



Research Article

Screening and Identification of Chitin Degrading Bacteria from Local Environment

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Authors' Contributions

AR designed the study, wrote the article and supervised the work. FM performed experiments, analyzed the results and wrote the article.

Keywords

Chitinase activity, Chitin, Zone of hydrolysis, pH, Temperature, Metal ions.

Abstract | In the present study, three bacterial isolates, named as H1, H4 and M4, were selected on the basis of chitinase assay and diameter of zone of hydrolysis. All the isolates were identified as *Bacillus* sp. on the basis of biochemical tests, and showed optimum growth at 37°C and pH of 7. All the bacterial isolates showed highest enzyme activity at pH 7. Isolates H4 and M4 showed maximum enzyme activity at 55°C while H1 showed highest activity at 70°C. Chitinase activities exhibited by H1 and M4 are stimulated by the presence of Zn²⁺ ions, while the presence of Ni²⁺, Cd²⁺ and Cu²⁺ did not show any positive effect. However in H4, presence of Zn²⁺, Ni²⁺ and Cd²⁺ showed positive while Cu²⁺ showed negative effect on the chitinase activity. The isolated strains have the ability to degrade complex chitin thus they could be used to degrade complex polysaccharides present in the organic wastes and would be helpful in cleaning the environment.

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Introduction

Chitin is considered as linear β 1,4 linked polymer of N-acetyl -D-glucosamine (GlcNAc) and it contains high percentage of nitrogen (6.89), that improves chitin's property to act as chelating agent (Khan *et al.*, 2013). Chitin is white, hard, non-elastic nitrogen polysaccharide present in outer skeleton as well as internal surface of non-vertebrates (Stawski, 2017), and is commercially obtained from wastes of crab shell and shrimp (Kaur and Dhillon, 2015). After cellulose, chitin is the next most available polysaccharide to extent to 10 gigas tons annually (Sillanpaa and Ncibi, 2017).

One of the major end products of chitin degradation by chitinase is chitobiose which is further degraded by chitobiase enzyme (Kim *et al.*, 2017). The monomeric unit of polymer chitin is N-acetyl-D-glucosamine which makes the external covering of crustaceans and insects (Bobbin-*ket al.*, 2015). Chitin can be obtained mainly from three sources; microorganisms, insects and crustaceans (Kumari *et al.*, 2015). The alpha (α) chitin is commonly obtained from crustaceans; β -chain chitin is attained from squid pen, while γ chain chitin is present in fungus and yeast (Rolandi and Rolandi, 2014). Arbia *et al.* (2013) reported that chitin extraction involves two steps; deprotenisation and demineralization, which can be achieved by chemical and biological methods. Chemical methods involve the use of acids and bases while biological methods involve the utilization of microorganisms. Chitin can be used as cationic

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agents for the polluted waste water treatment (Vakili *et al.*, 2014).

Chitin is medically important (Brkich *et al.*, 2018) as chitin and its derivatives have been designed to use as wound dressing, particularly for chronic wound treatment (Rajendran *et al.*, 2016), and as blood cholesterol control agent (Kunamneni and Ogaugwu, 2018). It is used in food industry to measure the contamination of food products and agriculture commodities (Manigandan *et al.*, 2018), and also used as soil repellent in textile industry (Pisitsak *et al.*, 2018).

Break down of chitin creates reducing conditions that can sustain anaerobic reductive processes similar to the denitrification process (Zhai *et al.*, 2018). Chitinase enzymes from animals, plants, insects and microorganisms with respect to their application and function have been widely studied (Griffith and Davis, 2018). Chitinolytic enzymes are synthesized from protozoans, and by different glandular tissues of the gut of many nematodes, polychaetes, oligochaetes and some arthropods (Miltko *et al.*, 2016). Chitinase enzymes have also been found in marine invertebrates, crustaceans such as prawn and molluscs (Rao *et al.*, 2017).

Normally in yeast, cell separation is achieved by small digestion of chitin septum by chitinase enzyme (Yusof *et al.*, 2017). Chitinotriosidases was considered as the first mammalian enzyme discovered (Turan *et al.*, 2017). Actinomycetes, particularly the genus *Streptomyces*, which are gram positive mycelial bacteria are ubiquitous in soil, and are well known producer of many extra cellular enzyme including chitinase (Sukalkar *et al.*, 2017).

The current study was aimed at isolating and characterizing bacteria from the local environment which are capable to degrade chitin into its products. Optimum growth conditions of the bacteria were determined and chitinase characteristics were also ascertained.

