Potent Chitinolytic Activity of a Marine Actinomycete, *Rhodococcus Sp.* SVL Isolated from Soils of Nizampatnam, Andhra Pradesh, India

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***Abstract:*-Eight predominant actinomycetes strains isolated from the marine soil samples collected from Nizampatnam coast of Andhra Pradesh demonstrated high chitinase activity on Colloidal chitin agar medium with average clearing zone of 20mm. A study was undertaken to screen these isolates for the production of chitinase. One of the strain produced higher amount of chitinase enzyme relative to the other strains thus the strain was identified based on 16s rDNA sequencing and designated as *Rhodococcus sp.*SVL. It grew maximally in shake flask and produced significant amount of chitinase at 35°C, pH 7.0. It has also shown a pronounced inhibitory activity against phytopathogenic fungi such as *Botrytis cinerea, Fusarium oxysporum* and *F. solani.* This is the first report of chitinase production from *Rhodococcus* sp.**

Key words: **Chitinase, *Rhodococcus* sp. SVL, colloidal chitin agar, optimization, Phytopathogenic fungi.**

I. INTRODUCTION

Chitin is a polymer of unbranched chains of β-1, 4-linked 2-acetamido-2-deoxy-Dglucose (GlcNAc; N-acetylglucosamine; NAG) and is the structural component of fungi as well as the shell or cuticles of arthropods. Chitinases (EC 3.2.1.14) are produced by many organisms, such as bacteria, higher plants and animals, and play important physiological and ecological roles (Gooday, 1990). Actinomycetes are an important group of microorganisms, not only as degraders of organic matter in the natural environment, but also as producers of antibiotics and other useful compounds of commercial interest (Bentley et al. 2002; Basilio et al. 2003; Saugar et al. 2002;).

In addition, actinomycetes are important for the production of enzymes, such as chitinase (*Streptomyces viridificans*), cellulases (*Thermonospora* spp.), proteases (*Nocardia* spp.), xylanases (*Microbispora* spp.), ligninases (*Nocardia autotrophica*), amylases (*Thermomonospora curvata*) and sugar isomerases (*Actinoplanes missouriensis*) (Solans and Vobis, 2003). Actinomycetes are a very sturdy group of microorganisms found in a myriad of environments including some extreme ones and are known to be major producers of chitinases. Chitooligosaccharides and their N-acetylated analogues are useful for applications not only due to their specific biological activities such as antimicrobial activity, antitumor activity and immuno-enhancing effects (Gohel et al. 2006) but also for their potential in drug delivery (Kadowaki et al. 1997), wound healing, dietary fiber and in wastewater treatments (Dixon, 1995; Flach et al. 1992). Some chitooligosaccharides such as ($GlcNAc_6$) and $GlcNAc_7$ have been reported to possess antitumor activity (Liang et al. 2007; Suzuki et al. 1986). Chitinase-producing organisms could be used directly as potential biocontrol agents or indirectly using their purified protein (Gomes et al. 2001; Gunaratna et al. 1994; Ueno et al. 1990) or through gene manipulation (Gupta et al. 1995; Singh et al. 1999).

The aim of this study was to screen the marine actinomycete isolates for their capacity to produce chitinase enzyme on Colloidal chitin agar (CCA) (Gupta et al. 1995). Among the several strains isolated, productivity studies were carried out with one potential isolate with a view to explore its commercial potential and also its prospective applications in various fields like biocontrol.

II. MATERIALS AND METHODS

2.1 Collection of soil samples:

Marine soil samples were randomly collected from the shore of Nizampatnam, A. P. (Latitude 15° 54' 0 N, Longitude 80° 40' E). Samples were collected in sterile polythene bags and transferred to the laboratory. They were dried at room temperature for 24-48h and then used for isolation.

2.2 Isolation of Actinomycetes:

Actinomycete strains were isolated by serial dilution agar plating technique on Yeast extract Malt extract Dextrose agar medium amended with increasing concentrations of sodium chloride and incubated at 28±2 °C. The soil sample was pretreated using physical method i.e., addition of calcium carbonate and dry heat of soil sample at 55 °C for 30min.

