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ARTICLE

Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase

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KEYWORDS

Microbial chitinase;
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Abstract Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. Chitinase enzyme has received increased attention due to its wide range of biotechnological applications, especially in agriculture for biocontrol of phytopathogenic fungi and harmful insects. In the present study, 58 bacterial isolates were screened for chitinolytic activity and on the basis of chitin hydrolysis zone 6 isolates were selected for chitinase production in broth media. Based on enzyme production, two most potent isolates identified as *Aeromonas hydrophila* HS4 and *Aeromonas punctata* HS6 were selected for further study. The effects of media composition and various fermentation conditions for optimization of chitinase production were studied. The maximum chitinase production was obtained at 37 °C and pH 8.0 after 24–48 h of incubation by HS4; and at 37 °C and pH 7 after 48 h incubation by HS6. Among the substrates colloidal chitin was the best for both the strains. Regarding carbon sources, starch (1%) was the best for both strains; while malt and yeast extract (1%) was found as the best nitrogen source for HS4 and HS6, respectively. Out of metal ions Mn^{2+} and Cu^{2+} enhanced enzyme production in the case of HS6. However, Co^{2+} was the most appropriate for HS4.

1. Introduction

Chitin is nitrogen containing polysaccharide consisting of β-1,4-linked *N*-acetyl-D-glucosamine which is chemically analogous to the cellulose, except that one of the hydroxyl groups of each glucoside residue is replaced by an acetylated or deacetylated amino group. Chitin is the second most abundant natural polymer and widely distributed as a structural component of crustaceans, insects, and other arthropods, as well as a component of the cell walls of most fungi and some algae. Approximately 75% of the total weight of shellfish, such as shrimp, crabs and krill are considered as waste, and chitin comprises 20–58% of the dry weight of the said waste [49]. About 1011 tons of chitin is alone produced annually in the aquatic biosphere [32,36]. Chitinase (EC 3.2.11.14) enzyme is capable of hydrolyzing insoluble chitin to its oligo and monomeric components found in a variety of organisms including viruses, bacteria, fungi insects, higher plants and

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animals and play important physiological roles depending on their origin [8,10,16]. Chitinases are constituents of several bacterial species; some of the best known genera include *Aeromonas, Serratia, Vibrio, Streptomyces* and *Bacillus* [7]. Chitinases can be classified as endochitinases or exochitinases. Endochitinases cleave chitin at internal sites to generate multimers of GlcNAc. Exochitinases catalyze the hydrolysis of chitin progressively to produce GlcNAc, chitobiose or chitotriose. Chitinase has a wide-range of applications such as preparation of pharmaceutically important chitooligosaccharides and *N*-acetyl D-glucosamine, preparation of single-cell protein, isolation of protoplasts from fungi and yeast, control of pathogenic fungi, treatment of chitinous waste, and control of malaria transmission [9]. Chito-oligomers produced by enzymatic hydrolysis of chitin are used in various fields like in medical, agricultural and industrial applications, such as antibacterial, antifungal, antihypertensive and as a food quality enhancer [5].

Optimization of media is very important to maximize the yield and productivity, and minimize the product cost [1]. The aim of this study was to isolate the most prominent chitinolytic bacteria from different soil samples with unique properties and optimize their fermentation conditions for maximum chitinase production.

2. Materials and methods

2.1. Sample collection and isolation of chitinolytic bacteria

A total of 15 different soil samples were aseptically collected from different regions of Lucknow, India. The location of collected soil was rhizosphere of maize, wheat and rice, fish market and pond. For screening of chitinase producing bacteria, the agar medium amended with colloidal chitin was used. The medium consists of (g L^{-1}): Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; yeast extract, 0.05; agar, 15 and colloidal chitin 1% (w/v). The colonies showing clearance zones on a creamish background were considered as chitinase-producing bacteria.

