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NOVEL ANTIFUNGAL CHITINASE FROM *AEROMONAS HYDROPHILA* HS4

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Abstract: Chitinase, a chitin degrading enzyme, has received increased attention due to its wide range of biotechnological applications including biocontrol of phytopathogenic fungi. The objective of the present study was to characterized the chitinolytic bacteria and evaluate the antifungal activity of chitinase produced by a newly isolated *Aeromonas hydrophila* HS4.

Key words: Chitinase, *Aeromonas hydrophila*, antifungal, *Shizophyllum commune.*

1. INTRODUCTION

Chitinase, a chitin degrading enzyme, found in a broad range of organisms *viz.* bacteria, fungi, higher plants, insects, crustaceans, invertebrates and some vertebrates [1]. Chitinolytic enzymes have wide-range of biotechnological and industrial applications including control of pathogenic fungi [2]. Among the known fungi, more than 8,000 can cause disease in plants and almost all the agricultural and horticultural crop species suffer severe yield losses due to fungal diseases [3]. In the Indian context, fungal diseases are rated as the most important factor contributing to yield losses in major cereal, pulse, fruits and oilseed crops. To control the plant pathogens, chemical pesticides are widely used in many countries including India. However, the non-degradable components of these compounds have accumulated over the years and entered the food chain causing higher toxicity in animals and environment [4-6]. The development of enzymatic and/or microbiological approaches for the control of plant pathogenic fungi is extremely recommended as they are safe, cheap and ecofriendly. Chitinase is considered to be important hydrolytic enzymes in the lysis of fungal cell walls [7, 8]. In the present study we evaluated the antifungal activity of chitinase produced by a newly isolated *Aeromonas hydrophila* HS4 against *Shizophyllum commune*.

2. Materials and methods

A. Bacterial culture and production media

The microorganism used in the study was isolated from soil sample of rice rhizosphere at Lucknow, India on the basis of maximum enzyme production in nutrient broth media [9]. Identification of the organisms was done primarily by studying the colony morphology followed by gram staining. Potential isolate was identified as *Aeromonas hydrophila* HS4 by using 16S rRNA analysis [10]. The chitinase activity was assayed by measuring reducing sugar released from colloidal chitin as per modified method of Toharisman et al. [11]. 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 equivalent per minute. Colloidal chitin was prepared from the chitin (Hi Media) by the modified method of Hsu and Lockwood [12]. The protein concentration was measured by using the method of Lowry et al. [13].

B. Characterization of identified isolate

The identified isolate was further characterized with respect to their antibiotics sensitivity and heavy metals tolerance capacity. Isolate was also checked for their plasmid mediated characteristics, such as whether antibiotic sensitivity, heavy metal tolerance and chitinolytic character are plasmid borne traits or due to chromosomal DNA, by curing of plasmid.

a. *Metal tolerance capacity*

Tolerance to metal ions was determined by minimal inhibitory concentration (MIC) technique

by agar dilution method (14). Stock solution of metals were prepared in a concentration of 10mg/ml and sterilized separately. Nutrient agar was amended with appropriate concentration of metal and poured into sterile petriplates and allowed to solidify. Test organisms were incubated at 37°C and examined for inhibition of growth. The metals used were iron (Fe), Manganese (Mn), Murcury (Hg), Cadmium (Cd), Copper (Cu), Magnesium (Mg), Cobalt (Co).

b. *Antibiotics susceptibility*

The antibiotics susceptibility of bacteria was studied on Muller-Hinton agar medium by disc diffusion methods (15). Muller Hinton Agar was prepared and poured into sterilized Petri plates. After solidification it was inoculated with test organisms by swabbing and disc of antibiotics were placed. The inoculated plates with discs were incubated at 37ºC and examined for inhibition of growth. Zone of inhibition was recorded after 24 hrs when lawn of bacteria was visible against clear zone of growth inhibition around the discs. Interpretation of results was done based on manufacturer's instructions (Hi-Media Pvt. Ltd, India). The antibiotics (ug) used were Nalidixic acid (30ug), Gentamicin (15ug), Tetracycline (10ug), Erythromycin (15ug), Ciprofloxacin (5ug), Doxycycline hydrochloride (30ug) and Ofloxacin (5ug).

