ISSN 0975-6299 Vol 2/Issue 2/Apr-Jun 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE MICROBIOLOGY

PRODUCTION OF CHITINASE BY STREPTOMYCES HYGROSCOPICUS VMCH2 BY OPTIMISATION OF CULTURAL CONDITIONS

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ABSTRACT

A total of 10 chitinolytic bacteria were isolated from 35 soil samples collected from different crop fields in Tamilnadu state, India. Among them, a strain designated as VMCH2 which produced highest chitinolytic activity in primary and secondary screening in colloidal chitin agar was selected and later identified as *Sterptomyces hygroscopicus*. The maximum chitinase production was observed in CCMB amended with 0.2% colloidal chitin at pH 7.0 and 35° C after 8 days of incubation. Under this optimized growth conditions, Streptomyces hygroscopicus - VMCH2 produced a total chitinase activity of 28. 09 units / ml against only 9.36 units / ml in the initial production medium stage.

ISSN 0975-6299 Vol 2/Issue 2/Apr-Jun 2011

KEYWORDS

Streptomyces hygroscopicus, chitinolytic bacteria, chitinase cultural conditions.

INTRODUCTION

Chitin, a linear Beta – 1,4 linked homopolymer of N-acetyl glucosamine is one of the 3 most abundant polysaccharides in nature besides cellulose and starch (DUO chuan 2006). The antifungal activity and highly biocompatible quality make the chitin and its derivatives particularly useful for biomedical applications such as wound healing, cartilage tissue engineering, drug delivery and nerve generation .Chitin's biodegradable and antifungal properties are also useful for environmental and agricultural uses and food technology and cosmetics **.**

Lysis of the host structure by secretion of extracellular lytic enzymes is one of the important mechanisms that are involved in the antagonistic activity of biocontrol agents. It is a major cell wall constituent of fungi, insect and crustacean shells. Interestingly the presence of chitin in the cyst wall of human pathogen, Entamoeba histolytica was demonstrated. Chitinases are produced by several bacteria. Chitinases have been isolated from variety of bacteria as well as Actinomycetes and some of them are reported to produce multiple forms of chitinases with different molecular masses. Glycol chitin is a soluble modified form of chitin, which has recently become a very useful substrate for activity staining but it is costlier than acid swollen chitin. The importance of chitin metabolism in nature and *in situ* gel activity staining technique, there is still no method available for detection of chitinase activity onto the solid plate method after polyacrylamide gel electrophoresis under native or denaturing conditions.

In this present study a number of chitin degrading streptomycetes were isolated from soil samples collected from agricultural fields of Tamilnadu, India. Among them a streptomycete isolate designated as VMCH2 which later identified as *Streptomyces hygroscopicus* produced optimum chitinase. Although chitinase production was reported in many different species of *Streptomyces* such as *Streptomyces lividans S. viridificans S. plicatus* and *S. halstedii* Our literature survey revealed that there was no report on the production of extracellular chitinase enzyme by *S. hygroscopicus*. Therefore the isolate was selected and its growth conditions were standardized in order to optimize the chitinase production.

MATERIALS AND METHODS

Preparation of colloidal chitin

5 Gms of chitin powder taken from crab shells sigma - USA] were added slowly to 60 ml of conc.HCl (Merck S.A) and left at the room temperature overnight with vigorous stirring. The mixture was added to 200ml ice cold 95% ethanol and incubated overnight at room temperature with vigorous stirring. The precipitate was collected by centrifugation at 5000g for 20 minutes at 4°C and transferred to a glass funnel with filter paper (80gm). The colloidal chitin was washed with the sterile distilled water until colloidal chitin became neutral (pH 7.0) , the colloidal chitin retained on a filter paper was removed, weighed and stored in a dark place at 4°C.

Isolation of soil - borne Actinomycetes :

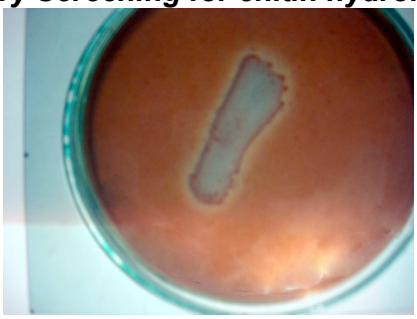
Thirty five random rhizosphere soil samples were collected in sterile polypropelline bags from paddy fields of different parts of TamilNadu. By the serial dilution- spread plate technique the actinomycetes were isolated. Dilutions (10^{-5} to 10^{-7}) were plated for isolation on agar media which were incubated at 30°C for 4 to 20 days to allow actinomycetes to sporulate. Desired colonies were picked and streaked for culturing pure colonies. Colonies of actinomycetes on the

agar plates were picked on the basis of morphological characteristics and purified on ISP -2 agar. For inducing sporulation purified colonies were sub cultured onto ISP-3.