2.3 Screening of Chitinase producing marine actinomycetes:

Screening for chitinase production for all the isolates was done by plate agar assay and followed by tests in broth for chitinase production. The composition of CCA includes (g l^{-1}): Colloidal chitin 15; $(NH_4)_2\ SO_4$ 1; $MgSO_4 \cdot 6H_2O$ 0.3;

$KH_2 PO_4$ 1.3; NaCl 80 and agar 20. Practical grade chitin flakes (Himedia, Mumbai, India) were used to prepare colloidal chitin as a substrate for growth and enzyme assay. Colloidal chitin was prepared according to Rojas Avelizapa et al. (1999). Enzyme production was carried out in shake culture (100 ml medium/500 ml Erlenmeyer flask) incubated at 28-30 °C in the incubator shaker at 150 rpm for 5 days.

2.4 Identification of the culture:

Genomic DNA of the strain was extracted using the method of Mehling et al. (1995). PCR mixture consisted of 2.5 µl of 10× buffer, 3.5 µl of MgCl2 (25 mM), 2 µl of dNTP (0.4 mM), 1 µl of 16S rDNA actino specific Primer - forward (10 pmol/µl), 1 µl of 16S rDNA actino specific Primer-reverse (10 pmol/µl), Taq polymerase (2 U/µl) and 2 µl template DNA. PCR amplification was carried out as follows: initial denaturation step at 94 ºC for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1min and extension at 72°C for 1 min, with a further 5min extension at 72°C. The PCR product was purified with Agarose Gel DNA Purification Kit (SoluteReady® Genomic DNA purification kit, PCR Master Mix, Agarose gel electrophoresis consumables and Primers from HELINI Biomolecules, Chennai, India). Finally, the deduced 16s rDNA sequence was compared with the sequences in GenBank (http://www.ncbi. nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST).

2.5 Chitinase assay:

Chitinolytic activity in the medium was determined by using colloidal chitin as a substrate (Thamthiankul et al. 2001) by measuring the release of reducing sugars (GlcNAc) with dinitrosalicylic acid method (Miller, 1959). One milliliter of reaction mixture contained 1ml of substrate (1% w/v) aqueous suspension of the colloidal chitin), 0.5 ml of 0.05 M citrate phosphate buffer (pH 6.0) and 1ml of the enzyme sample (culture supernatant). Reaction mixture was incubated in a water bath at 35°C for 60 min. Reaction was stopped by adding 3 ml of dinitrosalicylic acid followed by boiling in a water bath for 10 min. The reaction mixture was diluted to obtain a total volume of 10 ml with distilled water. The OD values were recorded 540 nm using UV Spectrophotometer. A standard curve for N - acetyl glucosamine was carried out in parallel to measure reducing sugar released. A unit of enzyme activity was defined as the amount of enzyme required to release 1µM of NAG h^{-1} at 37°C.

2.6 Antifungal Activity:

Czapek Dox (CD) agar medium was prepared and used for the cultivation of test fungi. Phytopathogenic fungi like *Botrytis cinerea, Fusarium oxysporum* and *F. solani* were selected for testing the antifungal activity. Spore suspension of test fungi was mixed with the cooled, molten CD agar medium and poured into petri dishes. Crude enzyme extract of the strain (50µL) was added to the well in the petriplates after solidification of the medium. The petriplates were incubated for 48-72h and observed for the inhibition zones around the wells.

III. OPTIMIZATION OF NUTRITIONAL AND PHYSIOLOGICAL CONDITIONS FOR CHITINASE PRODUCTION

3.1 Effect of Incubation period on Growth and Enzyme activity:

The strain was inoculated into 500 ml conical flasks containing 100 ml CC broth. They were incubated at 30°C for optimum yields on a rotary shaker at 180 rpm. At every 24 h interval, the flasks were harvested and chitinase enzyme production was estimated in terms of IU/5mL and the growth was measured in terms of dry weight of biomass.

3.2 Study of effect of chitin concentration on chitinase activity:

The crude extract of enzymes was incubated with different concentrations of chitin (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2%) at 35°C for 1 h in a temperature regulated water bath. The enzymes activity was measured. The experiment was performed in triplicate.

