JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. 4(1), p. 429-432

A Study on Fungal Antagonism by Chitinolytic Bacterial Isolates from Prawn Culture Farms of Ramanathapuram District, Tamil Nadu

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(Received: 26 November 2009; accepted: 21 April 2010)

Biological control of plant pathogens by soil-bacteria is a well established phenomenon and Chitinase production has been shown to play a role in the suppression of various diseases. The interest in chitin degrading enzymes and their application in management of fungal pathogens are significant because chitin is an important constituent of the cell walls of filamentous fungi, composed of β-1,4 linked units of the amino sugar N-acetyl-D-glucosamine (NAGA), speculated to play a vital role in fungal defense against harm and pressures. Chitinolytic bacterial strains were isolated from prawn culture farms of Ramanathapuram District, Tamil Nadu. Soil samples was serially diluted and inoculated on colloidal chitin agar (CCA) medium. Strains exhibiting clear zones around colony were picked and further subjected to antifungal activity by hyphal extension inhibition assay against *Rhizactonia solani, Trichoderma viride*, *Alternaria solani, Sclerotium rolfsii, Fusarium sp., Colletotrichum sp. and Aspergillus sp.* The potential Chitinase producing organism was identified to be *Bacillus subtilis JMC02* by 16SrRNA gene partial sequence.

Key words: Chitinase, chitinolytic bacteria, hyphal inihibition assay, fungal antagonism, *Bacillus subtilis* JMC02.

Chitin, a linear β-(1-4) – linked N-acetylglucosamine (GlcNAc) polysaccharide forms the structural component of the fungal cell walls[1] Chitinase enzymes (E.C 3.2.1.14), catalyze the biotransformation of chitin to its monomer or oligomeric components. These have been found in a wide range of organisms, including bacteria[2,3], plants[4,5] and fungi[6,7]. The production of chitinase by plants has been suggested to be a part of their defense mechanism against fungal pathogens. Chitinases produced by the bacteria seem to have a nutritional or scavenging role, as shown by their secretion into the medium[8]. In fungi, chitinase activity probably has a physiological role in hyphal growth and morphogenesis[9]. Several reports have suggested that chitinase producing bacteria can be effective as biocontrol of soil borne

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plant pathogenic fungi. The present study describes the fungal antagonism by a novel bacterium isolated from the prawn culture farms of Ramanathapuram district of Tamil Nadu, India against several phytopathogens.

MATERIAL AND METHODS

Site of sample collection

Ramanathapuram District of Tamilnadu, South India, is teamingly high with prawn culture farms. Therefore, due to abundancy of the Crustaceans in the sea shore and in the farm, the soil samples from these areas were collected aseptically and transported to the laboratory within six hours.

Preparation of Colloidal chitin

Around 5g of chitin powder was added slowly into 50 ml of 85% H_3PO_4 at 25°C under vigorous stirring for 2 hours. The suspension was poured into 500ml of 95% ice cold alcohol under vigorous shaking for 30mins. It was stored in -20°C until use. At the time of requirement 5ml of suspension was centrifuged and the precipitate formed was washed with 25ml of 0.1M sodium phosphate buffer (pH 7) for three times. The derived precipitate was dissolved in 45ml of 0.1M sodium phosphate buffer (pH 6).

Colloidal Chitin Media Formulation

Colloidal chitin prepared as mentioned above was added to the minimal agar medium in the concentration of 10% and sterilized. The sterile media was then cooled and poured on to the sterile petriplates and allowed to solidify. These plates were used for further studies in chitinase production and characterization.

Isolation of Chitinolytic bacteria

Chitin degraders were isolated by serial dilution of soil samples using the same water sample from which the soil was collected. It was then plated on 10% colloidal chitin agar (CCA) medium and incubated at 37°C for 72 hours. The organisms capable of degrading chitin with distinct zone of clearance on colloidal chitin agar (CCA) medium were selected and sub cultured in nutrient agar slants and maintained.

Optimization of Culture Conditions

The selected chitin degraders were analyzed for their biomass production at optimum conditions. This was carried out in colloidal chitin medium at various pH (3 to 10), Temperature (15°C, 30°C and 45°C), shaking (120 rpm) and Static Conditions. After one day of growth the cultures were harvested, centrifuged and the supernatant was used for chitinase assay.