Primary Screening for chitin hydrolysis:

Primary screening was performed by single line streak of spores in the center of CCA media containing colloidal chitin and incubated at room temperature. The zone of clearance due to chitin hydrolysis was recorded up to 5 days. The isolates producing clear zones over 0.5 cm alone were selected and subjected to secondary screening.

Fig 1
Primary Screening for chitin hydrolysis



Secondary screening for chitin hydrolysis:

Secondary screening was performed with the culture filtrates of the 10 selected streptomycete isolates using well diffusion method. All the 10 isolates were grown in SCA broth containing 0.1% chitin. 3% of the inoculum was suspended into the medium and incubated at 150 rpm in a rotary shaker at 35°C. After 8 days of incubation, the cultures were harvested, centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant was collected .Colloidal chitin (0.1%) agar plates were prepared and wells were made using 9mm sterile cork borer . 100µl of culture filtrate of each isolate was suspended in each well and incubated at 37°C. After 12 hours the development of clear zone around the well was observed.

Optimization of Cultural Conditions:

Effect of different nitrogen sources:

Effects of different nitrogen sources on the enzyme production by *Streptomyces hygroscopicus* were investigated. Results showed maximum chitinase activities obtained from optimized medium (9.36 units/ml). Chitinase secretion was increased using 0.2% colloidal chitin, as a sole nitrogen and carbon source (7.19 units /ml).Inorganic nitrogen source $(NH_4)_2SO_4$ was not suitable for enzymes production .Results in Fig 3 reflect the inducible and constitutive nature of chitinase enzyme with colloidal chitin.

Effect of the pH temperature & metal ions:

The effects of the pH, temperature and the metal ions on the chitinolytic enzyme production by *Sterptomyces hygroscopicus* were studied by growing cultures at temperatures between 15 and 40 degrees centigrade. Initial pH between 5.0 and 8.0 and the metal ions such as Mn^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+}, Na^{+}, Hg^{+} etc; . All experiments were carried out in 500 ml Erlenmeyer flasks containing 100ml culture medium incubated at 35°C (except for temperature experiments) at 150 rpm for 36 hours.

Medium:

Production medium designed by (Mitsutomi et al 1995) was used with slight modification: colloidal chitin - 0.1gm, NaNO3 – 2.0 gms, K_2HPO_4 – 1.0 gm, Mg SO_4. $7H_2O$ – 1.0 gm, $CaCO_3$ – 1.0 gm , $FeSO_4.7H_2O$ -0.01 gm, KCl – 0.5 gm, H_2O – 1000ml pH 7.0 . 3% of inoculum was inoculated into the medium and incubated at 150 rpm at 35°C. After 8 days of incubation, the cultures were harvested and centrifuged at 10000 rpm for 15 minutes and

the supernatant was used for the chitinase assay.

Chitinase Assay:

The reaction mixture contained 0.5ml of 0.01% colloidal chitin in sodium acetate buffer (0.05M pH 5.2) and 0.5ml culture filtrates was incubated at 37°C for 2 hours in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by the colorimetric method (Reissig et al 1955). The reaction was terminated by adding 0.1 ml of 0.08M potassium tetra borate, pH 9.2 to 0.5 ml of reaction mixture and then boiled in a water bath for 3 minutes. Then 3ml of diluted p-dimethylaminobenzaldehyde (p- DMAB sigma chemicals company, USA) reagent was added and again incubated at 37°C for 15 minutes. The released product in the reaction mixture was read at 585nm in a spectrophotometer (Hitachi, Japan) .Chitinase activity was determined using Na acetylglucosamine (Sigma chemicals company, USA) as the standard.

One unit of chitinase activity was defined as the amount of enzyme, which produces 1µ mole of N- actetylglucosamine in 1 ml of reaction mixture under the standard assay condition (Mathivannan et al 1998).

