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OPTIMIZATION OF CHITINASE PRODUCTION FROM *STREPTOMYCES MACROSPOREUS* M1.

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ABSTRACT: Chitinases catalyze hydrolysis of chitin and produce N- acetyl glucosamine and oligomers. Both chitinase and their products are commercially important in pharmaceutical, agriculture and in waste management. The present study concerned with optimization of chitinase production from *Streptomyces macrosporeus* M1 and its application as antifungal against phytopathogens. Chitinase production was optimized by studying effect of incubation period, temperature, initial pH of media, nitrogen source and metal salts under stationary conditions. The optimum chitinase production was found on 3 rd day of incubation, at temperature 40°C and pH 6. KNO_3 (0.2%) and $CaSO_4$ (10 mM) were act as effective nitrogen source and metal salt respectively for chitinase production. The combined effects of six parameters (pH, temperature, colloidal chitin concentration, nitrogen source, metal salt and incubation period) were studied in 12 different experimental combinations, set 9 showed 2.62 fold highest increase in production over control. Optimum medium for chitinase production composed of colloidal chitin 1.5 %, CaSO4 10 mM, KNO_3 0.2%, pH 6 and when incubated at 40°C for 3 days, yields 9.2 U/mL by *Streptomyces macrosporeus* M1. This produced chitinase showed antifungal activity against *Ustilago maydis, Rhizopus oryzae, F. oxysporium,* and *A. flavus* showing possibility of exploring it as biocontrol against phytopathogens.

KEYWORDS: Chitinase, *Streptomyces macrosporeus* M1, Optimization, Antifungal.

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1.INTRODUCTION

Chitin is linear polymer of N-acetyl glucosamine and it is component of fungal cell walls, arthropod exoskeletons, insect cuticles, shell fishes and plants. Chitinase enzymes (EC 3.2.1.14) are the

hydrolases family which catalyzes conversion of chitin to its monomers by breaking β- 1, 4 linkages. Chitinases from microorganisms has extensive applications in biocontrol, medical fields and degradation of pollutants, SCP production, biopesticides and protoplast isolation [1, 2]. Chitinase production from microorganisms is higher as compared with other organisms [3]. Among microorganisms actinomycetes have been widely distributed group in the production of metabolites including enzymes with high applicability [4]. *Streptomyces* sp were extensively studied for production of these economically important products because of their wide range of abilities of synthesis of useful secondary metabolites. The large population of *Streptomyces* showed chitin utilizing ability with highest production of chitinase [5]. *Streptomyces* sp. have been extensively studied and used as biocontrol agents against many plant pathogens [6]. A high toxicity of fungal pathogens and their increasing resistance to existing fungicides needs use of chitinase like enzymes for their control. New species of *Streptomyces* may have ability to produce chitinase with new properties of high productivity and novel kinetic properties. Chitinase production in microorganisms is controlled by growth factors as nitrogen, metal salts, temperature, pH, etc of production medium. Improved enzyme production can be achieved by manipulating growth conditions up to level that gives optimum production [7]. Knowledge of optimum parameters for enzyme production will give maximum production in less time with low cost for its large scale production and application.

This is the first report for study of chitinase production from *Streptomyces macrosporeus* M1 isolate from Arabian Sea (Mumbai) water sample. Therefore the present work conducted to study influence of different culture conditions on production of chitinase in submerged cultures and antifungal activity of produced chitinase.

2. MATERIALSAND METHODS

2.1. Microorganism identification

The potent chitinolytic actinomycetes *Streptomyces* sp. M1 was isolated from Arabian Sea, Mumbai water sample, India and identified by 16S rRNA sequencing [8]. The sequence data was used to carry out BLAST alignment search tool of NCBI Genebank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA5.