c. *Plasmid curing*

For plasmid curing 10ml of peptone water supplemented with 20-100µg/ml acridine orange was inoculated with 0.1 ml of overnight broth culture and incubated at optimum growth temperature for 24 hrs (16). Appropriate dilutions of the culture were plate on nutrient agar to obtain single colony isolates. After overnight incubation at 37°C resulting colonies were tested for loss of chitinase production on colloidal chitin agar plates. The colonies, which were are not forming clear zone around it, were regarding as the cured ones.

$$\% \text{ Curing} = \frac{\text{No. of organism cured}}{\text{No. of organism tested}} \times 100$$

C. Antifungal activity of crude chitinase

The optimized broth media was inoculated with 1% inoculums of *Aeromonas hydrophila* HS4 and incubated at optimized fermentation condition as described in our previous publication [9]. The fermented culture broth was used as source of crude enzyme. In order to evaluate antifungal activity of crude enzyme, the experiment was conducted according to the method of Prapagdee et al. [17] based on radial growth of a filamentous fungi. The *Shizophyllum commune* culture filtrate was added into warm molten PDA (45°C) to give final concentration at 10, 20 and 30% (V/V) and placed until solidified. The control plate was added equal volume of sterile distilled water instead of culture filtrate. Each plate was seeded with 6mm diameter mycelial plugs taken from the margin of 5 day old *Shizophyllum commune* plate. Inoculated plates were incubated at 28°C and fungal growth was recorded at every 24 hrs until those of the control plate reaching the edge of the plate. The fungal growth inhibition was expressed as the percentage inhibition of radial growth in comparison to control as shown in following equation.

$$\text{Inhibition of radial growth (\%)} = \frac{\text{Diameter of control sample} - \text{Diameter of test sample}}{\text{Diameter of control sample}} \times 100$$

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, positive isolate HS4 selected for further study.

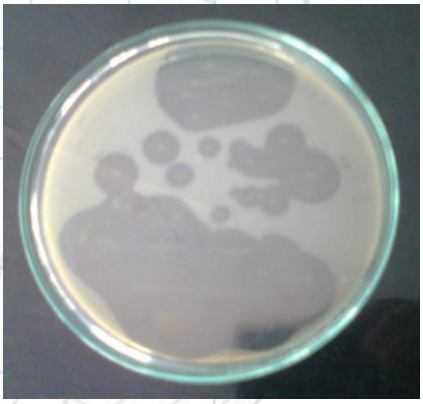

Fig 1: Screening of microorganisms for chitinase activity at (37°C)

After screening and observing the colony morphology, the culture giving the maximum zone of clearance was subjected to various biochemical tests like methyl red test, Voges-proskauer test, citrate utilization test, catalase or hydrogen peroxide test, Starch hydrolysis test, Gelatin test and motility test to confirm the characteristic features of the microorganism producing chitinase.

The isolate HS4 was gram negative, motile, non-spore forming and facultative anaerobes. The isolate HS4 showed positive result for the citrate utilization test, catalase production, methyl red

test and for the hydrolysis of chitin, starch, casein and gelatin.

A. Characterization of identified isolate

a. *Metal tolerance capacity*

The minimal inhibitory concentrations (MIC) of metals for Aeromonas hydrophila are given in Table 1. It is observed that both the strains have most tolerance capacity for Fe (60µg/ml) and Cd (60µg/ml), while least tolerance against Mn (10 µg/ml), Co (10 µg/ml), Mg (10 µg/ml) for *Aeromonas hydrophila.* Beside this, *Aeromonas hydrophila* have tolerance capacity for Hg (40µg/ml) and Cu (50µg/ml), respectively.

