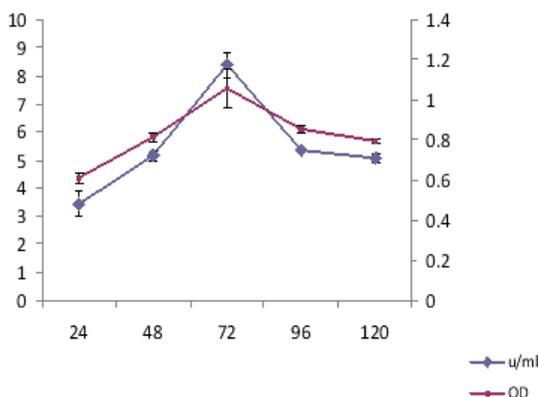


Fig. 1. Effect of course of time on chitinase and cell growth by *Aeromonas* sp. ZD_05

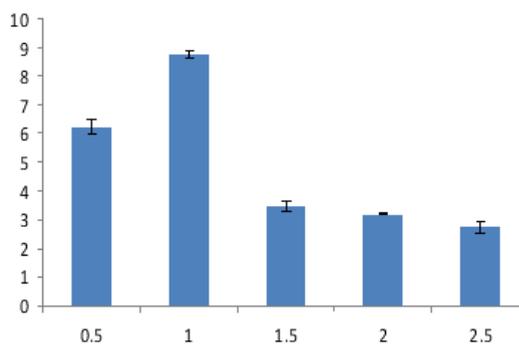


Fig. 2. Role of chitin concentration on chitinase production by *Aeromonas* sp. ZD_05

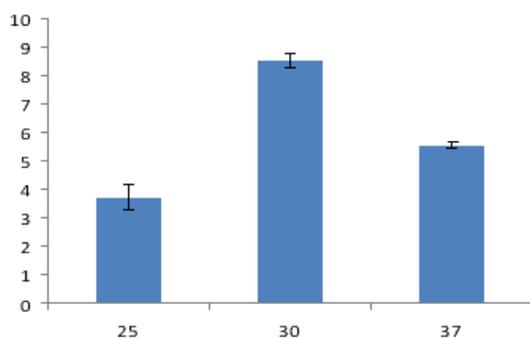


Fig. 3. Impact of temperature on chitinase production by *Aeromonas* sp. ZD_05

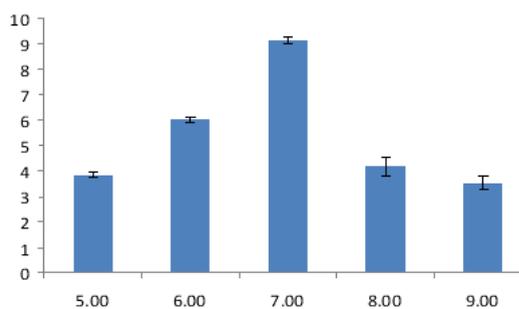


Fig. 4. Impact of pH on chitinase production by *Aeromonas* sp. ZD_05

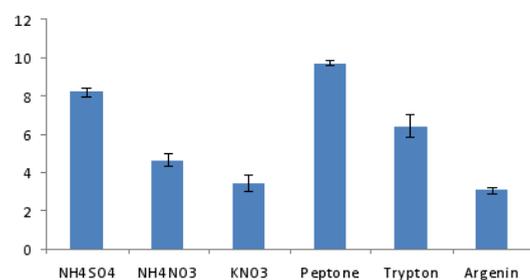


Fig. 5. Influence of additional nitrogen sources on chitinase production by *Aeromonas* sp. ZD_05

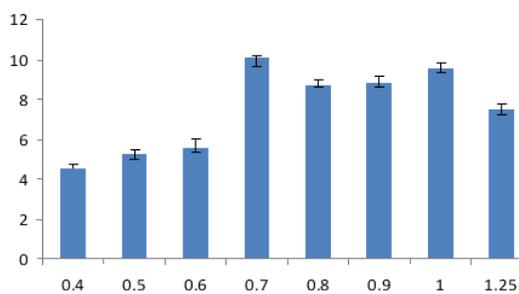


Fig. 6. Influence of different peptone on chitinase production by *Aeromonas* sp. ZD_05

obtained in the culture medium with pH 7 (Fig. 3) and optimum temperature at 30°C (Fig. 4). The obtained results are in agreement with several researchers who reported neutral or slightly acidic conditions are favorable for chitinase production^{5,17,19,20}. In contrast, Kim *et al.* claimed that *Streptomyces* sp. M-20 produce the optimum

chitinase at pH and temperature 5 and 30 °C, respectively²¹. In another study, Shanmugaiah *et al.*¹⁸, reported that the production of chitinase by *Bacillus laterosporous* MML2270 was optimal at basic condition (pH 8) and temperature 35°C, while Dai *et al.*²², reported that maximum production of enzyme by *Bacillus* sp. Hu1 was in nearly acidic

condition (pH 6.5) but surprisingly at high temperature 60 °C. Further study showed that the *Aeromonas* sp. ZD_05 was very sensitive to change of temperature. Any growth by this strain wasn't found in 42 °C. Maximum chitinase production by the strain ZD_05 was observed in 1% chitin amended basal medium incubated at pH 7 and temperature 30°C for 72 h.

Influence of different nitrogen sources on chitinase production

The influence of additional nitrogen sources on chitinase production was studied by supplementing different inorganic (0.05%, w/v) and organic nitrogen sources (0.1%, w/v). Among the various nitrogen sources in the basal medium, peptone was the most effective additives resulting in the increase of the enzyme production (Fig 5) and then ammonium sulfate came after as an inorganic nitrogen source. To evaluate the effect of peptone, different concentration was added to production medium (0.4 to 1.25 g/l). Maximum chitinase production by *Aeromonas* sp. ZD_05 was founded in media 0.7 g/l peptone (Fig. 6). In *Serratia marcescens*, chitin medium amended with peptone with chitin, enhanced the production of chitinase²³. Wang *et al.* reported that peptone (0.1 %) induced high levels of chitinase by *Bacillus sphaericus*²⁰. However, Feles *et al.* found that 0.5% of tryptone increased chitinase production by *Aeromonas* sp¹⁷. In general, chitinase production was improved much more by using organic nitrogen than inorganic nitrogen. This may be due to that organic nitrogen contains most types of amino acids and growth factors for the growth of bacterium that could be metabolized directly by cells, consequently promoting chitinase production. However, this study also showed that ammonium sulfate as an inorganic nitrogen source, could be a good alternative to peptone, especially for large scale of chitinase production purposes.

CONCLUSION

To our best knowledge, there are a few reports on indigenous chitinolytic bacteria inhabiting the Persian Gulf, and most of the studies are superficial. Therefore, this study provides a relatively high amount of extracellular chitinase in a simple medium in a relatively short time, leading to the chief advantages of chitinase production

by an indigenous *Aeromonas* sp. ZD_05. The level of production of chitinase can be enhanced by altering fermentation conditions. These properties make *Aeromonas* sp. ZD_05 a good candidate for industrial production of chitinase for fishery industries and management of chitinous waste. In future by purification and study of biochemical characterization of ZD_05 isolate could determine further its potential for industrial application.

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