2.2. Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin (Hi Media) by the modified method of Hsu and Lockwood [20]. In brief, chitin powder (40 g) was slowly added with 600 ml of concentrated HCl and kept for 60 min at 30 °C with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 2 l of water at 4–10 °C. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5 l of DW. Washing was repeated 3 times until the pH of the suspension was 3.5. After the above treatment, the loose colloidal chitin was used as a substrate [50].

2.3. Screening of chitinase producing bacteria

Screening was performed with bacterial isolates on the colloidal chitin agar medium incubated at 37 °C. Bacterial isolates were selected on the basis of a larger hydrolysis zone after 96 h of incubation and further screened for maximum enzyme production in nutrient broth media. The cultures were centrifuged at 10000 rpm for 15 min at 4 °C and the crude was used for chitinase assay.

2.4. Assays of chitinase activity and protein estimation

The chitinase activity was assayed by measuring reducing sugar released from colloidal chitin as per the modified method of Toharisman et al. [46]. Briefly, crude enzyme (150 µl) was added to the mixture consisting of 300 µl of 0.1% colloidal chitin and 150 µl of 0.1 M phosphate buffer pH 7.0. After incubation at 55 °C for 10 min, the reaction mixture was subjected to a refrigerated centrifugation at 10,000 rpm for 5 min. The resulting supernatant (200 µl) was added with 500 ml of DW and 1000 ml of Schales reagent then boiled for 10 min. After cooling, the absorbance of the mixture was measured at 420 nm. One unit of the chitinase activity was defined as the amount of enzyme which yields 1 µmol of reducing sugar as *N*-acetyl-D-glucosamine (GlcNAc) equivalent per minute.

2.5. Characterization of bacterial isolates

2.5.1. Identification of chitinolytic bacterium

The identification of bacterial isolates HS4 and HS6 was carried out according to the methods described in Bergey's Manual of Systematic Bacteriology [21].

2.5.2. Isolation of genomic DNA for 16S rRNA and PCR amplification

Total genomic DNA was extracted from the cells by using the phenol–chloroform method [35]. Isolated DNA was checked for its quality and concentration by agarose gel electrophoresis on UV transilluminator. 16S rRNA region was amplified with universal forward and reverse bacterial primers. The PCR amplification was performed using a PTC-150 Mini cycler (MJ Research), with a primary heating step for 2 min at 95 °C, followed by 30 cycles of denaturation for 20 s at 95 °C, annealing for 60 s at 55 °C, and extension for 2 min at 72 °C, then followed by a final extension step for 7 min at 72 °C. Each 25 µL reaction mixture contained 2 µL of genomic DNA, 14.25 µL of MilliQ water, 2.5 µL of 10× buffer (100 mM Tris–HCl, pH 8.3; 500 mM KCl), 1.5 µL of $MgCl_2$ (25 mM), 2.5 µL of dNTP's mixture (dATP, dCTP, dGTP, dTTP at 10 mM conc.), 1.0 µL of each primer (20 pmol/mL), and 0.25 µL of *Taq* DNA polymerase. The PCR-amplified product was analyzed on 1% agarose gel containing ethidium bromide (0.5 mg/mL) and 1 kb DNA molecular weight marker and documented using a gel documentation system. The PCR amplicon for the partial 16S rRNA gene was further processed for sequencing. Sequencing was carried out using the same set of primers in both the directions to check the validity of the sequence. Sequencing was done by Ocimum Biosolutions, India.

2.6. Optimization of enzyme production

2.6.1. Effect of media and incubation time on chitinase production

Six different broth media *viz*. nutrient broth (g L^{-1}: yeast extract, 1.5; NaCl, 5; beef extract, 1.5; amended with 1% colloidal chitin), luria bertaini broth (g L^{-1}: tryptone, 10; yeast extract, 5; NaCl, 5; amended with 1% colloidal chitin), M1