RESULTS

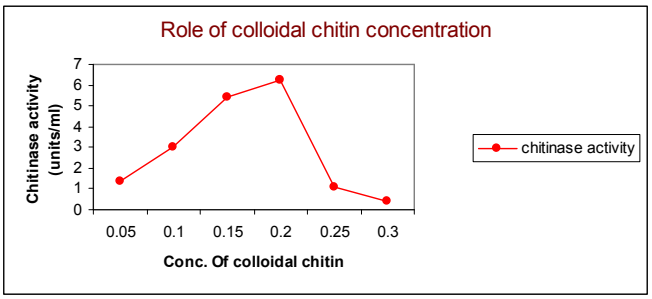
Isolation of chitinolytic streptomycetes:

A total of 36 chitinolytic streptomycetes were isolated from 35 soil samples with the pH between 6.0 and 6.5 and remaining streptomyces from pH 7.0 to 7.5 .Among the 35 chitinolytic streptomycetes only 10 isolates produced zone of clearance over 0.5cm. Interestingly, a streptomycete isolate obtained from the rice rhizosphere soil, designated as VMCH2 remarkably hydrolyzed the colloidal chitin and produced a prominent and maximum clear zone in CCA plate .Among the 10 chitinolytic streptomycetes tested in the secondary screening , the culture filtrate of the rice rhizosphere isolate VMCH2 produced clear zone of 1.3cm in CCA. The clear zones due to hydrolysis of colloidal chitin by the culture filtrates of the remaining 9 streptomycetes ranged between 0.5 and 0.8cm. The isolated VMCH2 is a gram +ve, chain like coiled spores forming, aerial mycelium with ash color, substrate mycelium with pink color, catalase and oxidase +ve reaction. Based on the physico - chemical tests, the isolated VMCH2 was identified as streptomycete. The morphological characteristics of VMCH2 is represented in Table: 1. Role of colloidal chitin conc. on chitinase are represented in Graph:1

Table: 1
Morphological characteristics of VMCH2.

CHARACTERISTICS	VMCH2
Gram stain	+
Spores	Spiral and coilled
Spore Mass colour	
Red	-
Grey	+
Mycelium pigment Red- orange	-
Diffusible pigment produced	+
Diffusible pigment	Pink
Melanin pigment	-
Degradation :	
Xanthine	+
Oxalate	-
Growth at 45°C	+
Growth at 37°C	+
Growth at 4°C	-

Graph:1
Role of colloidal chitin conc. on chitinase



Optimization of culture conditions for chitinase production:

Among the 3 media tested CCMB supported chitinase production of 9.36units/ml as compared to 2.54 and 7.89 units/ml respectively in CGMB, CMMB . Result on the effect of different concentration of the colloidal chitin on chitinase production. Among 6 different concentrations tested, colloidal chitin at 0.2% considerably enhanced the chitinase activity (6.219 units/ml) followed by 0.3%. Beyond 0.3% the substrate concentration decreased the enzyme activity. Enzyme activity of VMCH2 is represented in Table:2.

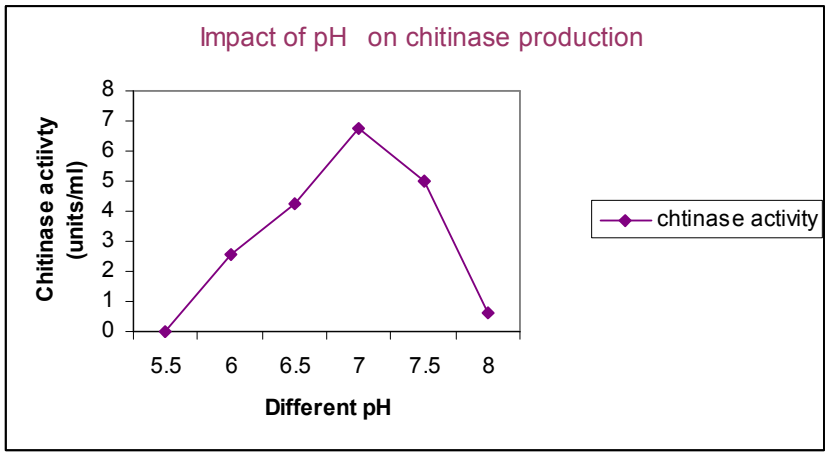
Table: 2
Enzyme activity of VMCH2.