3.3 Effect of pH on chitinase activity:

Crude extract of enzymes with substrate was subjected to different pH levels 2, 4, 6, 8 and 10. Level of pH, in the reaction mixture, ranging from 2 to 10 was adjusted by using four different buffers glycine HCl (pH range 2.2 - 3.4), acetate buffer (pH range 3.6 - 5.6), phosphate buffer (pH range 6.0 - 8.0) and glycine NaOH (pH range 8.6 - 11.2). The mixture was incubated at 37°C for 1 h in water bath. The reaction was terminated by the addition of DNS reagent and the activity was measured.

3.4 Effect of temperature on chitinase activity:

The crude extract of enzymes with substrate was incubated at temperatures ranging from 10 to 80 °C for 1 h in a water bath. The enzyme activity was measured after incubation. The experiment was performed in triplicate.

3.5 Effect of supplementation of Carbon and Nitrogen sources on growth and enzyme activity of the strain:

The carbon sources (1%) used were Glucose, Fructose, Galactose, Arabinose, Raffinose, Starch and Nitrogen sources (0.1%) tested were Peptone, Yeast extract, Tryptone, Ammonium nitrate, Potassium nitrite, Urea, L-asparagine and L – Tyrosine. Control was maintained for each set.

3.6 Effect of different concentrations of optimized carbon and Nitrogen sources:

Once the carbon and nitrogen sources have been optimized attempts were made to determine the effect of various levels of optimized carbon and nitrogen source. Increasing concentrations of carbon sources such as 0.25, 0.5, 1.0, 1.5 and 2% while 0.05, 0.1, 0.15, 0.2, 0.5, 1 and 2% of

nitrogen sources were added in addition to chitin in the medium. Triplicates were maintained for each set of experiment.

IV. RESULTS AND DISCUSSION

Chitinases are normally produced by a large number of microorganisms. Bacteria by the process of chitin decomposition release N-acetylglucosamine into the environment, which makes an important source of carbon and nitrogen for themselves and for many other organisms. Contribution of chitin to soil is in the form of animal biomass in the marine environment (Deshpande, 1986). Microbial production of chitinase had great attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method (Ahmadi *et al.* 2008). In the present study, an extracellular chitinase producing bacteria isolated from marine environment, identified as *Rhodococcus* sp. SVL was screened for chitinase production.

Eight predominant actinomycete strains were isolated from the marine soil samples collected from Nizampatnam coast. One strain was found to have potent chitinolytic activity and hence selected for further study of chitinase. The morphological, physiological and biochemical characteristics of the isolate were investigated and summarized in Table 1. The strain was acid fast and was negative for indole, methyl red, catalase test, weakly urease positive and positive for voge's Proskaeur, citrate utilization, oxidase and nitrate reduction test. The strain utilized a range of carbohydrates including glucose, sucrose, starch and arabinose. When sodium chloride tolerance of the strain was tested, the strain grew well only at a concentration of 8%, while the growth of the strain was moderate at other concentrations. The strain was also a producer of wide range of enzymes including amylase, cellulose, caseinase, chitinase and urease. A phylogenetic tree based on 16S rDNA sequence data was constructed (Fig. 1) and the strain was designated as *Rhodococccus* sp. SVL with an accession number JN256140.

Incubation time is characteristic of an organism which profoundly affects the enzyme production and duration of enzyme synthesis phase. In the present case, the strain gave maximum chitinase production at 96h (Table 2). When substrate concentration was optimized, with 1.2% colloidal chitin as the source of carbon, it produced high levels of chitinase in the culture medium (Fig. 3). Temperature and pH affect biological process through several mechanisms including enzyme induction or suppression, protein denaturation and altering cell viability (Nampoothiri et al. 2004). The optimum temperature where maximum chitinase activity observed was 35 °C. Higher or lower incubation temperatures adversely affected the production of chitinase (Fig. 4). The pH of the buffer in which the enzyme and substrate reaction occurred also affected chitinase activity. Maximum chitinase activity was at pH 8.0 (Fig. 5). Other marine bacterial chitinases showed broader pH optima (Hiraga et al. 1997; Ohtakara et al. 1979) or were more active in neutral or slightly alkaline conditions (Tsujibo et al. 1992; Wang et al. 1997).