Table 1: MIC of metals among chitinolytic strain

Strain	**Metals (µg/ml)**						
	Fe	Mn	Hg	Cd	Cu	Mg	Co
HS4	80	20	50	80	60	20	20

b. *Antibiotics susceptibility*

The results for antibiotic susceptibility are given in Table 2. When cultures were subjected to the various antibiotics, it was found that *Aeromonas hydrophila* is resistant to Erythromycin while it was sensitive to Nalidixic Acid, Gentamicin, Ciprofloxacin, Doxycycline Hydrochoride and ofloxacin. However, it shows intermediate characteristics for Tetracyclin.

Table 2: Antibiotic susceptibility pattern in chitinolytic strains

Antibiotics	Disc potency (ug)	*Aeromonas hydrophila*
Nalidixic Acid	30	S
Gentamicin	15	S
Tetracycline	10	I
Erythromycin	15	R
Ciprofloxacin	5	S
Doxycycline Hydrochoride	30	S
Ofloxacin	5	S

Resistant (R)/ Sensitive (S)/ Intermediate (I)

c. *Plasmid Curing*

Curing of plasmid was done using agent acridine orange (10-100µg/ml), which gets intercalated between the bases of DNA and inhibits replication of plasmid without inhibiting chromosomal DNA replication. Such inhibition can lead to loss of plasmid. Out of 50 colonies tested on CCA media after curing, only 8% colonies of *Aeromonas hydrophila* showed clear hydrolysis zone shows 16% curing and it indicated that the chitinase produced by *Aeromonas hydrophila* is a constitutive protein.

B. Enzyme production

The maximum chitinase production was obtained at 37°C and pH 7 after 48 hrs incubation [9]. The fermented broth was centrifuged at 10,000g for 10 minutes and clear supernatant was used as a crude enzyme.

C. Antifungal activity of chitinase

Chitinolytic enzymes are able to lyse the cell wall of many fungi. *Shizophyllum commune* is a filamentous fungi that include a number of economically important plant pathogenic species. The crude chitinase from *Aeromonas hydrophila* HS4 (10, 20 and 30%) was tested for antifungal activity by their ability to inhibit hyphal extension growth of *Shizophyllum commune*. The result of 30% culture filtrate showed stronger inhibitory activity (47.59%) towards potent phytopathogen, *Shizophyllum commune* in comparison of control after 3 days of incubation (Fig. 2). The culture filtrate of 10 and 20% also showed antifungal activity and inhibit 36.48 and 42.09% of fungal growth, respectively. The results from the experiment indicated that chitinase from *Aeromonas hydrophila* HS4 can be used as a biocontrol agent against phytopathogenic fungi.

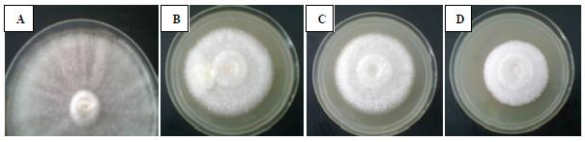

Fig. 2: Growth inhibitions of *Shizophyllum commune* by culture filtrate of *Aeromonas hydrophila* HS4. (a) Control, (b) 10%, (c) 20%, (d) 30% of culture filtrates.

Findings: The study shows one most attractive feature of *Aeromonas hydrophila* HS4 was its 16% plasmid curing behaviour in presence of strong curing agent, which reflects that protein is constitutive type. From the result it is also concluded that soil sample has proven as best source for isolation of bacteria for extracellular chitinase production. chitinase from 30% culture filtrate of *Aeromonas hydrophila* HS4 showed stronger inhibitory effect (47.59%) towards potent phytopathogen, *Shizophyllum commun*e.

Conclusion: The results concluded that the chitinase along with its producing microorganism,

Aeromonas hydrophila HS4, may be used as a biocontrol agent against phytopathogenic fungi.

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Competing interests: The author(s) declare that they have no competing interest.

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