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Chitinolytic *Aeromonas* strain PZ6: Isolation Characterization, and antifungal potential profile

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ABSTRACT
Chitin is extremely resistant long-chain nitrogenous polysaccharide of n-acetylglucosamine an organic amide derivative of monomer glucose. Natural source of chitin found in lower organisms, fungi, crustaceans (shellfish, lobster, crab, shrimp, oyster etc.), also major component of the exoskeleton of insects like, beetle, earwig etc. Chitinase enzyme modifies chitin into chito-oligomers (CHOS), dimers, and monomers (n-acetylglucosamine). A total 100 different soil samples were screened using colloidal chitin agar medium for chitinolytic activity. Based on maximum zone of chitin hydrolysis, morphological, and biochemical characterization, PZ6 isolate was selected for further study and submerged fermentation (smf). For species level identification 16s rRNA sequencing method was used. The potent isolated chitinolytic bacterium was found to be Aeromonas spp. The selected spp. was analyzed for antifungal activity against plant pathogenic fungi to exploit its industrial significance.
Keywords: *chitin, chitinolytic enzyme, shrimp shell, chito-oligomers (CHOS).*

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INTRODUCTION

Each year, plant diseases cause an estimated 10–15% loss of the world's major crops, with direct economic losses of billions of dollars [1]. Around 35% of these plant diseases are caused by pathogenic fungi [2]. Over 19,000 varieties of fungi are parasitic [1] and cause different types of diseases [3].The disease caused by plant pathogenic fungi can affect a wide range of crops, resulting in crop yield reductions, higher production costs, reduced fruit quality, and even a loss of income for farmers [4]. Phytopathogenic fungi are inhibited by chitin-degrading bacteria [5]. Most of the common bacteria synthesize chitinolytic enzymes, and some unknown species function effectively in degradation of chitin [6], [7]. Numerous chitinase enzyme producing bacteria were reported, like *Bacillus pabli k1* [8] , *Serratia marcescens gps5* [9], *Serratia marcescens bjl200* [10], *Paenibacillus sp. Ik5* [11], *Paenibacillus sp. Nbr10* [12], *Listeria monocytogenes st2* [13], *Paenibacillus sp. B2*[14], *Streptomyces sp. Th-11*[15], *Streptomyces sp. M-20* [16], *Streptomyces albolongusatcc 27414* [17], *Streptomyces anulatus cs242* [18], *Streptomyces thermodiastaticus* [19], *Streptomyces pratensis klsl55* [20], *Saccharomyces sp.* [21], *Bacillus cereus tku006* [22], *Aeromonas hydrophila* [23], *Aeromonas hydrophila sbk1* [24], *Micrococcus sp., Vibrio alginolyticus h-8* [25], as potent producer of chitinases. Due to their aerobic nature, Chitinase producers are rarely isolated from aquatic ecosystem [26], [24]. Many researchers are now interested in screening for chitinases to determine the most efficient and environmentally friendly means of degrading chitin containing pathogenic fungi [27]. Some plant pathogenic fungi produce negative effect on plants health like, *Fusarium oxysporum* and *Fusarium solani* causing loss in tomato cultivation [28], another common plant disease is red rot also known as "cancer" in sugarcane and its cause by *Colletotrichum falcatum* [29], [30]. In the present study we focused on isolation of chitinase-producing bacteria and depicted antifungal activity of PZ6 isolate against several phytopathogenic fungi.

MATERIALS AND METHODS

Primary and Secondary screening of chitin-degrading bacteria.

A total number of 100 soil samples were collected aseptically from diverse regions of Gujarat. This soil samples were collected from the fish market Lal Darwaja Ahmedabad, Dastan farm Ahmedabad, Gandhinagar, and coastlines areas of Khambhat, Somnath, Tithal, Valsad Gujarat. The minimal agar medium containing colloidal chitin was used for screening for chitinase-producing bacteria. Bacterial isolates were selected based on a larger chitin hydrolysis zone after 96 hours of incubation. Based on maximum zone of chitin hydrolysis PZ6 isolate was selected and further tested for maximum enzyme production in *colloidal chitin* broth (CCB). Six potential bacterial isolates were selected on the basis of chitin hydrolysis zone after 24 to 48 hours of incubation at 37°C.These isolates were further screened for maximum enzyme production using colloidal chitin broth.