Materials and Methods

Sample collection and bacterial isolation

To isolate chitinase producing bacteria, soil and water samples were collected in sterilized container. Soil samples were obtained from rice, wheat and maize fields, while water samples were collected of fish farm from Punjab University, Lahore. For the isolation of chitin degrading bacteria, L-agar medium supplemented with chitin (1g/100ml) was prepared, autoclaved and poured into four Petri plates. Then, 50 µl of each serially diluted sample was spread on chitin supplemented plates, and incubated at 37°C for 24h. Sixteen different colonies were randomly selected.

Colloidal chitin preparation

One gram of chitin flakes was taken in mortar and pestle, added 5ml of acetone and grinded gently for 10 min. Then 40ml concentrated HCl was added to the ground chitin and agitated for 3h to dissolve the chitin, and then placed at 4°C for overnight. The chitin solution was filtered and filtrate was made up to 250ml volume by using 50% ethanol with constant stirring. The solution was centrifuged at 10,000rpm for 20 min. The precipitates of chitin obtained were washed with distilled water until neutral pH obtained. Distilled water was added to form 2% colloidal chitin and this stock solution was stored at 4°C for further use.

Chitinase assay and screening of chitin degrading bacteria

Chitinase assay was performed to check the ability of the isolated bacteria to produce chitinase enzyme. The standard curve for chitinase assay was plotted. The reaction mixture contained 300µl of colloidal chitin in various concentrations i.e., percentage 0.0% (control), 0.1%, 0.2%, 0.4%, 0.6% and 0.8%, 150µl of 0.1 M phosphate buffer, 150µl of distilled water and was incubated at 55°C for 10 min. The reaction mixture was centrifuged at 10,000rpm for 5 min. The supernatant was added to 50ml of distilled water, 10ml of Schales reagent and then boiled for 10 min. Optical density of the reaction mixture was determined at 420nm. The extracellular enzyme activities of the bacterial isolates M1, M2, M3, M4, F1, F2, F3, F4, H1, H2, H3, H4, S1, S2, S3 and S4 was assayed by measuring reducing sugar released from colloidal chitin as per the modified method of Toharisman *et al.* (2005). Further screening was done by zone of chitin hydrolysis by using Congo red dye. On the basis of enzyme activity and hydrolysis zone H1, H4, and M4 were further selected for further study.

Biochemical characterization

The biochemical tests such as Gram staining, spore staining, mannitol salt agar and Voges-Proskauer were performed according to Cappuccino and Sherman (2001).

Determination of optimum growth conditions

Growth conditions (pH and temperature) of the selected bacterial isolates were determined. For the determination of optimum temperature, LB broth was prepared, and pH of the medium was adjusted to neutral. The broth 10ml was taken in each flask. Bacterial inoculum was prepared in 25ml of LB broth in 100ml flask. Suspension of the culture was prepared having O.D at 600nm and 100µl of inoculum was given in each flask. Flasks were incubated at 25°C, 30°C, 37°C and 50°C for 24 h. O.D was taken at 600nm and average of three reading was recorded.

The LB broth was prepared with different pH values (5,6,7,8,9). The pH was adjusted using HCl and NaOH. The broth (10ml) was taken in each flask. Bacterial inoculum was prepared in 25ml of LB broth in 100ml flask.

Suspension of culture was prepared of O.D 0.8 at 600nm and 100µl of inoculum was given in each flask. Flasks were incubated at 37°C for 24h. O.D was taken at 600nm and average of three readings was recorded.

Chitinase characterization

To determine effect of temperature on the chitinase activities, each reaction mixture contained 300µl of 0.1% colloidal chitin; 150µl of 0.1M phosphate buffer and 150µl crude enzyme. The reaction mixture was incubated at temperature 30°C, 45°C, 55°C, 70°C and 90°C separately for 10min. After incubation, the reaction mixtures were centrifuged at 10,000rpm for 5 min. The obtained supernatant 200µl was added with 50ml of distilled water, 10ml of Schales reagent and then boiled for 10 min. Optical density of there action mixture was measured at 420nm. One unit of enzyme activity was considered which yields 1µmol of reducing sugar as N-acetylglucosamine equivalent per minute under assay condition.

The optimization of pH for chitinase activity was done by incubating the enzyme extract at various pH (4,5,7,8,9) in different buffers. Then 150µl from each buffer was taken in separate eppendorf and 150µl crude enzyme extract and 300µl of 0.1% colloidal chitin was added to make volume of 600µl and incubated at 55°C for 10 min. After incubation, the reaction mixtures were centrifuged at 10,000rpm for 5 min. The obtained supernatant (200µl) was added with 50ml of distilled water, 10ml of Schales reagent and then boiled for 10 min. Optical density of the mixture was determined at 420nm.