(%: chitin, 1; K_2HPO_4, 0.1; $MgSO_4{\cdot}7H_20$, 0.05; at pH 7), M2 (%: colloidal chitin, 2; $MgSO_4{\cdot}7H_20$, 0.05; NaH_2PO_4, 0.5), M3 (g L^{-1}: chitin, 15; Urea, 0.32; $CaCl_2$, 0.1; $MgSO_4{\cdot}7H_20$, 0.08) and M4 (%: colloidal chitin, 0.3; K_2HPO_4, 0.1; $MgSO_4{\cdot}7H_20$, 0.01; NaCl, 0.1; $(NH_4)_2SO_4{\cdot}0.7$) were used to determine the growth of bacteria and chitinase production. The culture was inoculated (1%) and incubated at 37 °C for 24 h in a rotary shaker (120 rpm). After 24 h of incubation, the cultures were harvested, centrifuged at 10,000 rpm for 15 min and the supernatant used for chitinase assay [46]. For optimum incubation time, the bacterial culture was grown for 5 days and chitinase production was estimated every day.

2.6.2. Effect of temperature and pH on chitinase production

The effect of temperature on enzyme production was determined by incubating inoculated medium at different temperatures (18, 22, 37, 40, 50 and 55 °C) for optimized period of time. The effect of the initial pH value on the chitinase production was investigated by varying the initial pH of the culture medium from 4 to 12 and at optimized temperature and incubation period. Chitinase assay was performed as per standard protocol.

2.6.3. Effect of different substrates and their concentration on chitinase production

To find out the best substrate for enzyme production, the chitinase production was carried out by using different substrates (1%) in medium viz. fish shell powder (FS), colloidal chitin (CC) and chitin powder (CP) at previously optimized conditions. Different concentrations of substrates (0.1–1.2%) were applied in optimized media and condition to determine the best substrate concentration.

2.6.4. Effect of carbon and nitrogen sources on chitinase production

The effects of various carbon and nitrogen sources (1%) were used as additional supplement in media for maximum enzyme production. The supplemented media were inoculated with 1% inoculums and fermented at an optimized condition. Simultaneously media without any carbon and nitrogen source were used as control.

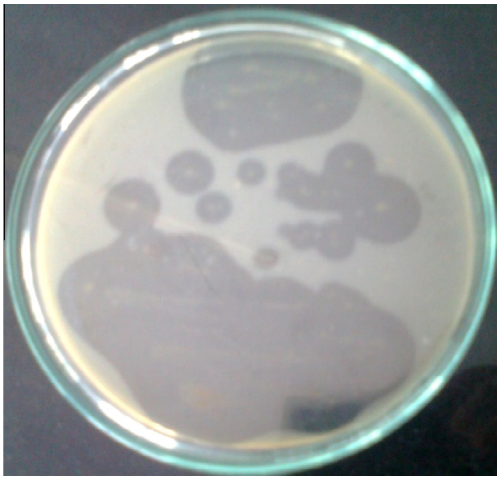

Figure 1 Bacterial colony showing clear hydrolysis zone on colloidal chitin agar.

Table 1 Production of chitinase at 37 °C after 24 h incubation.

Culture No.	Chitinase activity[a] (Unit/ml)
HS-2	3272
HS-4	5946
HS-6	6072
HS-8	4393
HS-9	4876
HS-12	5091

[a] Values are mean of 3 replications.

Table 2 Characterization of isolates.

Colony properties	HS-4	HS-6
Colony shape and size	Irregular , 2–3 mm	Circular, 2 mm
Elevation	Convex	Convex
Margin	Undulate, wrinkled surface	Entire edge, smooth surface
Gram reaction	Negative	Negative
Cellular morphology	Rod	Rod
Color	Creamish white	Creamish
Utilization of lactose, maltose, glucose, sucrose	+ve	+ve
Spore	−ve	−ve
Citrate utilization	+ve	−ve
Motility	+ve	+ve
Urease production	−ve	−ve
Catalase test	+ve	+ve
Starch hydrolysis	+ve	+ve
Triple ion sugar	−ve	−ve
Methyl red	+ve	+ve
Voges–Proskauer reaction	−ve	+ve
Growth at pH	6–7	5–9
Growth at temperature (°C)	22–40	22–37
Growth in NaCl	2–8%	2–6%

2.6.5. Effect of metal ions on chitinase production

Influence of various metal ions on chitinase production was determined by inoculating medium with different metal ions such as Mn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+} and Hg^{2+} in their chloride form except Cu^{2+} (copper sulfate). Chitinase assay was then performed as per standard protocol.