Among the seven carbon sources tested, CC broth amended with arabinose improved the enzyme production to a great extent (Fig 6). Enzyme production was nil when glucose was used as the additional carbon source in the medium. The data included in Fig 7 reveal that 0.5% arabinose along with 1.2% of colloidal chitin in the medium showed maximum chitinolytic activity. The nitrogen source that influences the enzyme production and growth of the strain was also optimized. Tryptone proved to be the most appropriate nitrogen source both for the growth and enzyme production (Fig. 8). The absolute level of optimized nitrogen source i.e. tryptone was 0.15% (Fig. 9). Narayana et al. (2009) also reported that optimization of nutritional and physiological factors improved chitinase enzyme production of *Streptomyces sp.* ANU 6277 isolated from the laterite soils of Acharya Nagarjuna University. After 3 days of incubation, growth inhibition appeared around the perimeter of the well containing the crude chitinase. The average diameter of inhibition zone on the tested fungal plates was 10.2 mm for *B. cinerea* and 10.5 mm for *F. oxysporum* and 9.8 mm for *F. solani*. Obviously, all the fungi tested were suppressed by the crude chitinase (50 µL), indicating that the obtained chitinase produced by the strain has the potential to be used as an antifungal agent. Many bacterial genera such as *Serratia* (Ordentlich et al. 1988; Sneh, 1981), *Aeromonas* (Inbar & Chet, 1991), *Chromobacterium* (Park et al. 1995), *Enterobacter* (Chernin et al. 1997), *Arthrobacter* (FraÈndberg and SchnuÈ rer, 1998; Sneh, 1981) and *Paenibacillus* (Singh et al. 1999) have been reported to be effective biological control agents of many soil- borne plant pathogens. Hoster et al. (2005) reported chitinase activity from the novel *Streptomyces* strain against *Aspergillus nidulans* and phytopathogens such as *Botrytis cinerea*, *Fusarium culmorum*, *Gulgnardia bidwellii*, and *Sclerotinia sclerotiorum*. Singh et al. (1999) used a chitinolytic *Streptomyces* sp. for the suppression of cucumber wilt caused by Fusarium oxysporum.

Elad et al. (1982) suggested that extracellular cell wall degrading enzymes are the main enzymes involved in the biological control of phytopathogenic fungi by *Trichoderma harzianum*. In addition, it has long been shown that actinomycetes, especially streptomycetes, are effective in the protection of plants against soil borne fungal pathogens (Crawford et al. 1993).

V. CONCLUSIONS

Among the eight predominant maine Actinobacteria isolated from soils of Nizampatnam coast, one strain designated as *Rhosococcus* sp. SVL fou nd to have potent chytinolytic activity. This is the first report of chitinase enzyme production from *Rhodococcus* sp. All the fungi tested were suppressed by the crude chitinase (50 µL) produced by

the strain, indicating that the obtained chitinase produced has the potential to be used as an antifungal agent.