Enzyme production

Cultivation of the isolate for chitinase production was carried out in 25ml liquid colloidal chitin medium in 100 ml Erlenmeyer's flasks for 72hours at 30°C on a rotary shaker (150 rpm). Cell growth was measured by absorbance at 540nm. The culture broth was centrifuged at 8000 rpm for 10 min and the supernatant was used for Chitinase assay.

Evaluation of Chitinase Activity

Chitinase activity was assayed with colloidal chitin as the substrate. Enzyme solution (0.5ml) was added to 0.5ml of substrate solution, which contained 1% of colloidal chitin in a sodium phosphate buffer (100mM, pH 8.0). The mixture was incubated at 37°C for 1hour. The reducing sugar released was measured by the DNSA method at 540nm using N-acetyl-D-glucosamine (GlcNAc) as standard. One unit of chitinase activity was defined as the amount of enzyme producing 1µmol of GlcNAc per hour under the specified conditions.

***In vitro* Antifungal Activity**

The *invitro* antifungal activity of chitinolytic bacterial isolates were studied against different phytopathogenic fungi namely, *Rhizactonia solani, Trichoderma viride*, *Alternaria solani, Sclerotium rolfsii, Fusarium sp., Colletotrichum sp. and Aspergillus sp.* Antifungal activity study was performed by hyphal extension inhibition assays. For this, potato dextrose agar (PDA) plate containing chitin (10%) was inoculated with fungi. At the centre, the chosen Chitinolytic bacterium was streaked and incubated at 30°C and allowed to over grow to visualize the hyphal inhibition.

Identification of the chosen Chitinolytic Bacterium by 16SrRNA Gene Amplification and Sequencing

DNA was extracted from pure culture of the chosen Chitinolytic bacterial isolate that showed prospective application. A partial DNA sequence for 16SrRNA gene was amplified by using ATG GAT CCG GGG GTT TGA TCC TGG CTC AGG(forward primer) and TAT CTG CAG

TGG TGT GAC GGG GGG TGG (reverse primer) [10]. Amplifications performed in 50µl reactions mixtures containing the template DNA, 40ng, 0.2µM, for each of the primers, dNTPs 200µM, Taq DNA polymerase 2.5U and 10X buffers 5µl. The mixture was subjected to the following amplification conditions; 2min at 94°C for 1min, and ended by a final extension step at 72°C for 7min. The PCR products mixture was purified and sequenced.

RESULTS AND DISCUSSION

The soil sample collected from the prawn culture farm was serially diluted and plated on colloidal chitin agar plates to screen for the growth of any chitinase producing organisms. Chitin being the primary source of carbon, only two different colony morphology could be identified and they were isolated as pure cultures for further analysis. These two bacterial isolates were named as JMC01 and JMC02 based on the name of the institution - ***J***amal ***M***ohamed ***C***ollege where the study was undertaken. The culture condition was optimized for various pH, Temperature, Shaking and Static conditions. The pH 7 and pH 9 was optimum for the biomass production of JMC01 and JMC02 respectively. Bothe the isolates demonstrated optimal growth under shaking condition maintained at 30°C. Supernatant obtained from the centrifugation of the chitinase broth after 24 hours incubation served as the crude source of the chitinase enzyme for various analyses. Kinetic studies of chitinase enzyme shows that the crude enzyme obtained from the broth was taken as the source of enzyme and