2.2. Optimization of submerged chitinase production by *Streptomyces macrosporeus* M1

For chitinase production, the culture inoculum was prepared by transferring a colony from *Streptomyces macrosporeus* M1 agar plates to 100 ml colloidal chitin broth in 250 ml flask, the culture broth was incubated on orbital shaking incubator at 30 ^{o}C, 150 rpm for 48 hours.

2.2.1. Effect of incubation period on chitinase production

The colloidal chitin broth (colloidal chitin- 5g, $MgSO_4.7H_2O$- 0.5g, K_2HPO_4- 0.7g, KH_2PO_4- 0.3g, $FeSO_4.7H_20$- 0.01g, $MnCl_2$- 0.001g, NaCl- 0.03%, yeast extract- 0.2% and distilled water 1000 ml)

was inoculated with 1% inoculums and incubated up to 6 days at 30 ^{o}C. For the determination of chitinase production 1 ml of aliquots was withdrawn after every 24 hours. The cell free supernatant was obtained and used as crude chitinase for chitinase assay [8].

2.2.2. Effect of initial pH and temperature on chitinase production

The initial pH of colloidal chitin medium were adjusted from 4 - 10, with 1 % inoculum of *Streptomyces macrosporeus* M1 was grown for 6 days at 30 ^{o}C. The chitinase production was estimated as described above. Similarly, the optimum temperature for chitinase production was observed by incubating the isolate at temperature ranging from 20 ^{o}C to 50 ^{o}C.

2.2.3. Effect of nitrogen sources on chitinase production

The effect of different nitrogen sources on chitinase production was studied by replacing yeast extract in colloidal chitin broth medium with 0.2% different nitrogen sources as urea, ammonium sulfate, casein, KNO_3, malt extract and peptone. The medium with different nitrogen sources were inoculated with 1% inoculum and incubated for 6 days. 1 mL of aliquots was withdrawn after every 24 hours for assessment of chitinase production.

2.2.4. Effect of metal salts on chitinase production

The effect of various metals as $CaSO_4$, $CuSO_4$, $BaSO_4$, $ZnSO_4$, $MnSO_4$, $FeSO_4$ and $MgSO_4$ was studied supplemented to colloidal chitin broth each at concentration of 10 mM.

2.2.5. Combined effect of process variables for optimized chitinase production

To evaluate the combined effects of optimized parameters as colloidal chitin concentration, nitrogen source (KNO_3), pH, temperature, metals ($CaSO_4$) on production of chitinase, 12 experimental sets were conducted as per table 1 [9].

2.3. Antifungal activity of chitinase

Antifungal activity was detected by well diffusion method [10]. The wells were prepared in PDA medium with sterile borer. 50 µL and 100 µL of crude enzyme samples were introduced in the well in each fungal plate and heat inactivated (boiled) enzyme kept as control. The plates were incubated for 72 hours at 30 ^{o}C. The diameter of zone of inhibition was recorded. The test pathogens used for assay are *Ustilago maydis, Rhizopus oryzae, F. oxysporium* and *Aspergillus flavus* which are collected from MTCC, India.

3. RESULT AND DISCUSSION

3.1. Microorganism identification

The 16S rRNA sequence compared with the database and it gave 99 % similarity with *Streptomyces macrosporeus* NBE12. Based on this, *Streptomyces* sp. M1 was named as *Streptomyces macrosporeus* M1 (Figure 1).

3.2. Optimization of chitinase production

Chitinase production by *Streptomyces macrosporeus* M1 starts within 24 hours of incubation and reaches to maximum up to 3rd day after that its production gradually reduces. Chitinase production

starts early may be because of chitin is the only source of carbon in the medium which is utilized for growth (figure 2). Similarly, *Streptomyces* strain S_{242} was produced optimum chitinase on 4th day of incubation [11]. Maximum chitinase production of *Streptomyces* sp. PTK19 was recorded on 6th days after incubation and then decreased [6]. Production optimization studies by three actinomycetes revealed highest chitinase production on 5th day of incubation which is constant up to 6th day [12].

