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CHITINASE PRODUCTION BY RHIZOBACTERIAL STRAINS ISOLATED FROM ROOT NODULES OF VIGNA TRILOBATA CULTIVARS

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ABSTRACT

Rhizobacterial strains were isolated from root nodules of Vigna trilobata (L) Verdc. plants raised in soils collected from geographically different areas in A.P. India. Among the twenty one strains only four strains were proved to be positive for chitinase activity on chitin agar plates. The four strains were identified as Ensifer xinjiangense MRR 110 (KC415691) and Agrobacterium tumefaciens MRR 111(KC415692), and Bacillus altitudinus MRR122(KC503884), Paenibacillus sp. MRR 124(KF621017) after 16S rDNA sequencing. The non rhizobial strain Paenibacillus sp. MRR 124 showed the highest chitinase (0.46 U/ml) production based on maximum clear zone/colony size ratio on chitin agar plates and chitinase activity in culture filtrate. Chitinase production increased with increase in incubation time up to 72h and declined afterwards in all the four strains. Neutral pH was found to be the best for chitinase activity by these strains. Arabinose as carbon and peptone as nitrogen source supplemented to the chitin medium supported maximum chitinase activity. The results obtained in the present study showed that the non rhizobium strain Paenibacillus is a potent producer of chitinase and can be exploited as bio control agent.

KEYWORDS: Agrobacterium Tumefaciens, Chitinase, Paenibacillus

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Original Article

INTRODUCTION

Chitin is a homopoloymer of N-acetyl-D-glucosamine residues linked in β 1-4 linkage. Chitin derivatives are oligosaccharides of 2 to 20 N-acetyl glucosamine residues in length and having low molecular weights, offer chemical and biological properties other than those of original polymer, such as water solubility and signalling functions during symbiotic interactions in plants. Chitin and all its derivatives share a high nitrogen content (6.14-8.3%) and high thermal and chemical stability (Yen and Mav, 2007). Recent findings demonstrate that chitin and its derivatives can improve legume- Rhizobium symbiosis. Chitinase activity is stimulated in the nodule cortex and in the infected zone of some ineffective soybean nodules (Staehelin et al., 1992; Pasniske et al., 1994).

The major source of chitinase (E.C. 3.2.1.14) is microorganisms and it represents a vast renewable fermentation feedstock of both carbohydrate and nitrogen sources. Enzymes capable of bio converting chitin to low molecular weight fermentable products potentially have significant commercial value. Chitin-producing strains utilize chitin, colloidal chitin, or other chitin derivatives as a major carbon source for chitinase production. Chitinase enzyme has a very broad application as bio control agent, morphogenesis, and bio conservation of waste containing chitin, pollution degradation, mosquito control, fungal biomass estimation, protoplast isolation and bio-pesticides. It is also used along with antifungal agents and also skin lotions, and creams for fungal infections. Due to its broad range of applications in agriculture (Wang and Hang, 1997) and pollution degradation, these exists a strong interest to enhance the chitinase production for industrial purposes.

Chitinase have received increased attention due to their wide range of biotechnological applications especially in agriculture for bio control of fungal phytopathogens (Mendonsa et al., 1996; Pinto et al., 1997).

Plant growth promoting rhizobacteria directly or indirectly promote plant growth by the production of phytohormones, bio control of phytopathogens and improvements of nutritional status of the plant (Chandra et al., 2007). Several reports have indicated that some strains of *Rhizobium* acts as bio control agents due to the production of antibiotic-like substance (Krishnan et al., 2007), siderophores (Arora et al., 2001), while some strains also showed antagonism towards plant pathogenic fungi in dual cultures (Gupta et al., 2005; Chandra et al., 2007).

Species of *Aermonas, Alteromonas, Bacillus, Enterobacter, Pseudomonas, Serratia, Streptomyces* and *Vibrio* were reported to produce chitinase (Bhattacharya et al., 2007) however, in the present study legume nodulating rhizobacteria - *Ensifer xinjiangense* and *Agrobacterium* species were reported to produce chitinase for the first time.