3. Results and discussion

A total of 58 morphologically different chitinolytic bacteria were isolated from 15 soil samples collected from different habitats of Lucknow, India. On the basis of colloidal chitin degradation (Fig. 1) and zone of clearance (>0.2 cm) on CCA plate, six colonies were selected for secondary screening in broth media and tested for enzyme activity. Based on maximum chitinase production after 24 h of incubation, two potential isolates HS4 and HS6, isolated from the soil of rice rhizosphere and fish market respectively, were selected for further studies (Table 1).

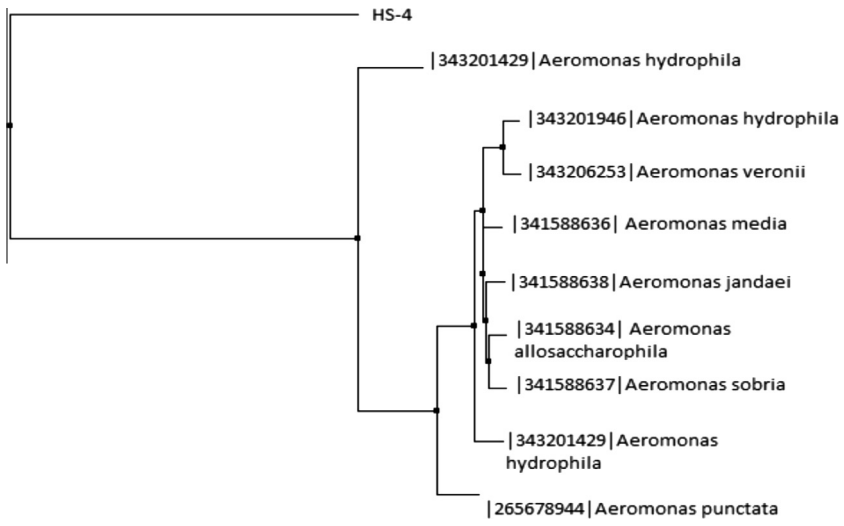

Figure 2 Phylogenetic tree of strain HS4 showing the similarity with *Aeromonas hydrophila*.

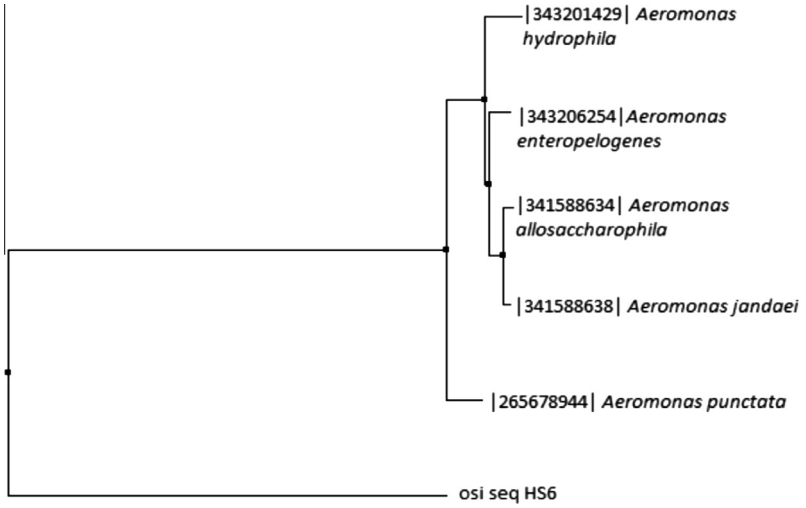

Figure 3 Phylogenetic tree of strain HS6 showing the similarity with *Aeromonas punctata*.