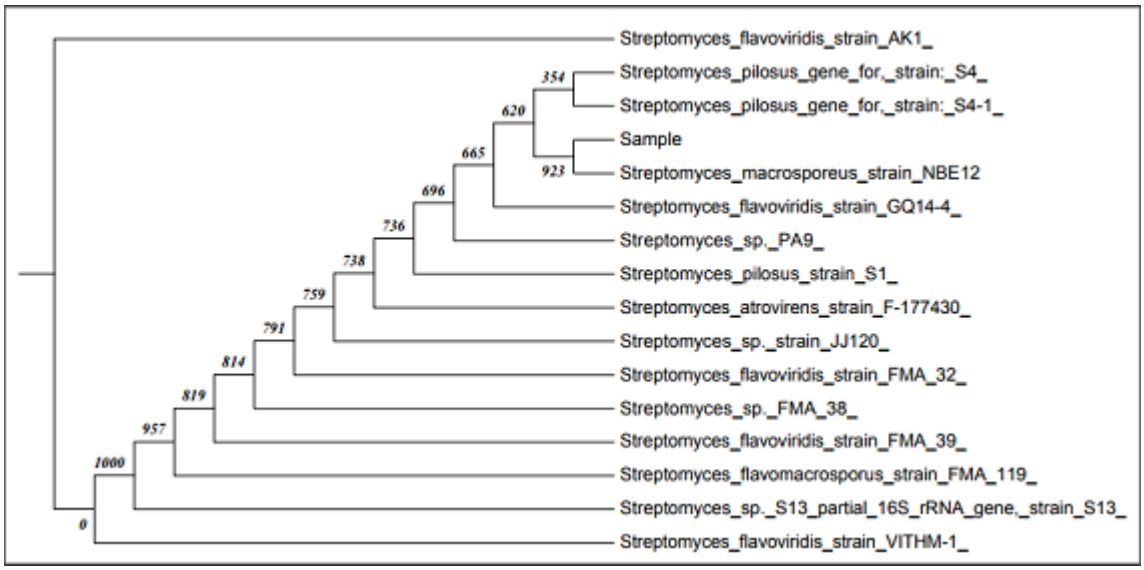


Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences of *Streptomyces* sp. M1.

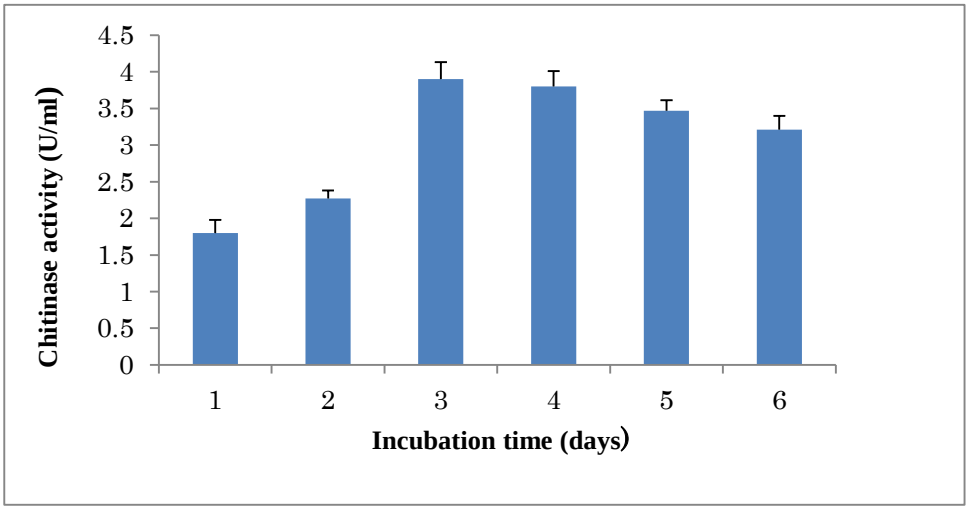


Figure 2. Effect of incubation period on chitinase production.