The genus *Vigna* comprises of nearly 150 species is one of the major nodulating genera in the family *Leguminoseae*. *Vigna trilobata* commonly called as Pillipesara, mainly cultivated as short term forage crop in India. A perusal of literature on *Vigna-Rhizobium* interactions reveals that the studies on nodulation, isolation, cultural and biochemical studies was carried out mainly on few species of *Vigna* viz. *V. mungo, V. unguiculata* and *V. radiata*. The studies on cultural and biochemical characterization of the *Rhizobium* spp. associated with *V. trilobata* were very merge. Hence it was proposed to carry out the optimization of chitinase production by *Rhizobium* and non-*rhizobium* strains from *Vigna trilobata* and finally deriving the efficient strain.

MATERIALS AND METHODS

Isolation

Rhizobial strains were isolated from the root nodules of *Vigna trilobata* plants raised in earthen pots filled with soil collected from twenty one districts of Andhra Pradesh and maintained properly in the botanical garden of our university. For the isolation, pink coloured healthy root nodules were collected by gently uprooting the plants growing in soils from Cadapah, Ananthapur, Prakasam and Nellore district soils, twenty one days after sowing, surface sterilized with 0.1% mercuric chloride and washed several times with sterile distilled water. Bacterial suspension was prepared by crushing these nodules with sterile glass rod using sterile distilled water. A loopful of suspension was streaked on to yeast extract mannitol agar media plates with 0.1% Congo red and incubated at room temperature for 3 days. After incubation, the white translucent, convex, colonies with high mucilage were isolated and pure cultures were maintained after subculturing the same medium. Pure cultures of all the twenty one isolates were authenticated as *Rhizobium* by performing the appropriate biochemical tests (Somasegaran and Hoben, 1994), and nodulation ability on homologous hosts by plant infection tests (Vincent, 1970). However, 16S rDNA sequencing (Macrogen, South Korea) studies revels that the rhizobia associated with the root nodules belongs to both rhizobial and non rhizobial species. Hence, the sequences of the strains used in the present study *Ensifer xinjiangense* MRR 110 (KC415691) and *Agrobacterium tumefaciens* MRR 111(KC415692), and *Bacillus altitudinis* MRR122 (KC503884), *Paenibacillus* sp. MRR 124 (KF621017) were deposited in the Gene bank.

Chitinase Activity

In YEMA medium, mannitol was replaced with 10 g/L chitin, and it was used to screen the *Rhizobial* strains for chitinase activity. Each strain was separately inoculated on to the plates containing colloidal chitin and incubated at room

temperature for up to 7 days. Lysed hyaline zone around the *Rhizobial* growth was considered as positive evidence for chitinase activity (Cody, 1989; Wirth and Wolf, 1990). The potency of the isolates for chitinase production was determined on the basis of ratio of zone of clearance (CZ) to colony size (CS) (Cody, 1989).

Enzyme Source

Each *Rhizobial* strain was inoculated into 50 ml of the liquid medium and incubated at room temperature on rotary shaker at 200 rpm. The culture filtrate was collected by centrifuging the culture broth at 7000 rpm for 30 minutes and used as extracellular enzyme.