Morphological and biochemical characterization

The identification of isolate PZ6 was carried out based on their physiological, biochemical, and morphological characteristics according to the method describe in Bergey's Manual of Determinative Bacteriology [31]. The selected strain PZ6 was streaked on the chitin agar plate and cultivated for 24h at 37°C.Then, colony characteristics, including colony color, size, texture, shape, surface condition, pigment production status, etc., were recorded. Several biochemical tests were performed for biochemical identification of PZ6 isolate.

Preparation of colloidal chitin and colloidal chitin agar (CCA) plates:

The modified Hsu and Lockwood method were used to prepare colloidal chitin by using chitin powder (Hi media) [32]. For colloidal chitin making 40g of chitin powder was slowly mixed with 600 ml of hydrochloric acid and held at 30°C for 60 min with vigorous stirring. Chitin was precipitated as a colloidal suspension in 2 liters of water. The suspension was recovered using suction filtration on course filter paper and then washed with 5 liters of distilled water. The procedure was repeated three times until the pH of the suspension reached 4. The loose colloidal chitin was utilized as a substrate following the process as mentioned above [33].

Identification and phylogenetic analysis of the bacteria

Molecular identification was done by 16srRNA analysis. High yield of chitinase producer PZ6 was selected for the study. 125 ng of extracted DNA was used for amplification along with 10 pmol/µl of each primer added. PCR was performed in a 50 µl reaction mixture (125 ng genomic DNA) under the following cycling condition: 94°C for 3 min. Initial denaturation was done at 94°C for 1 min., further steps involve denaturation, 55°C for 1 min. annealing, 72°C for 2 min. Extension done at 72°C for 7 min, (Total 35 cycles).Agarose gel electrophoresis was carried out to detect PCR products. Sequencing was performed with the help of ABI 3130 genetic analyzer with a big dye terminator cycle sequencing kit v.3.16s forward - GGATGAGCCCGCGGCCTA and 16s reverse - CGGTGTGTACAAGGCCCGG, were used for the sequencing reaction. New sequences have been deposited in GenBank. A phylogenetic tree was created using weighbor with an alphabet size of 4 and a length of 1000[34], [35]. The analysis protocol was performed using software bdtv3-kb-devono. (seq-scape -v 5.2 software).

Enzyme assay and protein estimation

The protein content was estimated by Lowry's method by using bovine serum albumin (BSA) as standard in 20–200 µg/ml concentration [36]. Enzyme activity of chitinase was estimated based on the release of reducing sugar from colloidal chitin. Crude sample, precipitated enzyme, and purified enzyme (0.5ml) was added with equal volume of 1% (w/v) colloidal chitin in 0.1 M phosphate buffer with pH 7. After incubation at 37°C for 1 hour the reaction mixture was added with 1ml of DNS reagent and boiled for 5 min. One unit (u) of chitinase activity considered as the production of one µmol of N-Acetyl-D-glucosamine (GlcNac) per minute using a standard curve of N-acetyl-D-glucosamine (GlcNac) [37].