(Vertical bars represents standard deviation, n= 3).

Initial pH of the production medium changes due to synthesis of new metabolites during chitinase production. Optimum pH for chitinase production was found as 6 with 4.21 U/ml chitinase activity **(**Figure 3**)**. As pH increases or decreases to optimum chitinase production reduces. Studies by Saadoun et al showed maximum chitinase activity at pH 7. In contrast, *Streptomyces albus* strain FS2 gives optimum chitinase production at pH 8 [13]. The optimum temperature for chitinase production is 40 ºC as 5.16 U/ml which is also constant at 45 ºC (Figure 4**)**. Opposingly, *Streptomyces* strain S_{242} showed optimum chitinase production at 35 ºC and 40 ºC chitinase become completely inactive

[11]. Optimum temperatures for chitinase production by *Streptomyces* sp were lies between 30 ºC to 40 ºC [14, 15, 16].

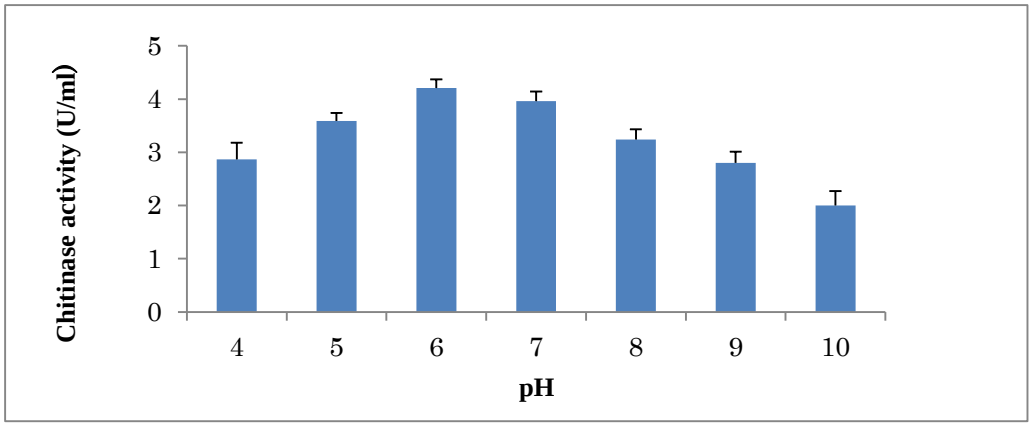


Figure 3. Effect of pH on chitinase production.

(Vertical bars represents standard deviation, n= 3).

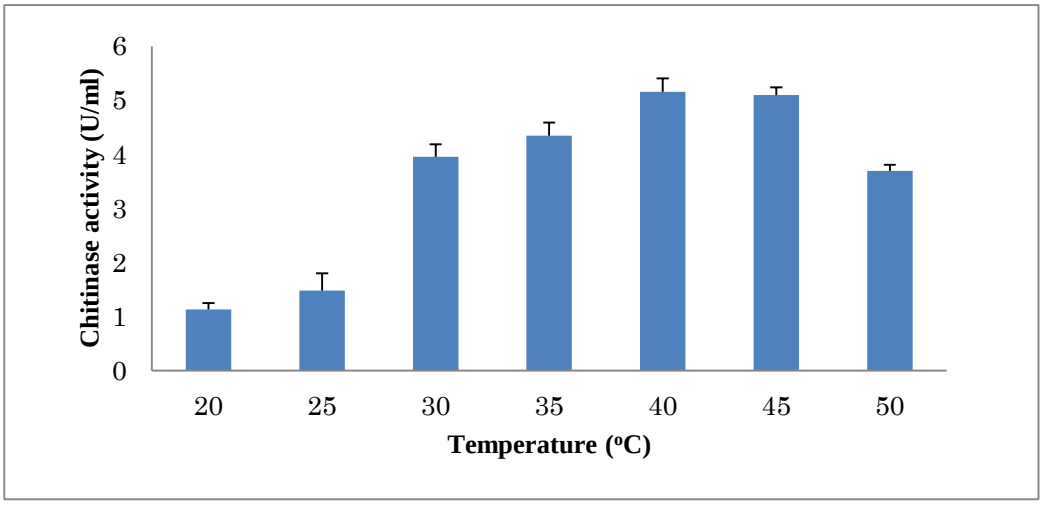


Figure 4. Effect of temperature on chitinase production.