Chitinase Assay

The chitinase was assayed by the method described by Vyas and Desh Pande (1989). Chitinase activity was determined by incubating 1 ml of crude enzyme with 1 ml of 1% colloidal chitin in 0.05 M phosphate buffer pH 7.0 at 35 °C for 1 h. After centrifugation, 1 ml of reaction mixture was taken and to this 1 ml of distilled water was added, boiled in a glass ball –covered centrifuge tube for 10 minutes and then centrifused. From the supernatant 0.5 ml of aliquot was taken and to this 0.1 ml of Potassium tetraborate was added and boiled for exactly 3 minutes in a water bath. After cooling, 3 ml of P-Dimethyl Amino Benzaldehyde (P-DMAB) reagent was added, and the absorbance was read at 585 nm against the blank prepared without chitin or enzyme. The amount of N-acetyl-D-glucosamine released in the supernatant was determined using N-acetyl-D-glucosamine as the standard. One unit of the chitinase activity was defined as the amount of the enzyme products 1µ mole of N- acetate glucosamine in 1 ml of reaction mixture under the standard assay condition (Mathirannan et al., 1988). Stranded graph was prepared with curve for authentic N-acetyl D-glucosamine to convert the absorbency values to micro moles of N-acetyl D-glucosamine liberated from colloidal chitin.

Optimization of cultural characteristics

To study the effect of incubation time on Chitinase production the enzyme was estimated at different incubation periods (24, 48, 72, 96 and 120 h) for all the strains individually. For the effect of carbon sources on chitinase production the medium was supplemented with mannitol, glucose, sucrose, raffinose, arabinose, and galactose separately and chitinase production was calculated for all the strains after 72h incubation. To test the effect of Nitrogen sources the medium was supplemented with different nitrogen sources (0.1%) KNO_3, $(NH_4)_2 SO_4$, L-asperagine, $NaNO_3$ and Yeast extract for each strain and enzyme was estimated after 72h of incubation at room temperature. By adjusting the pH of the medium from 6 to 10 using 0.1N HCl / NaOH the effect of initial pH on chitinase production was studied.

RESULTS AND DISCUSSIONS

Four of the twenty one strains screened showed the positive results for chitinase activity by the production of clear zone surrounding the colony on chitin agar plates after 72 h of incubation. Among the strains *Agrobacterium tumefaciens* MRR 111 showed maximum CZ/CS ratio followed by *Bacillus altitudinis* MRR122 and *Paenibacillus* sp. MRR 124 (Table 1). However, maximum enzyme activity of 0.46 U/ml was recorded with *Paenibacillus* sp. MRR 124. Though CZ/CS ration was high, *A. tumefaciens* could not show maximum enzymatic activity as there was synchronization between size of the colony and activity.

Effect of Incubation Period

Our results (Figure 1) elucidated that the incubation period influences the enzyme production, where the chitinase activity increased steadily and reached maximum (0.46U/ml) at 72 h of incubation. Further increase in the incubation period led to a reduction in chitinase production. This was in conformation with the previous reports of Ahmadi et al., (2008) in *Paenibacillus chitinolyticus* JK2 isolated from seafood industrial wastes. The *Paenibacillus* sp. MRR 124 in the present study produced more amount of the enzyme at 72 h than the previous report. Meija saules et al., (2006) reported maximal chitinase production by *Serratia marcescens* WF at pH 6.5, and temperature of 28°C during fermentation up to 72 h. Bhattacharya et al., (2012), also reported that incubation period influences the enzyme production in *Serratia marcescens*, where in the chitinase activity increased steadily and reached maximum of 10.87 U/ml at 72 h of incubation. Wiwal et al.,(1999) reported maximum chitinase production by *Bacillus circulance* at 96h, while *in Pseudomonas* sp. and *Pseudomonas dispersa* production was highest at 144h incubation period. Though much variation in enzyme production was reported previously, all the four strains belongs to different genera showed highest enzyme production at 72 hours incubation.

Effect of pH

All the four strains exhibited the maximum enzyme activity at neutral pH with a progressive increase in enzyme activity from pH 4.0 to 7.0 (Figure 2). Similar result was also reported previously for the strains *Bacillus* sp. 13.26 (Purwani et al., 2004) and *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997) respectively. Organisms exhibiting different pH optima and decrease or increase in pH on either side of the optimum will affect the growth was previously reported by Bhattacharya et al., (2012).

