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Isolation of Chitinase Producing *Streptomyces Sp.* M1 for Recycling of Fungal Biomass

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ABSTRACT

Chitinases are glycosyl hydrolase which catalyse degradation of chitin. Microorganisms produce variety of chitinases that exhibits different substrate specificity and applicability in various fields. In the present study 107 actinomycetes obtained from water and soil samples of various habitats of Maharashtra, were screened for chitin hydrolysis activity on colloidal chitin agar. 30 isolates with prominent chitin hydrolysis ability were subjected for quantitative screening in colloidal chitin broth. 10 isolates with highest chitin hydrolysis used to study effect of fungal, insect, shrimp chitin and crab chitin on chitinase production. The isolate M1 from Mumbai sea water showed a good chitinase activity with all types of substrates. The highest activity was observed in the presence of fungal chitin- 3.8 U/ml followed by insect chitin- 3.7 U/ml, shrimp chitin- 3.5 U/ml and crab chitin- 3.4 U/ml. Isolate M1 is further identified microscopically and 16S rRNA sequencing as *Streptomyces* sp.

Keywords: Chitin, Chitinase, *Streptomyces* sp, substrate specificity.

I. INTRODUCTION

Chitin is N-acetyl glucosamine polymer which is estimated to be produced *as second most abundant polysaccharide* followed by cellulose. Chitin occurs in 3 allomorphs α, β and γ. These forms of chitin have been found in different parts of the same organism which shows that they have different functions and not used for grouping of animals. In insects, α form is found in chitoneous cuticle whereas the other forms are found in cocoons (Kenchington, 1976; Peters, 1992). In animals chitin chains are associated with proteins and $CaCO_3$ and in fungi associated with glucans in the form of microfibrills.

Chitinases (EC 3.2.1.14) are enzymes that hydrolyse chitin and yields chitooligosaccharides and N acetyl glucosamine (Patil, 2000; Dahiya, 2006). These products found increasingly application in diverse fields of medicine, waste management, food technology and agriculture (Rafaelas, 2012). They are widely applied in biological control of fungal pathogens and insects, in preparation of mycolytic enzymes, formation of fungal protoplast (Bansode, 2006; Wang, 2009).Chitinase are produced by many organisms such as bacteria, virus, higher plants and animals and play important physiological and ecological roles. Production of chitinase is higher in microbes as compared to other chitinase producers (Matsumoto, 2014). From several microbial sources fungi, bacteria and actinomycetes are extensively studied sources for chitinase production. Among bacterial sources species of *Serratia, Vibrio* and *Bacillus* has shown highest chitinase activity, while from several fungi *Myrothecium, Trichoderma* sp and *Aspergillus* sp have proved to be promising in chitinase production (Stoykov, 2015). Bacterial and fungal chitinases belongs to 18 glycosyl hydrolase whereas chitinase from actinomycetes belongs to 19 chitinasefamily which is found in plants. These 19 family chitinases are known to have more antifungal activity as compared to other. Nearly 90 % of actinomycetes have ability to utilize chitin. *Streptomyces* species among actinomycetes are known as highest chitinase producers. Until now several species of *Streptomyces* are reported for chitinolytic enzyme production as *S. antibioticus, S. griseus, S. plicatus, S. lividans, S. aureofaciens, S. halstedii and S. Virdificans* (Kolla, 2009). Among rare actinomycetes *Nocardiopsis, Streptosporangium* sp, *Micromonospora* and *Microbiospora* have been reported as chitinase producers (Nawani, 2007; Shirlin, 2016). Fungi are commonly used in many industrial fermentations, where after production of products, the fungal biomass is

discarded as waste. Such type of discarded biomass can be reused by using chitinolytic enzymes as substrate for production of economically important chitooligosaccharides.

Since large number of chitinases are being reported but there are certain limitations of them for various application as same enzymes are unable to act effectively on chitin from different sources (Sharma, 2011). Due to extracellular location of chitinase, the actinomycetes from new environmental conditions may give enzymes with biochemical properties which are important for their industrial applications. So there is need for new chitinases from new habitats having high and broad spectrum of activity.

In view of this, present study planned for the isolation and screening of broad substrate specific chitinase producing actinomycetes from various environments and identification of potential actinomycetes.

