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Original Research Paper

Phylogenetic Identification of DNase Secreting Soil Bacteria Antagonistic to Fungus *Aspergillus*

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ABSTRACT

Contamination of major cash crops by mycotoxigenic fungi in developing countries, including India, is becoming one of the major areas of concern. Continual use of synthetic pesticides to fulfil the increasing demand for consumption of major legumes has negative impact on human health and has major environmental risks associated with it. Microbes living in vicinity to these mycotoxin producing fungi have been found to be a promising substitute against harmful pesticides. The main objective of the present work was to isolate the bacterial strain which has a promising potential to act as an antagonist against the fungal pathogen *Aspergillus* which is considered to secrete Aflatoxin, the most potent mycotoxin. Large number of bacteria were isolated randomly from the rhizosphere of groundnut crop sown in Jamia Hamdard (New Delhi, India) randomly by serial dilution method, and screened for their antagonistic effect against fungus *Aspergillus.* Initial screening results show that, 14 of the isolates exhibit promising antagonism on dual culture media. Optimization of growth conditions was carried out at different pH, temperature and salt (NaCl) concentration. *In vitro* antagonism assay comprising of mycelial inhibition assay on to PDA using the scale of 1-4 (where 1 indicates bacterial colony completely overgrown by fungi, 2 indicates overgrown but bacterial colony visible, 3 indicates growth of fungus to the edge of the bacterial colony and 4 indicates inhibition zone) and inhibition of mycelial growth as observed by a significant drop on mycelial dry weight by co-inoculation of bacterial culture with fungal spores in M1 media. One of the bacterial isolates MAK603 exhibiting the maximum antagonistic effect was selected after optimization for further studies. The mechanism of action of MAK603 was studied *in vitro* for the production of hydrolytic enzymes. Phylogenetic analysis using 16 S rDNA amplification was carried out to establish its identity.

INTRODUCTION

In the 20th century, an increasing number of pesticides, based on biocidal molecules, were the means for a substantial increase in food and fibre production and quality. Because of the health and environmental concerns, continued extensive use of such molecules is intensively debated and substitutes are often urgently required (Gerhardson 2002). Besides crop plant resistance, various biological control methods based on natural pest suppressing organisms are regarded as main alternatives. Several approaches and concepts also have been tested and commercial organism-based preparations are steadily increasing. At present, they are not comparable to pesticides in meeting efficacy, market and other expectations, but they still have a promising future, especially where genetically modified organisms can be used. The agricultural sector that does not use pesticides for control of plant diseases is increasing and there is an urgent need for alternative methods. A deeper understanding of the importance of the soil and root microbiota in cropping systems and new molecular techniques for the study of microorganisms, as well as the possibility of manipulating their actions, has opened up new prospects for biological solutions (Johansson et al. 2003). One of these areas is the use of preparation of microorganisms as a biological means of controlling fungal diseases (Weller 1988, Thoma-show & Weller 1996). It is well demonstrated that many soils and rhizosphere bacterial isolates have antagonistic effects against plant pathogenic fungi when tested in dual cultures (Weller et al.1994). These bacterial strains are capable of directly antagonizing fungal pathogens by competing for niche and essential nutrients or by producing fungitoxic compounds (Pitt et al. 1994); they may also activate host defence system (Ongena et al. 2005). However, further biotechnological efforts are required to give them the status of being practical substitutes to pesticides.

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. It grows by producing thread like branching filaments known as hyphae (Payne 1998). Growth of fungus on a food source leads to contamination with aflatoxin (toxic and carcinogenic compound). Persons infecting with *Aspergillus* have often reduced or compromised immune system (Richard & Payne 2003).

Aspergillus is considered to contaminate a major part of cash crops like legumes, groundnut, corn, etc. in an agriculture based country like India. (Stephane et al. 2005). Most recently, research has been directed towards developing biological control methods for these harmful pathogens.

Rich diversity of the microbial world provides a seemingly endless resource for this purpose. Increasing the abundance of a particular strain in the vicinity of the plant can suppress disease without producing lasting effects on the rest of the microbial community or other organisms in the ecosystem (Gilbert et al. 1993). Biological control is more durable than the control achieved by synthetic chemicals because of the complexity of the organism's interaction, involvement of numerous mechanisms of disease suppression by a single microorganism and also the adaptive nature of most biocontrol agents to the environment (Cook 1993). Therefore, to continue agricultural growth and to feed the growing human population in a healthy environment, complimentary and new alternatives to the present dominance of chemical applications needed to be developed.

Although the number of products containing biocontrol formulation is increasing, even then they represent only 1% of agricultural chemical sales (Fravel 2005).

The main objectives of this research was to isolate the microbes from the soil of the crop which are more prone to attack by these fungi, to evaluate their disease suppressive ability and their phylogenetic assessment using 16S rDNA sequencing by PCR amplification. By using modern molecular biotechnology, future microbial gene based biocontrol may be integrated with plant breeding by transgenic manipulations to produce purified antifungal gene products as a safe chemical control (Cook 2000, Mcspadden & Fravel 2002).

MATERIALS AND METHODS

Microbial strains, culture media and conditions: Peptone, yeast extract, beef extract, agar, dextrose and other microbiological media used were either from DIFCO or HI-MEDIA and all other chemicals used were from Sigma.