In vitro antifungal activity

The antifungal activity of the bacterial chitinase was evaluated against the phytopathogenic fungi *Colletotrichum gloeosporioides, Trichoderma asperelloides,* and *Fusarium oxysporum*, which are common contaminant of fruits such as mango, papaya, citrus, cashew, muskmelon, tomato, eggplant, pepper, etc. [28], [29]. These fungi can be easily cultivated on Sabouraud Dextrose Agar. Agar cup method was used to evaluate the antifungal activity [38]. The Sabouraud Dextrose Agar (SDA) media used and was prepared as per the standard composition given by Himedia. The culture filtrate of *Aeromonas* PZ6 strain (20µl) was placed in an 8mm well and allowed to diffuse through the well into the media seeded with fungal conidia, the plates were sealed with paraffin and incubated at 28$\pm$2ºC for 48 to 96hr. Antifungal activity was studied using antifungal drug Luliconazole (500ppm) as positive control for negative control well was loaded with sterile distilled water [39]. The zone of inhibition and the diameter of these zones were measured in mm.

RESULTS AND DISCUSSION

Screening chitinase producing strain

A total of 100 morphologically different chitinolytic bacteria were isolated from soil sample collected from different habitat of Gujarat, India. On the basis of colloidal chitin degradation and zone of clearance on CCA plate (Table 1), [40], Six isolates were selected for secondary screening in broth media and tested for enzyme activity, Designated as PZ1, PZ2, PZ3, PZ4, PZ5 and PZ6 (Table 2). The isolate PZ6 was identified as a potent chitinase producer on a plate supplemented with 1% colloidal chitin and incubated at 37°C for 24 to 48hours [23], [24], [41].

Table 1. Chitin hydrolysis efficiency of selected bacterial isolates.

Bacterial isolate	Zone of clearance (CZ/CS) *
PZ1	0.81
PZ2	0.90
PZ3	0.75
PZ4	0.68
PZ5	0.40
PZ6	1.40

*CZ = Colony size + Hydrolysis zone, CS = Colony size

Table 2. Chitinase activity of selected bacterial isolates

Culture No.	Chitinase activity (U*)
PZ1	4342
PZ2	4855
PZ3	3248
PZ4	2316
PZ5	1246
PZ6	5800

U*= Unit (One unit of chitinase activity considered as the production of one µmol of N-Acetyl-D-glucosamine (GlcNac) per minute) [47].

Morphological and biochemical identification of PZ6 isolate

The organisms were identified primarily by studying colony morphology followed by Gram staining [42]. Isolate produced circular colony, the elevation was convex, had smooth margin with creamy white color appearance. Cells having round end with diameter of 0.7-1.0µm x 1.5-1.8µm and were observed. Isolate PZ6 was Gram negative, motile, non-spore forming, and facultative anaerobic. Isolate PZ6 showed positive result for viz., citrate utilization test, catalase production, methyl red test, nitrate reduction test, and for the hydrolysis of chitin, starch, casein, Urea, and gelatin (table 3). The strain is capable of fermenting sugars like glucose, lactose, mannitol, and sucrose. For all the other tests, it depicted negative results. Biochemical characteristics of the isolate were found to be consistent with those described in Bergey's Manual for *Aeromonas sp.* [43]. Most of this species impart constitutive as well as induced chitinase production [23], [43].

Table 3. Biochemical and morphological profile of isolate PZ6

Characterization test	Bacterial reaction
Cell shape	Rod
Cell size	Small
Cell arrangement	Single and paired
Gram reaction	Gram-ve
Motility	+ve
Catalase test	+
Voges-Proskauer test	+
Methyl red test	+
Hydrolysis of Casein	+

Gelatin	+
Starch	+
Chitin	+
Urea	+
Utilization of citrate	+
Triple sugar iron test	+
H_2S production	-
Lipase	+
β – galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Tryptophane deaminase	-
Nitrate to nitrite	+
Gas from nitrate	-
Formation of indole	-
Acid from sugar fermentation	
Glucose	+
Sucrose	+
Mannitol	+
Lactose	+
D-melibiose	-
L-arabinose	+

Phylogenetic analysis

Multiple sequence alignment tools were used for phylogenetic analyses [44]. The BLAST program used to compare the sequences to the reference sequences [45]. Phylogenetic trees were constructed using neighbour joining after identifying the closest phylotypes [34]. The selected isolate PZ6 was closely identified as *Aeromonas* spp., 97% similarity with *Aeromonas spp (*Fig 3.1). (Sequence id: NR_042155.1).