II. METHODS AND MATERIALS

Collection of Samples

Three rhizosphere soil samples from sugarcane, banana and turmeric cultivated agriculture fields were collected from Nanded district (19' 16^{o} N, 77' 27^{o} S) Maharashtra by composite random sampling. Two fresh water samples from Godavari River, Nanded (19'7^{o} N, 77'17^{o} S) and pond, SRTMUN, Nanded (19'6^{o} N, 72'17^{o} S) were collected. Two marine sample from Mumbai (19'4^{o} N, 72'52^{o} S) and Ratnagiri (16'59^{o} N, 73' 18^{o} S) were also collected. All samples were collected in sterile polythene bags and stored at 4 ^{o}C till further processing.

Enrichment and isolation of actinomycetes

1 gm of each soil samples was suspended in 10 ml of sterile physiological saline and vortexed at high speed and the clear suspension, supernatant was used for isolation of actinomycetes. Both soil suspensions and water samples were subjected for heat treatment at 70 ^{o}C for 15 minutes in water bath. 1 ml of each sample was inoculated in starch casein broth containing nystatin and tetracycline (50µg/ml each). The flasks were incubated at 30 ^{0}C for 7 d. 0.1 ml aliquot of each enriched sample was spreaded on starch casein agar (SCA) (Mohseni, 2013). Plates were incubated at 30 ^{0}C for 7 days. A total of 107 well isolated morphologically different colonies were selected and purified on same medium by repeated sub-culturing of actinomycetes. The purified cultures were maintained on SCA slants at 4 ^{0}C.

Screening of Actinomycetes Chitinolytic Activity

Actinomycetes isolates were screened for chitinase production on colloidal chitin agar as per the method described by Bansode et al, (2006). Colloidal chitin was prepared by adopting modified protocol of Murthy et al, (2012) from chitin flakes (Himedia). For qualitative screening, each isolate was spot inoculated on colloidal chitin agar plates. The plates were incubated at 30 ^{0}C for 7 days. The chitinolytic activities of isolates were determined by the presence of clear zone of chitin hydrolysis around colony growth. The isolates showing significant size of zone were further subjected for quantitative screening. The isolates were individually inoculated in colloidal chitin broth and incubated at 30^{0} C for 5 days with monitoring of enzyme production at 24 hours intervals. The individual culture broths were centrifuged at 8000 rpm for 10 minutes at 4^{0} C. the cell free supernatants were used as source of crude chitinase.

Chitinase Assay

The chitinase activity of crude enzyme was determined by reduction of 3, 5-dinitro salicylic acid in the presence of N acetyl glucosamine released by enzymatic hydrolysis of colloidal chitin (Rojas- Avelizapa, 2001). The reaction mixture used in assay was composed of 1 ml crude enzyme solution; 1 ml colloidal chitin (0.5% aq) and 1 ml citrate buffer (0.1 M, pH 7.0). The assay mixture was incubated at 37^{o}C for 60 minutes. After incubation, the reaction was terminated by adding 1 ml dinitrosalicylic acid reagent. The reaction mixture was kept in boiling water bath for 5 minutes the absorbance was recorded at 530 nm. One unit of chitinase activity (U) was defined as the amount of enzyme required to release 1 u mol of N acetyl glucosamine per ml per minute under standard assay conditions.

Effect of Substrate on Chitinase Production

Fungal chitin, insect chitin, shrimp chitin and crab chitin were used to study their effect on chitinase production by selected actinomycetes.

Fungal chitin extracted by protocol given by Krishnaveni et al (2015). For fungal (*Aspergillus flavus*) submerged cultivation, 5 % spore suspension was inoculated in 1000 ml potato dextrose broth medium and flasks were incubated at 28^{o}C on orbital shaker at 150 rpm for 96 hours. The mycelium was harvested, washed twice with distilled water and air dried in oven at 40^{o}C and subjected to deprotinization with NaOH (2 % w/v) at 90^{o}C for 2 hours. The crude chitin was separated by centrifugation at 8000 rpm for 15 minutes. The precipitate was washed with distilled water and decolorized with ethanol and acetone, air dried at room temperature.

For extraction of insect chitin, collected insects (Family-Sphaeriusidae) were washed several times with water and kept at 50 ^{o}C overnight in oven. The dried insect shells were grinded in mixer. The obtained powder was deminerialized with 4 M HCl at 75^{o}C for 2 hour, and then rinsed with distilled water. Deprotinization was carried out by using alkaline treatment with 4 M NaOH solution at 150^{o}C for 2 hours. The crude chitin was filtered and decolorized with H_2O_2 and 33% HCl (9:1 v/v), finally washed with distilled water and dried at 50^{o}C in dry heat oven (Kaya, 2013).

The isolates were inoculated in colloidal chitin broths prepared with different chitin sources and the flasks were incubated under previous stated conditions and subjected for chitinase extraction. The chitinase assay was performed as per the method described earlier.