The reaction mixture contained 300µl of 0.1% colloidal chitin, 150ul of crude enzyme, and 150µl of phosphate buffer. To each reaction mixture different metal ions in the form of salts CuSO₄·5H₂O, ZnCl₂, NiCl and CdCl₂ were added separately, in such a way that their final concentration in the reaction mixture was maintained at 0.1mM. Control of the reaction was containing no metal ions in it. The reaction mixture was then incubated at 55°C for 10 min. After incubation at 55°C, the reaction mixture was centrifuged at 10,000rpm for 5 min. The obtained supernatant was added with 50ml of distilled water, 10ml of Schales reagent and then boiled for 10 min. Optical density of the mixture was measured at 420nm.

Chitin hydrolysis zones visualization by using Congo red dye

The minimal salt medium, having 0.5% colloidal chitin, was prepared, poured into three plates and labeled it as M4, H1, and H4. The plates were spot inoculated with labeled bacterial cultures having OD 0.8 and were incubated at 37°C for 48 h. After incubation, the plates were flooded with 0.1% Congo red dye and kept for 15 min. The Congo red solution was then poured off and plates was further treated by flooding with 1MNaCl for 15 min. The zone of hydrolysis was then visualized in each plate.

Statistical analysis

Three independent measurements were taken for each experiment and data shown are average values of means ± standard deviation (SD). Control group was treated identically without exposure to any treatment.

Results and Discussion

Screening of chitinase producing bacterium

Soil and water samples were collected for the isolation of chitinase producing bacterial strains. In the first step of screening, 16 bacterial isolates were randomly picked and their chitinase activities were determined (Figure 1). In the second step, diameter of zone of hydrolysis on the basis of chitinase activity was measured. After comparing the enzyme activities and zone of hydrolysis of the 16 isolates, only three bacterial strains were selected for further investigation and designated as H1, H4 and M4 (Table 1).

Table 1: Chitinase activity and diameter of zone of hydrolysis in the presence of chitin by bacterial isolates.

Bacterial isolate	Zone of hydrolysis(nm)	Enzyme activity (O.D)
M4	1.0	0.292
H1	1.5	0.520
H4	2.0	0.648

Chitin, which is found abundantly in nature, is degraded by microorganisms including bacteria. They produce chitinases to degrade chitin to meet their nutrition requirements. Chitinases play an important role in the breakdown of chitin found in nature (Salwan and Sharma, 2018). The bacterial chitinases belong to the family of glycosyl hydrolases (Kumar *et al.*, 2018). Bacterial chitinases also contribute in the generation of nitrogen and carbon in the environment (Eichsorst *et al.*, 2018).

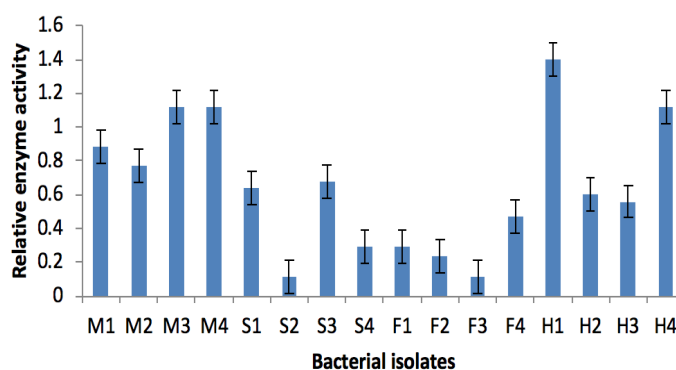


Figure 1: Comparison of enzyme activity of selected bacterial isolates under assay standard condition.

Bacterial identification and growth conditions

All the bacterial isolates were gram positive (Figure 2), spore former, and mannitol positive. H1 and M4 were positive for Voges-Proskauer test while H4 was negative.

Morphological characteristics are also given in Table 2. On the basis of biochemical characterization, H1 and M4 belong to *Bacillus subtilis* while H4 belongs to *Bacillus megaterium*. Bacillus genera include many species such as *B. fastidiosus*, *B. alvei*, *B. coagulans*, *B. marinus*, *B. mycoides*, *B. subtilis*, *B. cereus*, *B. alcalophilus*, *B. pumulis*, *B. circulans*, *B. licheniformis*, *B. polymyxa*, etc. which already have been reported to produce large variety of enzymes including chitinases (Bal et al., 2009). Although some work has been reported in literature with regard to *B. cereus*, but scanty information is available regarding chitinase production by *B. megaterium*. In the present study, all the isolates showed maximum growth at 37°C and at pH 7 (Figure 3a, b).