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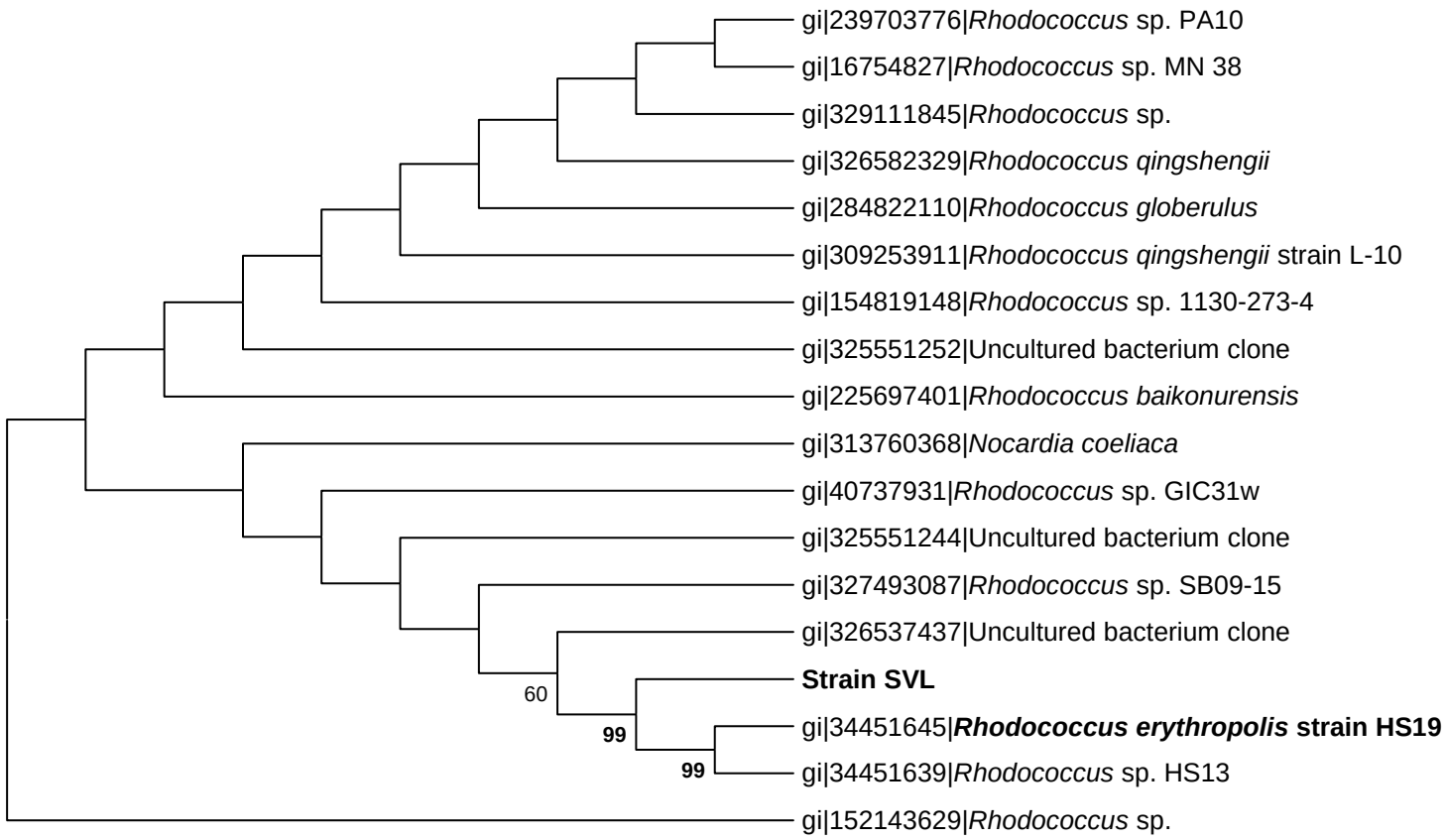

Fig. 1 Phylogenetic tree of *Rhodococcus* sp. SVL
The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap values above 50 were shown

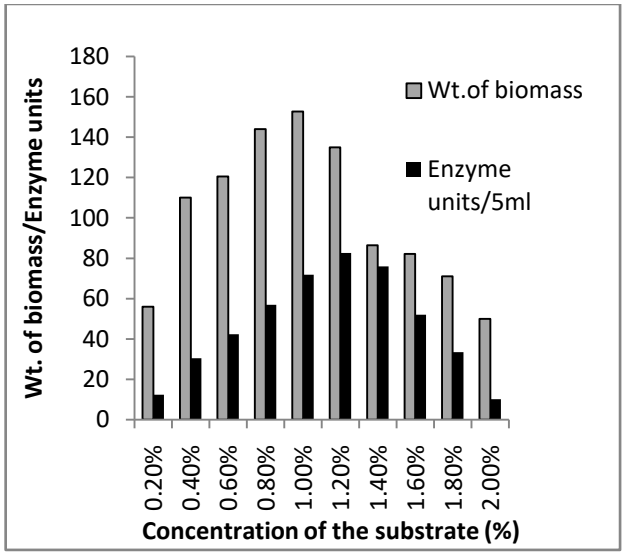

Fig. 2 Effect of Substrate concentration on growth and enzyme activity of *Rhodococcus* sp. SVL