Identification of Potent Chitinase Producer

Of the 10 isolates, M1 Mumbai marine water isolate showed highest chitinase activity with all chitin sources and was selected as efficient isolate for further studies. The isolate M1 identified microscopically and by 16S rRNA sequence analysis. For 16s rRNA sequencing DNA extraction was carried out using Hipura bacterial genomic DNA purification kit (Himedia). The isolated DNA was subjected to PCR amplification using Biometra thermal cycler (T personal-48). The pair of primers used for amplification is 235f-CGCGGCCTATCAGCTTGTTG and 878f CCGTACTCCCCAGGCGGGG.

III. RESULTS AND DISCUSSION

Enrichment of all water and soil samples from various habitats of Maharashtra for isolation of actinomycetes was performed in colloidal chitin broth medium in which colloidal chitin act as sole source of carbon. A total of 107 actinomycetes were selected, of which 57 isolates were from water samples (16 marine and 41 fresh) and 50 from soil samples (14 banana, 17 turmeric, 19 sugarcane) on starch casein agar medium (Table- 1). Similarly, Saadoun et al, (2009) obtained 231 *Streptomyces* isolates from different soil samples in Jordon, screened for chitinase production which was further subjected for chitinase screening. Mane et al (2009) also used 80 actinomycetes isolated from Krishna River, Satara (M.S), India for chitinase production.

All the actinomycetes were screened for chitinase production on colloidal chitin agar plates. The chitinase positive isolates were selected on the basis of zone of chitin hydrolysis around spot. Primary screening results showed that out of 107 isolates only 30 isolates have detectable chitinolytic activity. Of these selected isolates 7 were from fresh water, 5 marine water sample, 7 each from turmeric and sugarcane cultivated rhizosphere soil and 4 isolates were from banana rhizosphere soil (Table-2). Pattanapipipaisal et al (2012), primary screening of 283 strains for chitinolytic activity showed presence of 45 strains with significant chitinase production (>5 mm zone). These 30 chitinolytic actinomycetes were further subjected to quantitative screening. Quantitative screening of isolates revealed highest chitinase production by 10 isolates as G12, G25, M1, M3, M9, B10, B16, T17, S14 and S17, in the range of 2.9 U/ml to 3.7 U/ml. Chitin utilizing ability of both marine and fresh water isolates were higher as compared to soil sample isolates (that might be due to amount of chitin present in specific habitats). *Streptomyces* species PTK 19 isolated from shrimp cell decomposed soil showed maximum chitinase production (1.84U/ml) on 0.4% colloidal chitin concentration (Thiagarajan et al, 2011). Mane and Deshmukh (2009) reported chitinase activity of *S. canus*, *Micromonospora brevicatiana* and *S. psedogriseolus* isolated from Krishna river as 3.1 U/ml, 3.4 U/ml, and 4.2 U/ml respectively. The highest

chitinase producing 10 isolates were subjected to check the effect of different chitin substrates on production of chitinase.

Table1: isolation of actinomycetes from various sites of Maharashtra

No	Sample	No. of isolates	Chitinolytic actinomycetes	% of Chitinolytic actinomycetes
1	Mumbai marine water	11	8	72
2	Ratnagiri marine water	5	3	60
3	Godavari fresh water	25	20	80
4	Pond fresh water	16	8	50
5	Turmeric rhizosphere soil	17	14	82
6	sugarcane rhizosphere soil	19	15	79
7	Banana rhizosphere soil	14	11	78

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Table 2: Screening for chitinase production

No.	Actinomycetes Isolate	Zone Of Chitinolysis (mm)	Chitinase production (U/ml)
1	G4	15	2.7
2	**G12**	**18**	**2.9**
3	G14	19	2.5
4	G20	18	2.4
5	**G22**	**16**	**3.4**
6	**G25**	**19**	**3.5**
7	**M1**	**19**	**3.6**
8	**M3**	**16**	**3.7**
9	M4	20	2.5
10	M9	17	2.2
11	R2	17	2.2
12	P6	18	2.6
13	T1	17	2.3
14	T4	18	2.6
15	T8	16	2.0
16	T10	17	2.2
17	T14	18	2.6
18	T16	19	2.7
19	**T17**	**18**	**3.0**
20	S4	16	2.3
21	S10	19	2.7
22	S12	15	2.1
23	**S14**	**17**	**2.9**
24	S16	18	2.6
25	S17	19	2.7
26	**S19**	**17**	**3.7**
27	B2	16	2.4
28	**B10**	**17**	**3.2**
29	B11	17	2.5
30	**B16**	**18**	**3.6**