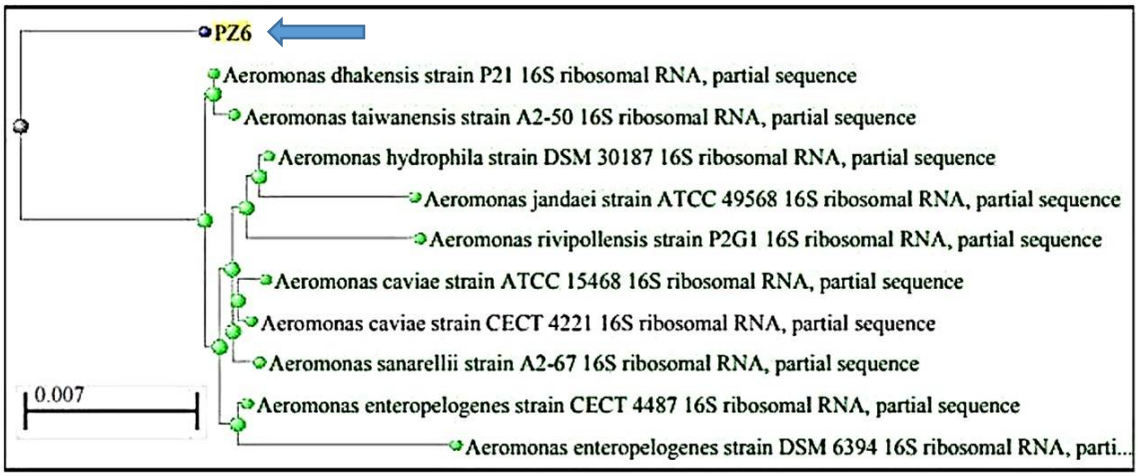

Figure 1. Phylogenetic tree of the strain PZ6

Chitinase enzyme activity:

Total six isolates tested were able to produce chitinase enzyme at different strength [46]. The selected isolates PZ1 to PZ6 showed different chitinolytic capacities, PZ6 exhibiting maximum chitinase activity under submerged fermentation broth with optimized parameters (Table 2). One unit of chitinase activity considered as the production of one µmol of N-acetyl-D-glucosamine (GlcNac) per minute [47].

Antifungal activity

The culture filtrate of *Aeromonas*sp PZ6 was active against the selected phytopathogenic fungi. Among these the chitinase developed maximum inhibitory activity (Table 4) on *Fusarium oxysporum* (20 mm in diameter), followed by *Colletotrichum gloeosporioides* (15 mm in diameter) whereas *Trichoderma*

asperelloides showed no clear antagonistic effect of the chitinase enzyme. Several studied recorded for bacterial antagonism against phytopathogens [48].

Table 4. Antifungal activity of crude chitinase derived from PZ6

Sr. No.	Organism	Code	Zone of Inhibition (mm)
1	*Colletotrichum gloeosporioides*	B1	15 ± 0.050
2	*Trichoderma asperelloides*	B1	00
3	*Fusarium oxysporum*	B1	20 ± 0.050

CONCLUSIONS

Many soil microorganisms have been identified and used for effective crumple of chitin waste. Among various living sources of chitinase bacterial chitinase enzyme consider to be a significant candidate to recycle naturally abundant chitin waste. In present study we have isolated and characterized chitinolytic bacterial strains. The potent chitinolytic strain was identified as *Aeromonas spp.* PZ6, a soil bacterium capable of depicting higher chitinase activity. The chitinase enzyme derived from *Aeromonas spp. PZ6* conferred antifungal activity against phytopathogenic fungi *Fusarium oxysporum* and *Colletotrichum gloeosporioides.* This suggests that these bacteria could be used in a wide range of environmental friendly ways to degrade plant pathogenic fungi.

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