3.1. Characterization of bacterial isolates

3.1.1. Identification of bacteria

The isolates HS4 and HS6 were identified as *Aeromonas hydrophila* and *Aeromonas punctata*, respectively. The identity was further confirmed by 16S rRNA analysis. The strains were gram negative, motile, non-spore forming and facultative anaerobes (Table 2).

3.1.2. Analysis of DNA sequences

The homology of the partial 16S rRNA gene sequence of the isolates was analyzed using the BLAST algorithm in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses were conducted using a multiple sequence alignment tool (Clustral W). Only the highest-scored BLAST result was considered for phylotype identification. BLAST showed that the isolate HS4 linear DNA has maximum homology (99%) with *A. hydrophila* (Fig. 2) while HS6 having maximum homology (99%) with *A. punctata* (Fig. 3).

3.2. Optimization of enzyme production

3.2.1. Effect of media and incubation period

Among all the tested media, LB with colloidal chitin was more productive in both the strains such as *A. hydrophila* HS4 (64.41 U/ml) and *A. punctata* HS6 (59.41 U/ml) (Fig. 4). Karunya et al. [22] also observed that presence of colloidal chitin in LB supports maximum chitinase production from *Bacillus subtilis*. The effects of incubation time on chitinase production are shown in Fig. 5. *A. hydrophila* HS4 produced highest chitinase after 24 h and remains constant up to 48 h (80.9 U/ml) while *A. punctata* HS6 produced maximum chitinase (82.36 U/ml) after 48 h of incubation. Enzyme production gradually decreased in both strains after

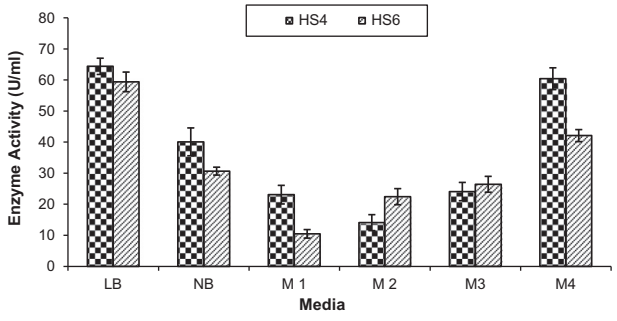

Figure 4 Effect of media on chitinase production by HS4 and HS6.

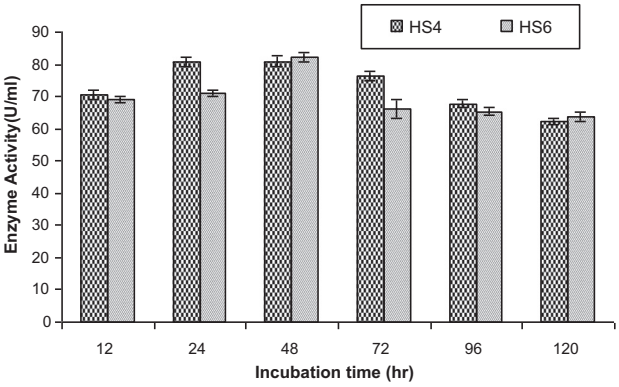

Figure 5 Effect of incubation period on chitinase production by HS4 and HS6.

48 h. One of the reasons for decreased production may be the lack of nutrients or production of toxic chemicals in the medium resulting in the inactivation of secretary machinery of the enzymes [31]. Similar observations were also reported by Nawani et al. [30] with *Microbispora* sp. Wang and Hwang [48] reported that *B. cereus, B. alvei* and *B. sphaericus* produced highest chitinase after 48 h of incubation. Although Faramarzi et al. [11] reported maximum chitinase production at 36 h of incubation by *M. timonae*. However, at 72 h of incubation, maximum chitinase production was observed by *Penicillium aculeatum* [6] and *Trichoderma harzianum* [37].