Effect of carbon sources on Chitinase production

Chitin medium along with arabinose was found to increase chitinase activity in all the four *rhizobiacterial* strains, but supplementing with other carbon sources actually decreased the enzyme activity, when compared with chitin as sole carbon source (Figure 3). The non-*rhizobial* strain *Paenibacillus* sp. MRR 124 showed the maximum chitinase production of 1.91 U/ml followed by *Ensifer xinjiangense* MRR 110 with 1.88 U\ml when arabinose was used as carbon source. The fact that the arabinose in combination with chitin doubles the chitinase production has been reported earlier in *Streptomyces viridificans* (Gupta et al., 1995). Addition of easily metabolised sugars reduced chitinase production but supported growth, whereas control containing colloidal chitin as the sole carbon (control). Addition of glucose, sucrose, raffinose, and galactose decreased chitinase production in all the four *rhizobiacterial* strains.

Effect of Nitrogen Sources on Chitinase Production

Among various nitrogen sources tested, peptone was identified as the best nitrogen source producing highest level of chitinase (3.21 U/ml). In confirmation to our findings similar results have been reported in previous studies. (Figure 4) Addition of yeast extract has been reported to increase chitinase activity in *Serratia marcescens, Alcaligenes xylosoxydans* and *Paenibacillus sabina* JD 2 (Moneral and Reese, 1969; Vaidya et al.,2001). Gohel et al., (2006) also reported the significant influence of yeast extract, urea and peptone on chitinase production by *Pantoea dispersa*. The chitinase activity was lower with nitrogen sources such as ammonium sulphate, L-asperagine, potassium nitrate, and sodium nitrates when compared to control.

CONCLUSIONS

This was the first report of occurrence of nonrhizobial strains- Bacillus altitudinis and Paenibacillus sp. in the root nodules of Vigna trilobata. All the four rhizobacterial strains showed highest production of chitinase when chitin medium was supplemented with arabinose and peptone as carbon and nitrogen sources respectively at pH 7 after 72 hours of incubation.

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APPENDICES

Table 1: Ratio of Clear Zone/Colony Size (Cz/Cs) and Chitinase Activity of *Rhizobacterial* Strains from *Vigna Trilobata*

S. No.	Strain Names	Cz/Cs	Chitinase Activity (Units/Ml)
1	***Ensifer xinjiangense* MRR 110**	1.5	0.30
2	***Agrobacterium tumefaciens* MRR 111**	2.5	0.20
3	***Bacillus altitudinis* MRR 122**	2.0	0.40
4	***Paenibacillus* sp. MRR 124**	2.0	0.46

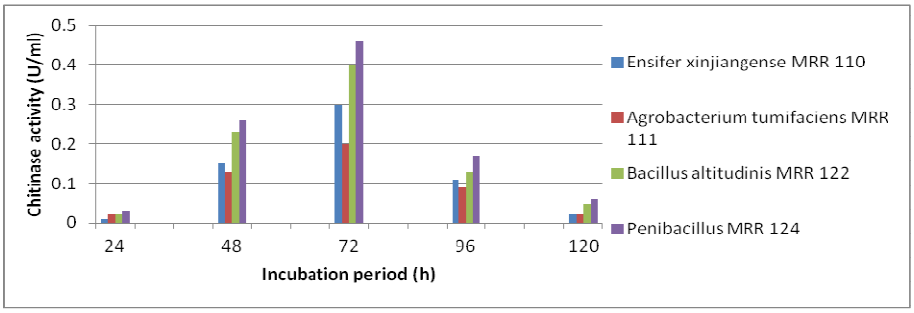

Figure 1: Effect of Incubation Period on Chitinase Production (U/Ml) By *Rhizobacterial* Strains from *Vigna Trilobata*