Enzyme activity	
Lipolysis	+
Gelatin reduction	+
Nitrate reduction	+
H_2S production	+
Urease	-
Xylanase	-
CMC	+

Among different pH tested pH 7.0 in modified SCB medium favoured the chitinase production at the maximum of 6.724 units/ml as against 0-5.031units/ml in rest of the pH. Interestingly, there was no chitinase production at pH 4.0. Impact of PH on chitinase production is represented in Graph:2. Quantitative assay determined by hydrolysis of colloidal chitin has also revealed that the chitinase production was high in culture filtrate of streptomyces at pH 7.0 than the culture filtrate of the other pH. Among different temperature tested, Streptomycin hygroscopicus produced maximum chitinase activity of 4.061units/ml at 35°C.The chitin activity in rest of the temperature ranged between 0 and 3.269 units/ml. It has been observed that in both the lower and higher temperatures (20 & 40°C) , the chitinase activity was sharply decreased . It was observed that Streptomyces hygroscopicus VMCH2 produced maximum chitinase of 9.36units/ml on the 8th day, when the organism grows in all. Other standardized parameters such as SCB medium , 0.2% colloidal chitin as substrate pH 7.0 and temperature 35°C. Impact on temperature on chitinase production is represented in Graph:3. The chitinase activity was declined in subsequent ages and only 1.54 units/ml activity was measured on 13 th day . Utilization of Nitrogen and carbon source by VMCH2 is represented in Table:3. Impact of nitrogen sources on chitinase production is represented in Graph:4.Effect of production of chitinase on different media is represented in Graph:5.

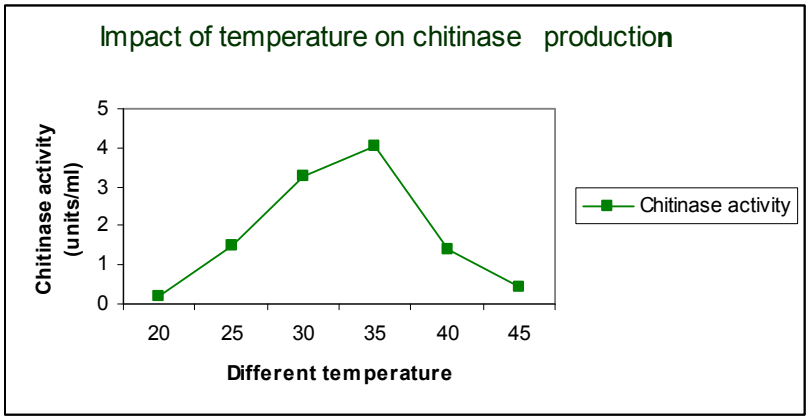
Graph:2

Impact of PH on chitinase production.



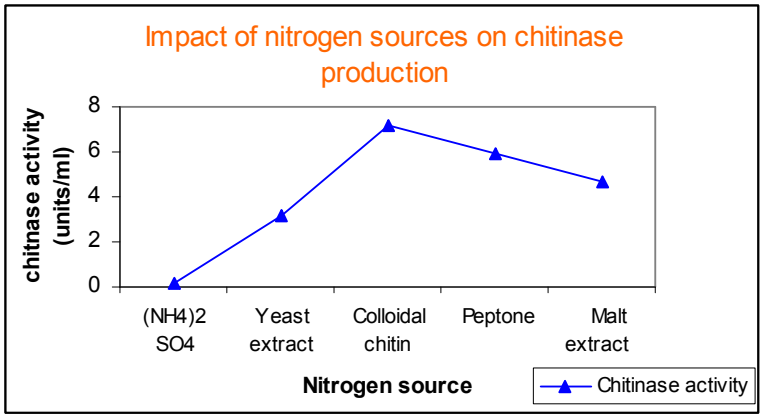
Graph: 3

Impact of temperature on chitinase production



Graph: 4

Impact of nitrogen sources on chitinase production.



Graph: 5

Effect of production of chitinase in different media.