(Vertical bars represents standard deviation, n= 3).

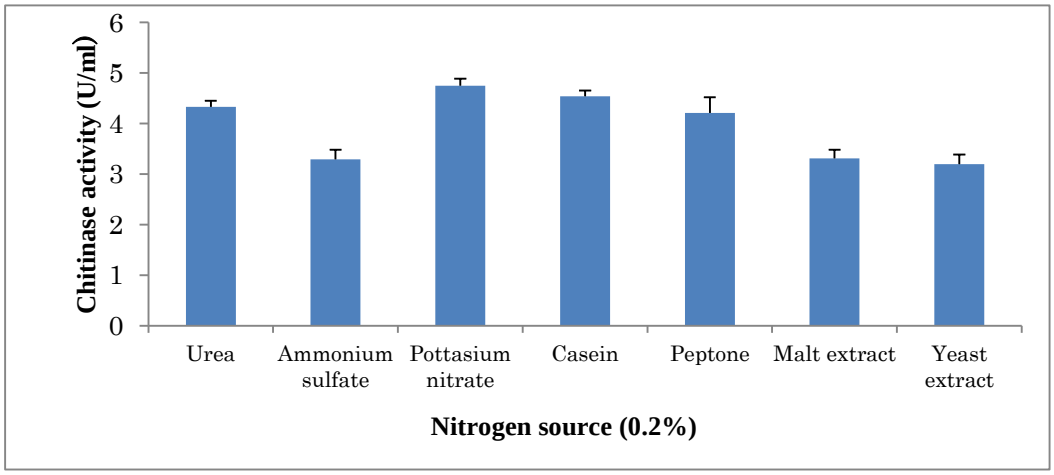


Figure 5. Effect of nitrogen source on chitinase production.

(Vertical bars represents standard deviation, n= 3)

Among all nitrogen sources chitinase production was found to be higher with KNO_3 (4.75 U/ml)

which is followed by casein (4.54 U/ml). Lowest chitinase production was found in presence of ammonium sulfate (3.29 U/ml) (Figure 5). *Streptomyces* SJKP9 showed maximum chitinase production in presence of yeast extract in production medium [17] where as *Streptomyces* sp PTK19 gives maximum level of chitinase production induced by peptone [6]. Presence of metal salts significantly affects chitinase productions because of metals are involved in the metabolic activities. Highest chitinase production was found in presence of $CaSO_4$ (5.44 U/ml) which is followed by $MnSO_4$ (5.21 U/ml) and lowest chitinase production is observed with $ZnSO_4$ (2.8 U/ml) (Figure 6). Similarly, Patel et al, and Gohel et al also reported significant increase in chitinase production in presence of Ca ion [18, 19].