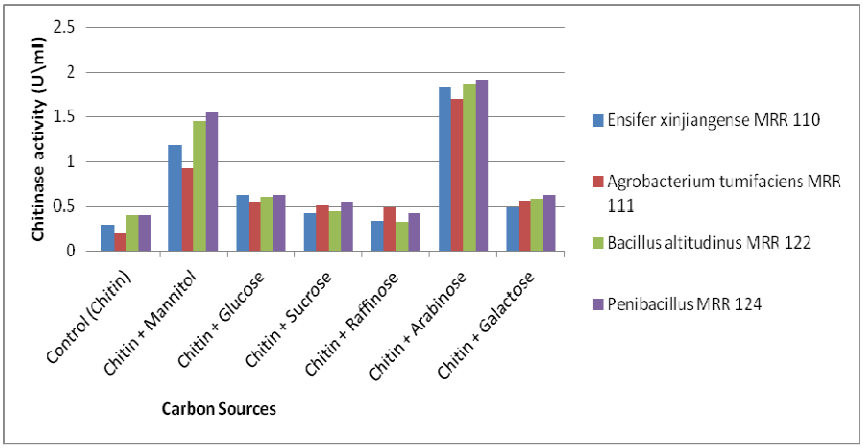

Figure 2: Effect of Ph on Chitinase Production (U/Ml) by *Rhizobacterial* Strains from *Vigna Trilobata*

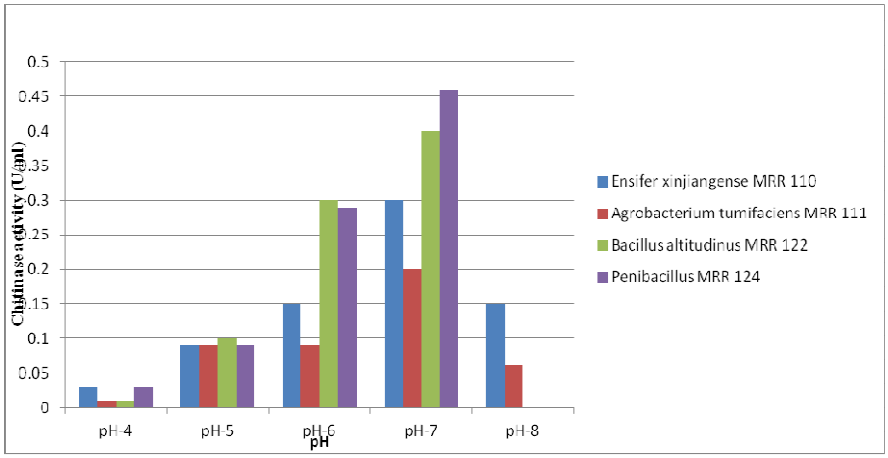

Figure 3: Effect of Carbon Sources on Chitinase Production (U/Ml) By *Rhizobacterial* Strains from *Vigna Trilobata*

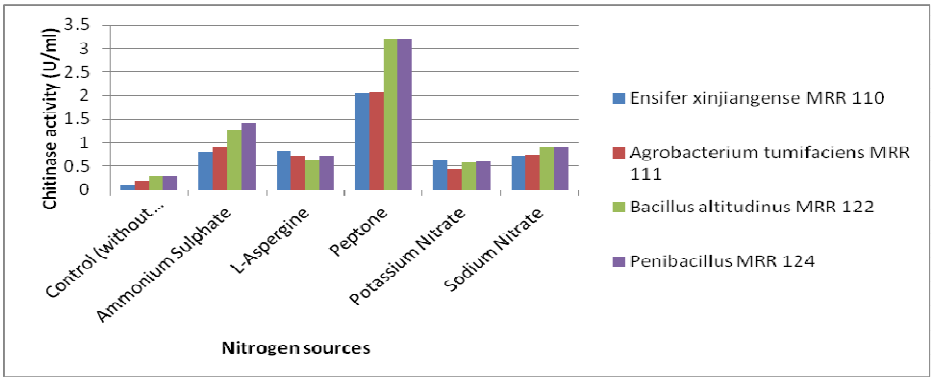

Figure 4: Effect of Nitrogen Sources on Chitinase Production (U/Ml) by *Rhizobacterial* Strains from *Vigna Trilobata*