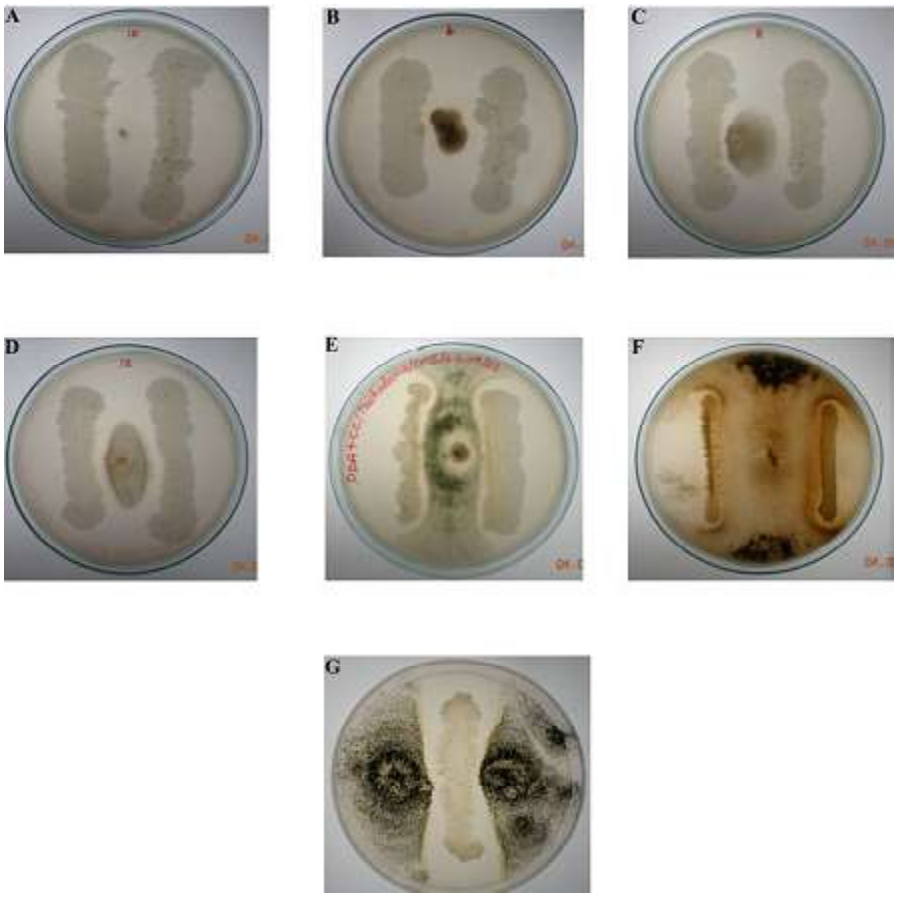

Fig. 1. Showing the hyphal extension inhibition activity of Bacillus subtilis JMC02 against various phytopathogens: (a) *Sclerotium rolfsii* (b) *Alternaria solani* (c) *Fusarium* sp., (d) *Colletotrichum* sp., (e) *Trichoderma viride* (f) *Rhizactonia solani* and (g) *Aspergillus* sp

kinetic studies were performed. The chitinase activity of JMC01 and JMC02 was found to be 0.2 and 0.34µmol/Min respectively.

As the prime target is to screen for the fungal growth inhibition, the *invitro* antifungal activity of chitinolytic bacterial isolates were studied on seven different fungi such as *Rhizactonia solani, Trichoderma viride*, *Alternaria solani, Sclerotium rolfsii, Fusarium sp. Colletotrichum sp. and Aspergillus sp.* Hyphae extension assay was done in PDA plate containing chitin, with test fungi and the bacterium streaked in the same plate for visualizing fungal antagonism. Among the two isolates, JMC02 exhibited inhibition of fungal hyphae extension where as JMC01 did not show any significant activity. Among the various plant pathogens tested, JMC02 was found to be effective in inhibiting the growth of *S. rolfsii* (Fig. 1A) at the maximum followed by *Alternaria sp.,* (Fig. 1B) *Fusarium sp.,* (Fig. 1C) and *Colletotrichum sp.* (Fig. 1D). Inhibition of the growth of *Trichoderma* (Fig.1E), *R. solanii* (Fig. 1F) and Aspergillus sp., (Fig. 1G) was only to a moderate level by JMC02. Based on the fungal antagonism, JMC02 was selected for 16S rRNA Gene Amplification and Sequencing for the identification of the isolate.

Nucleotide sequence accession number

The sequence data of the 16S rRNA genes of strain JMC02 was determined (using the BLAST tool on http:// www.ncbi.nlm.hih.gov) to be *Bacillus subtilis JMC02*. The sequence data has been deposited in the GenBank nucleotide sequence databases under accession number GQ120620. In conclusion, the use of 16S rRNA as molecular markers has become a routine technique for microbial ecologists, which can circumvent the limitations of traditional biochemical tests in the assessment of the biodiversity of microbial communities [10]. Through this technique, the strain JMC02 was assigned to the genus *Bacillus*. The potential use of the chitinase producing organisms have been examined and have yielded significant knowledge regarding their role in inhibiting few important fungal phytopathogens in this study directing us to the next level in the explorations.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. M. Sheik Mohamed, Principal, Dr. M. M. Shahul Hameed, Head, Dept. of Biotechnology, and the Management, Jamal Mohamed College (Autonomous), Tiruchirappalli – 620 020, Tamil Nadu, for their constant support and provision of the facilities.

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