Table 3: Screening of different chitin substrates on production of chitinase

N0	Isolate	Chitinase Activity (U/ml)			
		Shrimp chitin	Crab chitin	Insect chitin	Fungal chitin
1	G12	2.9	2.9	2.9	3.8
2	G25	3.4	3.2	3.9	3.5
3	**M1**	**3.5**	**3.4**	**3.7**	**3.8**
4	M3	3.6	3.0	3.8	2.8
5	M9	3.7	2.8	2.8	3.0
6	B10	3.6	3.4	2.7	2.9
7	B16	3.2	3.1	3.3	3.1
8	T17	3.0	3.5	3.3	3.3
9	S14	2.9	3.4	3.1	3.3
10	S17	3.7	3.5	3.7	3.4

All selected isolates showed ability to use types of chitin sources in varying efficiencies (Table-3). The isolate M1 from Mumbai sea water sample was found to be able to utilize all types of chitin sources with highest chitinase production in presence of fungal chitin- 3.8 U/ml followed by insect chitin- 3.7 U/ml, shrimp chitin- 3.5 U/ml and crab chitin- 3.4 U/ml (Fig-1).

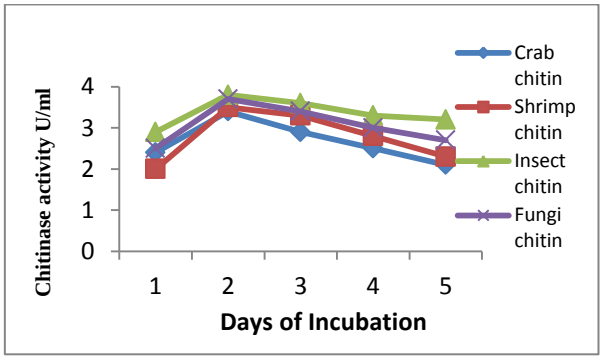

Figure 1: Chitinase production by isolate M1.

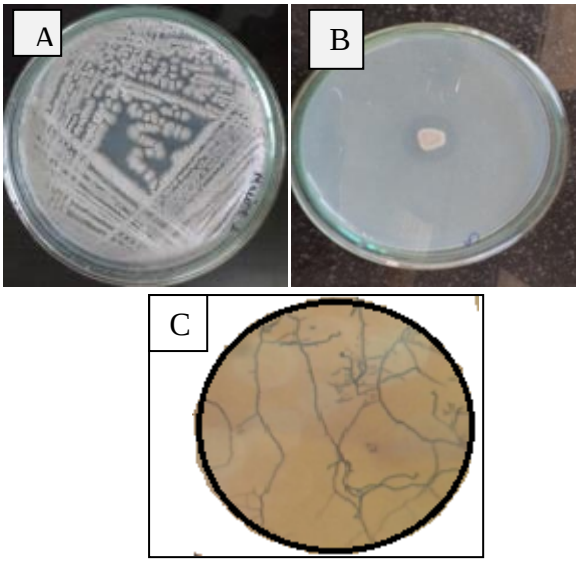

Figure 2: A. Isolate M1 on starch casein agar.
B. Isolate M1 showing zone of chitinolysis.
C. Microscopic observation of isolate M1.

Highest chitinase production from M1 was followed by Godavari fresh water isolate G25 and sugarcane rhizosphere isolate S17. The M1 was selected as potent chitinase producer and was identified microscopically and by 16S rRNA sequencing (Fig-2). The microscopic examination of M1 showed presence of spirals and hook, which are characteristics of *Streptomyces* genus. Similarly, 16S rRNA sequencing reveals maximum similarity of M1 belongs to genus *Streptomyces*. Until now chitinolytic Streptomyces are reported from various habitats like mangoline soil, river water, animal dung, rhizosphere and sea shores. Streptomyces species are considered as potential chitinase producers. However, rare actinomycetes as *Nocardiopsis*, *Streptosporangium* are not considered as major source of chitinase (Tsujibo, 2003).

IV. CONCLUSION

The present findings suggest that actinomycetes from soil and water samples are good source of chitinase. *Streptomyces* sp M1 isolated from Mumbai marine water sample have highest potential for chitinase production. Chitinase from *Streptomyces* sp M1 is produced in short incubation period with broad substrate specificity and also showed highest activity with fungal chitin. So chitinase from *Streptomyces* spM1 can be act as good candidate for its application in recycling of industrial fungal biomass waste with production of economically important chitooligosaccharides. However, this chitinase requires further studies of kinetics and other factors for its large scale application.

V. REFERENCES

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