Table 2: Morphological and biochemical characteristics of bacterial isolates.

Physiochemical characteristics	<i>B. subtilis</i> (H1)	<i>B. megaterium</i> (H4)	<i>B. subtilis</i> (M4)
Shape	Irregular	Circular	Irregular
Texture	Smooth	Rough	Smooth
Elevation	Umbonate	Raised	Umbonate
Margin	Entire	Undulate	Entire
Color	White	Yellow	White
Gram's staining	+ve	+ve	+ve
Spore staining	+ve	+ve	+ve
Mannitol salt agar test	+ve	+ve	+ve
Voges-Proskauer test	+ve	-ve	+ve

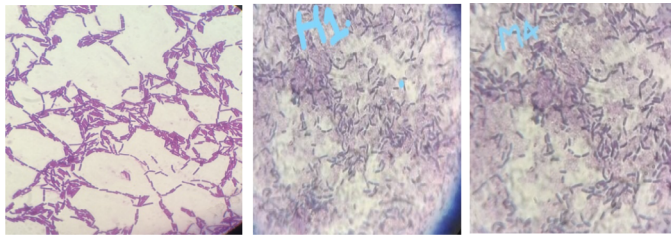


Figure 2: Gram's staining of the bacterial isolates H4, H1 and M4 respectively.

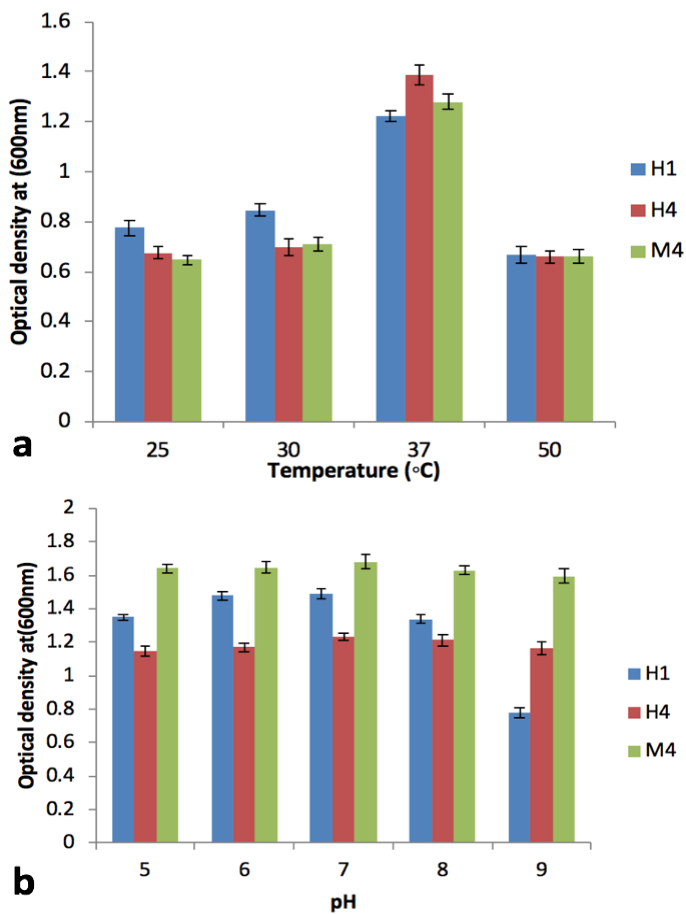


Figure 3: Determination of optimum temperature (a) and pH (b) of bacterial isolates.