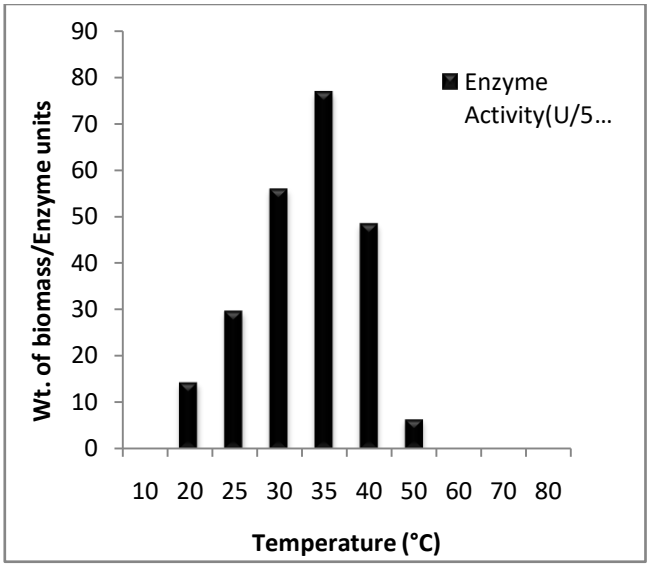

Fig. 3 Effect of temperature on chitinase activity of *Rhodococcus* sp. SVL

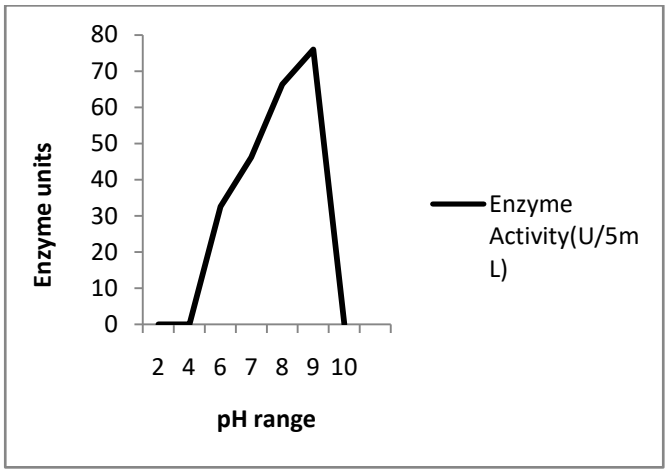

Fig. 4 Effect of pH on chitina se activity of *Rhodococcus* sp. SVL

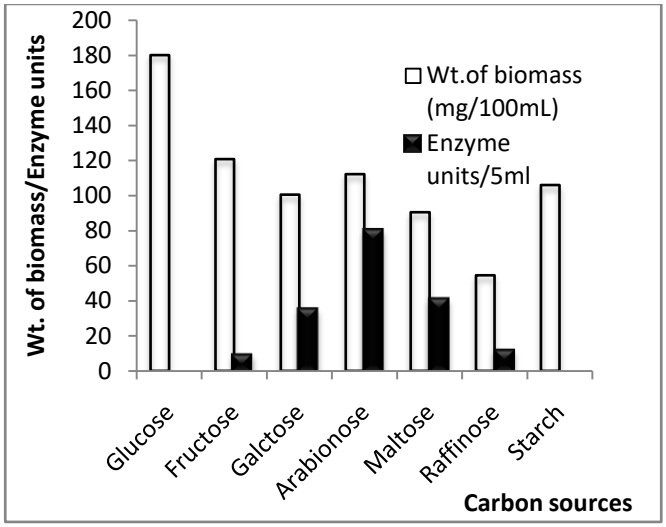

Fig. 5 Effect of Carbon sources on growth and enzyme activity of *Rhodococcus* sp. SVL