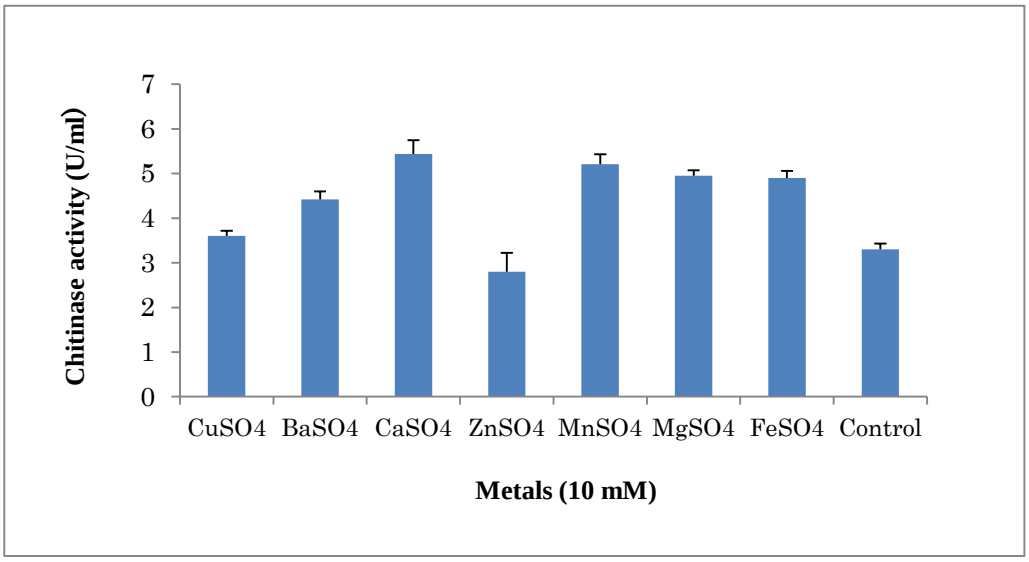


Figure 6. Effect of metal salts on chitinase production.
(Vertical bars represents standard deviation, n= 3)

The combined effects of selected parameters were studied in 12 experimental sets and respective chitinase production was presented in table 1. The highest enzyme units were observed in 9th experimental set with 9.2 U/ml. The composition of optimal medium designed as per 9th experimental set is highlighted in table- 1, showed 2.62 fold increase in chitinase production over control medium used for chitinase production by *Streptomyces macrosporeus* M1.

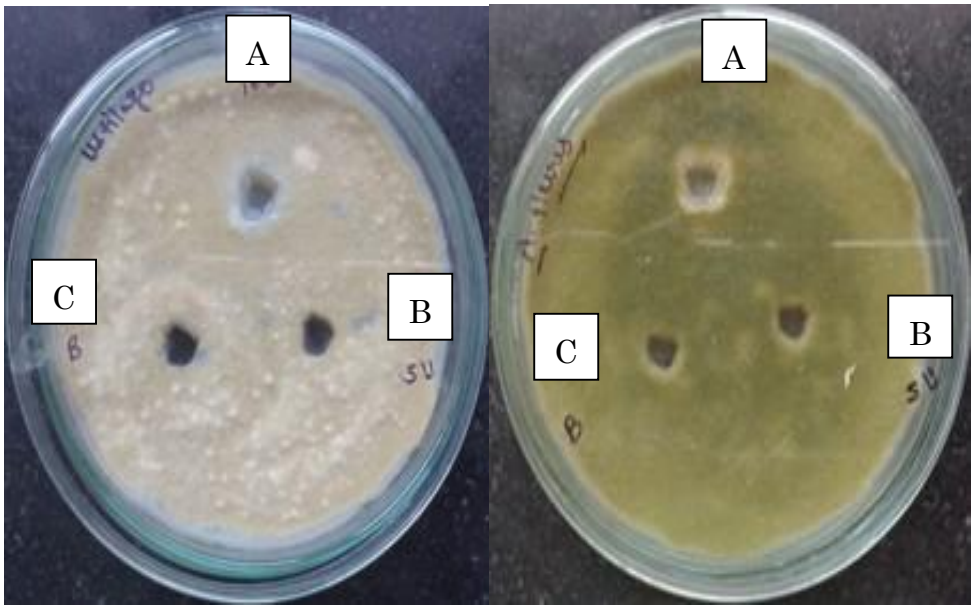
Table 1. Combined effects of process variables on chitinase production.