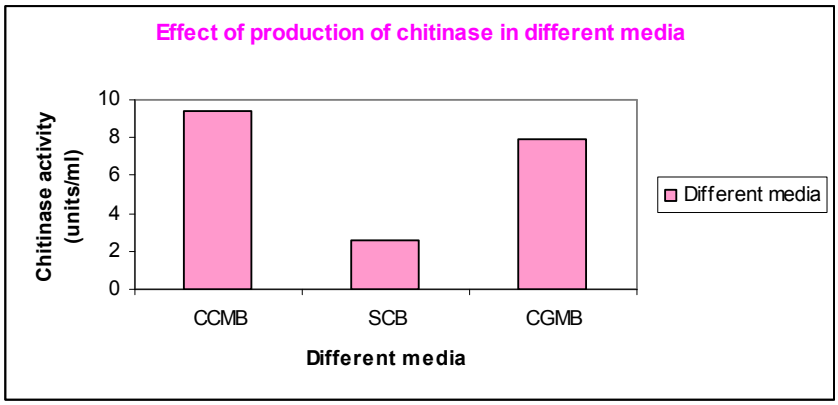


Table: 3

Utilization of Nitrogen and carbon source by VMCH2.

Utilization of nitrogen sources:	
L Hydroxyproline	-
L-Phenylalanine	+
KNO_3	+
L-Proline	-
Utilization of carbon sources	
Sucrose	+
Xylose	+
Mannitol	+
Lactose	+
Rhamnose	+
D-Fructose	-
Glucose	+

DISCUSSION

Previous reports have shown that species of streptomyces are known to produce chitinolytic enzyme. Present study shows that only 10 strains showed chitinolytic activity of the total 35 Streptomyces strains screened. It means that not all streptomyces strains can be considered as a potential chitinase producer. Streptomyces hygroscopicus was found to be most active organism when colloidal chitin was added to the medium as sole carbon and nitrogen source. In this study on chitinase production, extra cellular chitinase activity was determined. There was no chitinase, until 49 h of incubation, after which the amount increased from 96 to 240 h. The chitinase activity remained until

288 h. similar observations had been made by Young and Bell (1985) and Neugebour, (1991) during production of chitinase from *S. marcescens* and *S. lividans*, respectively.

The growth of the culture was slow at the beginning and was exponential after 96 h. Enzyme production increased was in exponential phase and more amounts were detected in the stationary growth phase. Maximum enzyme activity was detected in the decline growth phase. Chitinases are fairly stable over broad pH range. The pH stability of chitinase varies from organism to organism. Chitinase from *Streptomyces* are

found to be stable over a pH range of 4.0 to 10.0. Chitinases of *S. marcescens* and *Serratia liquifaciens* have pH optima between 5.0 to 6.0 (Brurberg et al., 1996). *Streptomyces. erythraceus* chitinases has an optimum pH 5.0 (Hara et al., 1989). According to our study chitinase from *S.hygroscopicus* have optimum activity at pH 7. The temperature optima for chitinases range from 40 - 60°C depending on source. Optimum temp for *S. lividans* is 50°C. *S. erythraceus* chitinase has optimum activity at 60 - 70°C but the enzyme was not stable above 60°C (Hara et al., 1989). According to our study, *S.hygroscopicus* showed maximum activity at 40- 60°C. In this study on chitinase production, extra cellular chitinase activity was determined. There was no chitinase, until 49 h of incubation, after which the amount increased from 96 to 240 h. The chitinase activity remained until 288 h. similar observations had been made by Young and Bell (1985) and Neugebour, (1991) during production of chitinase from *S. marcescens* and *S. lividans*, respectively. The growth of the culture was slow at the beginning and was exponential after 89 h. Enzyme production increased was in exponential phase and more amounts were detected in the stationary growth phase. Maximum enzyme activity was detected in the decline growth phase. Chitinases are fairly stable over broad pH range. The pH stability of chitinase varies from organism to organism. Chitinase from *Streptomyces* are found to be stable over a pH range of 4.0 to 10.0. Chitinases of *S. marcescens* and *Serratia liquifaciens* have pH optima between5.0 to 6.0 (Brurberg et al., 1996). *Streptomyces erythraceus* chitinases has an optimum pH 5.0 (Hara et al., 1989). According to our study chitinases from *S. hygroscopicus* have optimum activity at pH 7. The temperature optima for chitinases range from 40 -60°C depending on source. Optimum temp for *S. lividans* is 50°C. *S. erythraceus* chitinase has optimum activity at 60 - 70°C but the enzyme was not stable above 60°C (Hara et al., 1989). According to our study, *S.hygroscopicus* showed maximum activity at 40 - 60°C.

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