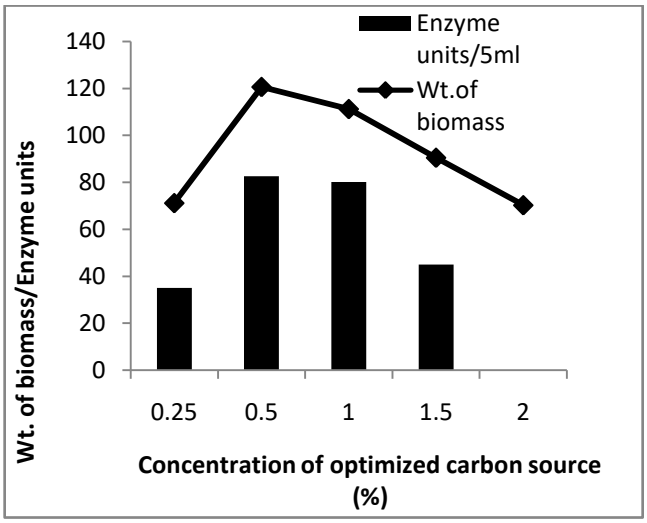

Fig. 6 Effect of different concentrations of optimized carbon source on growth and enzyme activity of *Rhodococcus* sp. SVL

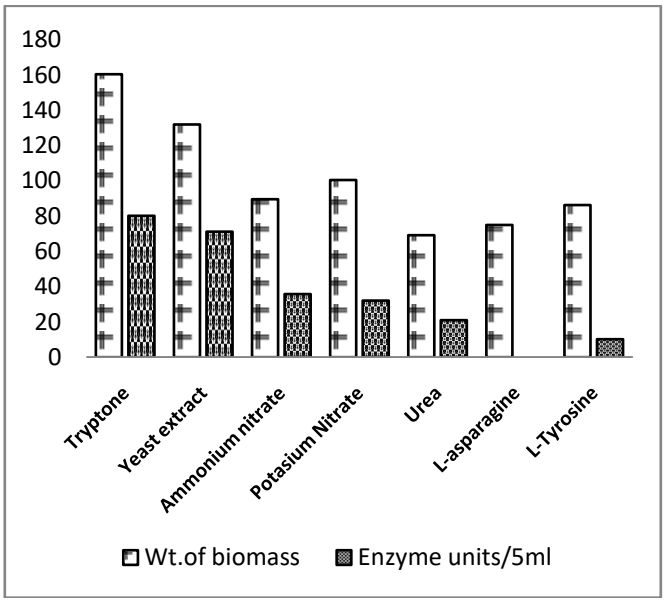

Fig. 7 Effect of Nitrogen sources on growth and enzyme activity of *Rhodococcus* sp. SVL

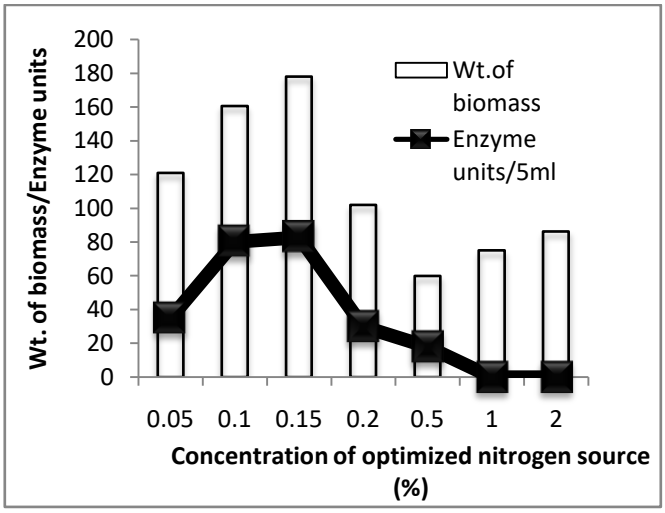

Fig. 8 Effect of different concentrations of optimized Nitrogen source on growth and enzyme activity of *Rhodococcus* sp. SVL