Sr.no.	Substrate concentration (%)	KNO3 (%)	CaSO4 (mM)	pH	Temperature (ºC)	Incubation (Hours)	Enzyme Units (µg/mL)
CONTROL	1	-	-	7.0	30	-	3.5
1	1.5	1	10	7.0	45	72	6.53
2	1.5	1	5	6.0	40	72	7.76
3	1.5	0.5	10	7.0	45	48	7.76

4	1.5	0.5	5	6.0	45	48	8.2
5	1.0	1	10	6.0	45	72	8.2
6	1.0	1	5	7.0	40	72	6.65
7	1.0	0.5	5	7.0	45	48	7.34
8	1.0	0.5	10	6.0	40	48	6.70
9	**1.5**	**0.5**	**10**	**6.0**	**40**	**72**	**9.2**
10	1.5	1	10	7	40	48	5.7
11	1.0	1	10	7	40	72	5.6
12	1.0	0.5	5	6.0	40	48	8.9

3.3. Antifungal activity

Crude chitinase showed zone of growth inhibition around well, on agar plates containing test fungi (figure 7). The growth of all test fungi inhibited in presence of 100 µL of enzyme but absent for 50 µL of enzyme. Whereas, boiled enzyme had no inhibitory activity against phytopathogens. Growth of *Rhizopus oryzae* highly inhibited by chitinase as compared to other test fungi. This is might be due to antifungal potential of chitinase is related to the chitin content of the fungus. Antifungal activities of chitinase includes inhibition of germ tube elongation, spore germination, spore bursting [20]. Antifungal potential of various *Streptomyces* species were reported against *Aspergillus* sp., *Botrytis cinerea*, *Fusarium* sp., *Alternaria* sp., *Phythium* sp., *Sclerotinia* sp. [17, 21].



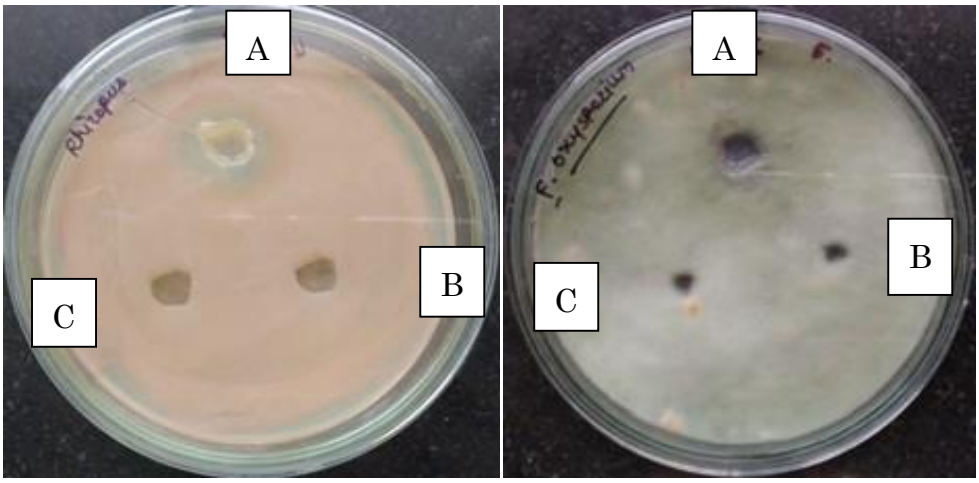


Figure 7. Antifungal activity of *Streptomyces macrosporeus* M1 crude chitinase.
(A: 100µL of enzyme, B: 50 µL of enzyme, C: boiled enzyme.)

4. CONCLUSION

In the present study, chitinase producer strain *Streptomyces macrosporeus* M1 has been optimized for improved chitinase production. *S. macrosporeus* M1 showed highest chitinase production in shortest time of incubation as compared with other actinomycetes. The study also showed that, crude chitinase has potential inhibitory activity against phytopathogens, so it could be used as antifungal agent in agriculture.

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CONFLICT OF INTEREST:

The authors have no conflict of interest.

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