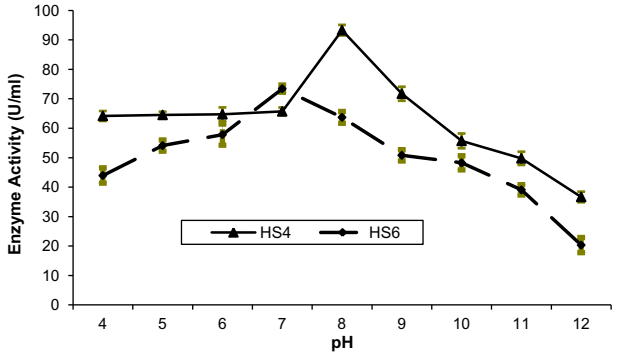

Figure 6 Effect of pH on chitinase production by HS4 and HS6.

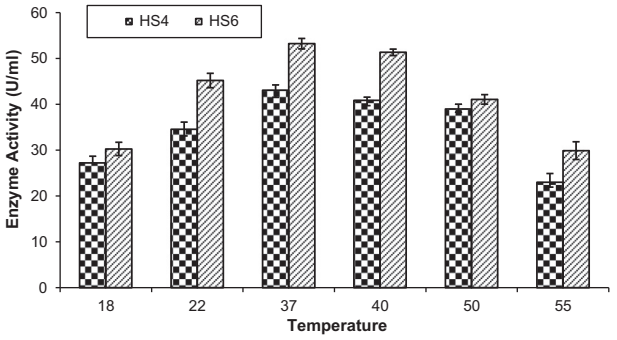

Figure 7 Effect of temperature on chitinase production by HS4 and HS6.

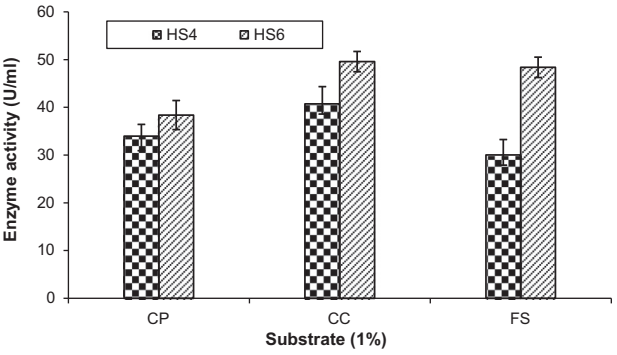

Figure 8 Effects of different substrates on chitinase production by HS4 and HS6.

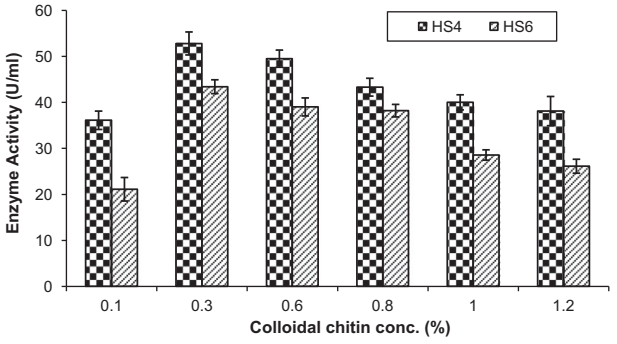

Figure 9 Effect of substrate concentration on chitinase production by HS4 and HS6.

3.2.2. Effect of pH on chitinase production

In order to evaluate the effect of pH of media on the chitinase production, bacterial cultures were grown at different pH (4–12). Among the tested pH, pH 8 for *A. hydrophila* HS4 (93.27 U/ml) and pH 7 for *A. punctata* HS6 (73.43 U/ml) supported the maximum chitinase production (Fig. 6). From the above results, we can conclude that pH of media not only helps in the production of chitinase but also plays an important role in cell growth.

Like *A.* hydrophila HS4, previous reports also suggested that *Bacillus laterosporous* [38], Micrococcus sp. AG84 [4], *Alcaligenes xylosoxydans* [47], *Serratia marcescens* XJ-01 [51],

Aeromonas sp. JK1 [2] and *Bacillus pabuli* [12] are capable of producing a high amount of chitinase at alkaline condition. The result of HS6 strain is also supported by other publications [2,25,26,29,33,40,41]. Furthermore, Hiraga et al. [19] reported chitinase production in a broad range of pH-value (5–8) by *A*. hydrophila H-2330.