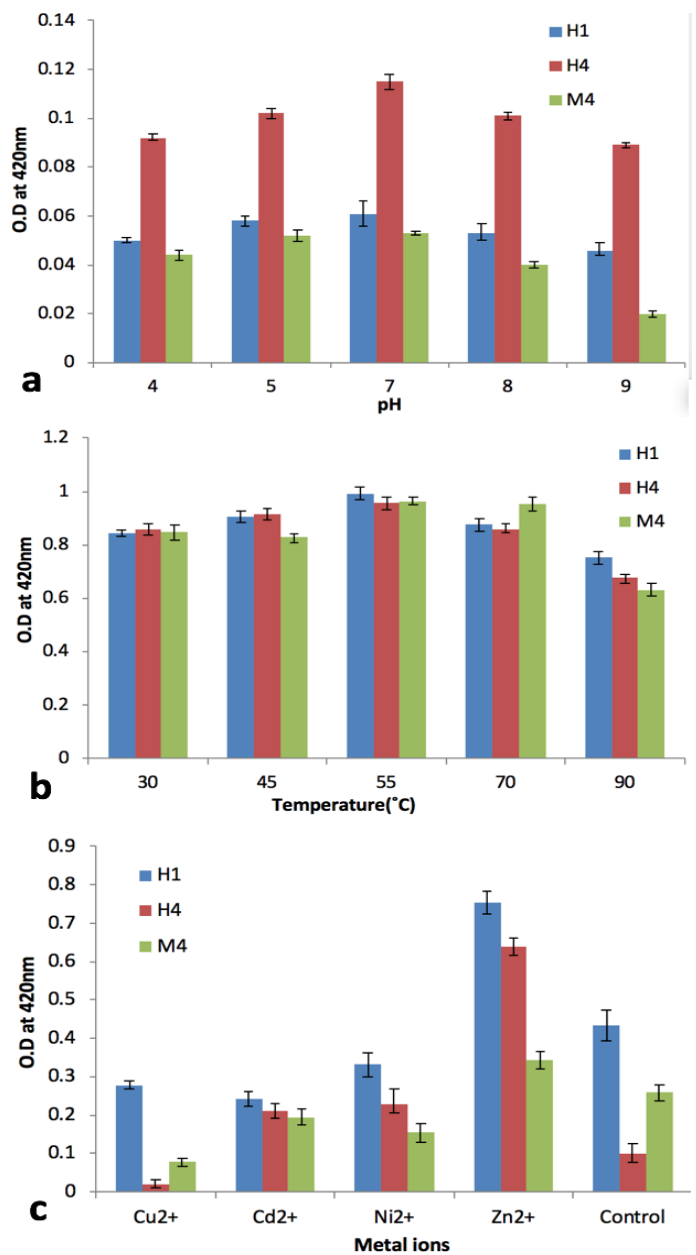


Figure 4: Effect of pH (a), temperature (b) and metal ions (c) on enzyme activity of bacterial isolates.



Figure 5: Zone of chitin hydrolysis of the bacterial isolates after the application of Congo red dye.

Chitinase characteristics

Chitinase from all the strains work efficiently at pH 7 (Figure 4a). Two isolates, H4 and M4, showed the maximum activity at 55°C which indicates that these isolates are mesophiles while H1 showed maximum activity at 70°C which depicts that H1 is extremophile (Figure 4b). Zn^{2+} enhances the chitinase activities in case of H1 and M4 while all the other metal ions, Ni^{2+} , Cd^{2+} and Cu^{2+} did not show any positive effects. However, in case of H4, metal ions such as Zn^{2+} , Ni^{2+} and Cd^{2+} showed stimulatory effects while Cu^{2+} did not show positive effect on chitinase activity (Figure 4c).

Chitinases considered as glycosyl hydrolases with the size ranging from 20kDa to 90kDa (Chen *et al.*, 2018). Chitinases can be categorized into two major classes which include endochitinases and exochitinases (Xu *et al.*, 2018). Chitinases have hydrolytic capacity towards chitin (Spasic *et al.*, 2018), and the breakdown of chitin by chitinase enzymes consequently produce low molecular mass multimers of GlcNAc such as chitotriose, chitotetrose and dimer diacetylchitobiose (Vaijyanthi *et al.*, 2016).

Visualization of chitin hydrolysis zones using congo red dye

The bacterial isolates H1, H4, and M4 were spot inoculated on the minimal medium, containing 2% colloidal chitin. Then incubated, flooded with 0.1% Congo red dye and zones of hydrolysis were measured (Figure 5). Bacterial isolates H1, H4, and M4 showed hydrolysis diameter of 0.9, 0.8 and 1.0 mm, respectively.

In conclusion, all bacterial isolates showed optimum growth at 37°C and pH of 7. All the bacterial isolates showed maximum chitinase activity at pH 7. Both *B. megaterium* (H4) and *B. subtilis* (M4) showed maximum enzyme activity at 55°C while *B. subtilis* (H1) showed highest activity at 70°C. In case of H1 and M4, Zn^{2+} showed stimulatory effect on enzyme activity while Ni^{2+} , Cd^{2+} and Cu^{2+} did not show any positive effect. While for *B. megaterium*, Zn^{2+} , Ni^{2+} and Cd^{2+} showed positive while Cu^{2+} showed negative effect on the enzyme activity. The isolated strains have promising potential to degrade complex chitin and ultimately will be helpful in cleaning the environment.

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