3.2.3. Effect of temperature for chitinase production

Temperature affects various biological processes, therefore the growth of bacteria and enzyme production are also affected with the change in incubation temperature. To evaluate the optimum growth temperature for chitinase production, the cultures of *A. punctata* HS6 and *A. hydrophila* HS4 were grown at 18–55 °C. Chitinase production was maximum at 37 °C with both strains *A. hydrophila* HS4 (43.08 U/ml) and *A. punctata* HS6 (53.22 U/ml) (Fig. 7). The enzyme production was decreased above 40 and 50 °C by *A. punctata* HS6 and *A. hydrophila* HS4, respectively. Narayana et al. [28] and Sudhakar and Nagarajan [44] also reported maximum chitinase production at 35 °C by *Streptomyces* sp. ANU6277 and *T. harzianum*, respectively. Other reports also concluded maximum enzyme production from *Streptomyces* sp. in between 30 and 40 °C [15,18,24,38,43].

3.2.4. Effect of different substrates on chitinase production

Among the various substrates like fish shell (FS), chitin powder (CP) and colloidal chitin (CC), colloidal chitin was found to be a best substrate for chitinase production by both the strains (40.74 and 49.6 U/ml by *A. hydrophila* HS4 and *A. punctata* HS6, respectively) (Fig. 8). Similar observation has also been reported with *Streptomyces viridificans* [18], *Streptomyces lydicus* WYEC108 [25] and *Acremonium obclavatum* [17]. Faramarzi and coworkers [11] also showed that colloidal chitin acts as a sole carbon and nitrogen source for chitinase production. A study on chitinase production from *Streptomyces* sp. confirmed that the presence of colloidal chitin along with sucrose doubled the enzyme production [43]. Karunya et al. [22] and Andronopoulou and Vorgias [3] also reported a similar result with *Bacillus subtilis* and *Thermococcus chitonophagus*, respectively.

3.2.5. Effect of substrate concentration on chitinase production

Different concentrations of colloidal chitin were used to elucidate the best concentration for maximum chitinase production which can be exploited at the industrial level. The results showed both the strains produced enzyme maximally at 0.3% of colloidal chitin (Fig. 9). Accordingly *A. hydrophila* HS4 and *A. punctata* HS6 produced 52.8 and 43.4 U/ml of enzyme, respectively. Our results are also supported by the findings of Souza et al. [42] and Karunya et al. [22] who reported the maximum chitinase production at 0.3% colloidal chitin.

3.2.6. Effect of carbon sources on chitinase production

A total of seven different carbon sources (1%) namely mannitol, glucose, fructose, sucrose, lactose, starch and dextrose were tested for maximum chitinase production. Among the carbon sources, starch supported maximum chitinase production for *A. hydrophila* HS4 (97.35 U/ml) and *A. punctata* HS6 (89.87 U/ml) followed by lactose and fructose (Fig. 10). The same findings were also reported with *Streptomyces aureofaciens* CMUAc130 [45], *S. marcescens* [40] and *Streptomyces* sp. ANU [28].

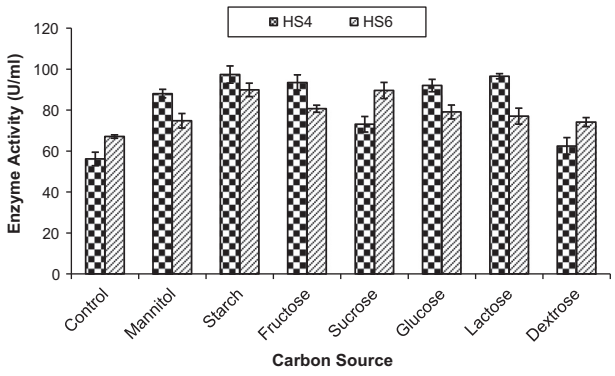

Figure 10 Effect of carbon sources on chitinase production by HS4 and HS6.

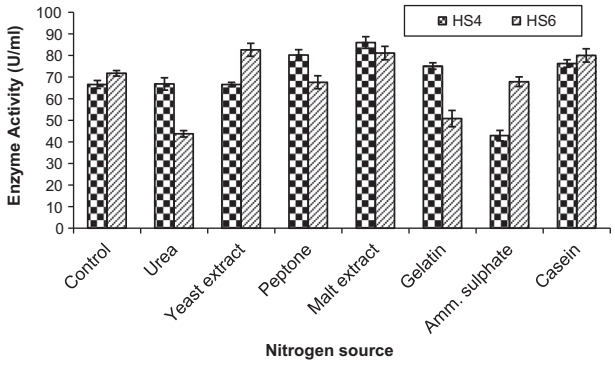

Figure 11 Effect of nitrogen sources on chitinase production by HS4 and HS6.

Table 3 Effect of metal ions on chitinase production by HS4 and HS6.

Metal ions	Chitinase activity[a] (Unit/ml)	
	HS-4	SH-6
Control	51.77	48.22
Mn^{+2}	84.67	92.71
Co^{+2}	87.05	48.54
Ca^{+2}	40.2	49.93
Cu^{+2}	72.81	85.43
Fe^{+2}	75.24	40.12
Mg^{+2}	45.63	45.14
Hg^{+2}	59.22	29.28

[a] Values are mean of 3 replications.

3.2.7. Effect of nitrogen source for chitinase production

As per the results, the addition of malt extract, peptone, geletin and casein in *A. hydrophila* HS4 and yeast extract, malt extract and casein in *A. punctata* HS6 had a significant effect on chitinase production among the various tested nitrogen sources (Fig. 11). Malt extract in *A. hydrophila* HS4 (86.01 U/ml) and yeast extract in *A. punctata* HS6 (82.64 U/ml) were the

most favorable nitrogen source. Rattanakit et al. [34] observed the same result with urea, yeast extract and peptone which had a repressive effect on chitinase production by *Aspergillus* sp. Sl-13. Urea enhanced in chitinase production by *Paenibacillus* sp. D1 [39] and *Pantoea dispersa* [14]. In contrast to the present study, ammonium sulfate was found to be effective to enhance the production by *Aspergillus sp*. Sl-13 [34] and *Aeromonas* sp. JK1[2]. The results of the present study with *A. punctata* HS6 were supported by other studies also [25,27,28,37,47]. In our findings, urea, peptone, gelatin and $(NH_4)_2SO_4$ repressed the enzyme production but these nitrogen sources enhanced enzyme production in the case of *Paenibacillus* sp. D1[39], *A. xylosoxydans* [47] and *Aeromonas* JK1 [2].

3.2.8. Effect of metal ions on chitinase production

Metal ions play an important role in maintaining the structure and configuration of enzymes [23]. The effect of various metal ions on chitinase production by *Aeromonas hydrophia* HS4 and *A. punctata* HS6 was investigated. The results showed that chitinase production was enhanced by the addition of Co^{2+} and Mn^{2+} in the culture media of *Aeromonas hydrophia* HS4 and *A. punctata* HS6, respectively (Table 3). However, enzyme production was inhibited by Ca^{2+} and Mg^{2+} in the case of *Aeromonas hydrophia* HS4 and by Fe^{2+}, Mg^{2+}, and Hg^{2+} in the case of *A. punctata* HS6. The present result is also supported with previous studies where chitinase production was enhanced by other microbes [2,13,39].

4. Conclusion

Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. The result concluded that *A. hydrophila* HS4 and *A. punctata* HS6 are novel mesophilic bacterial strains that have the ability to produce a huge amount of chitinase in short time. Both the isolated strains of *Aeromonas* sp. have the ability to produce chitinase between temperature 22 and 40 °C which is the field temperature for the cultivation of most of the crop in India, so it may be applicable to field condition against plant pathogenic fungi which is the major problem for agricultural food production. This enzyme may also be useful in the management of sea food waste industries.

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