Isolation of Bacterial cell from soil: The antagonistic bacteria selected was isolated from groundnut rhizosphere using dilution plating technique i.e., 1g of soil was taken into sterile polypropylene tube and 10 mL of sterile distilled water was added to it, tube was shaken on orbital shaker for 10 min to fully disperse soil particles; following shaking sample was diluted 3 times in a 10-fold series (i.e., dilution of 10^0,10^{-1} and 10^{-2}) upon each dilution, isolation of bacteria was made on nutrient agar medium followed by incubation at 37°C for O/N, and visibly different bacteria were selected and streaked onto individual NA plates. Isolates were stored at 4°C (Wakelin et al. 1998) and routinely grown on nutrient agar at 28°C for 24 hours.

Fungal strain (*Aspergillus flavus*): *Aspergillus flavus* strain No. MTCC 277 was obtained from IMTECH Chandigarh, India and grown and maintained on potato dextrose agar (PDA).

***In vitro* screening for antagonistic activity:** Isolated bacterial strains were tested for antagonistic potential by dual culture technique on PDA as described earlier (Montealegre et al. 2003). A. flavus spores were spread on one half of PDA plate while the other half was spread with freshly prepared bacterial cells followed by incubation at 28°C ± 2°C for 5 days. Several bacterial strains exhibited antagonistic potential but one of them (MAK603) gave the best antagonistic effect against *A. flavus* and was selected for further investigations.

Mycelial inhibition assay: The bacterial isolate MAK 603 was spot inoculated at equidistant point around the edges of PDA plates with centrally placed mycelial plug of the pathogenic fungus *A. flavus* (mycelium side down) (Wilson Boyd 2000). Plates were assessed after 6 days using a scale from 1-4, where 1 would indicate bacterial colony completely overgrown by fungi, 2 would indicate overgrown but bacterial colony visible, 3 would indicate grown to the edge of bacterial colony, 4 would indicate inhibition zone i.e., no growth of fungus around bacterial colonies.

Effect of co-inoculation on dry weight of mycelia: Fungal spores were collected from freshly prepared culture of *A. flavus*, and suspension was prepared. 50 microlitres (containing 1×10^6 spores) of this suspension was co-inoculated with 50 microlitres (containing 1×10^6 cfu) of freshly grown bacterial culture in M1 media (sucrose 200 g/L, yeast extract 20 g/L, $MgSO_4$ 10 g/L, $ZnSO_4$ 26 g/L, $CuSO_4$ 2.6 mg/L, $FeSO_4$ 5mg/L, $Co(NO_4).6H_2O$ 1mg/L) followed by incubation at 28°C for 5 days. After incubation, the mycelia was collected from both control as well as test flasks by filtration using sterile Whatman filter paper, mycelia were dried in oven and properly dried mycelia were weighed (Vaidya et al. 2001).

Optimization of growth conditions: Selected bacterial strain MAK 603 exhibiting the maximum antagonistic activity was tested for growth conditions at different temperatures, pH and salt (NaCl) concentration and was also characterized morphologically and phenotypically by Gram's staining reaction.

Determination of IAA: Assay for the production of Indole Acetic Acid (IAA) was carried out in order to assess the plant growth promoting potential of bacterial isolate MAK603 by rapid *in situ* assay (Bric et al. 1991). In brief, freshly prepared bacterial culture MAK603 was spread on to the nutri-

ent agar plate and incubated overnight at 37°C. After proper incubation, the plates with growth were covered by Whatman paper (No. 1) which imprints the bacterial colonies on to it, this Whatman paper was then treated with Salkowski reagent (2% 0.5 M ferric chloride in 35% perchloric acid), followed by incubation at room temperature till adequate colour development occurs. The colorimetric estimation of production of indole acetic acid was also carried out by using the colorimetric assay (Gordon & Weber 1950).

Determination of phosphate solubilizing activity: Bacterial strain MAK603 was spot inoculated on Pikovskaya medium containing tricalcium phosphate [$Ca_3(PO_4)_2$] followed by incubation at 30°C for 7 days (Pikovskaya 1948). Plates were then observed for zone of clearance around the inoculated area on to the Pikovskaya plate.

Detection of siderophores and hydrogen cyanide (HCN) production: Siderophore production by bacterial isolate MAK603 was determined by Chrome Azurol S (CAS) assay (Schwyn & Nielands 1987). In brief, production of siderophore was checked both in liquid as well as solid medium as freshly grown culture of strain MAK603, was spot inoculated on to CAS agar plate followed by incubation at 30^0C. Similarly, to check the production of siderophores in solution (0.5 mL) of supernatant of freshly grown culture of MAK603 was mixed with 0.5 mL CAS assay solution, the absorbance was measured at 630 nm (colour shift from blue to orange). The cyanogenic activity was assessed on King's B agar media by spot inoculation of freshly grown bacterial culture on King's B agar media (Lorck H 1948).

Analysis of extra-cellular nuclease activity: Bacterial isolate was tested for extracellular hydrolysis of DNA which was being tested onto the DNase agar plate prepared from the DNase test agar medium. 4.2 g of DNase test agar was dissolved in 100 mL of double distilled water and autoclaved. Bacterial strain MAK603 was streaked on to it and incubated at 37°C (O/N). After proper incubation, the plates were assessed for area of hydrolysis by flooding with 1N hydrochloric acid (HCl), to precipitate out the hydrolysed DNA (Jeffries et al. 1957).

Bacterial genomic DNA isolation: Bacterial isolate MAK603 was grown in nutrient broth at 37°C and cells were harvested after 24 hrs and immediately processed for genomic DNA isolation by standard procedure (Sambrook et al. 1989). The concentration and purity of DNA was determined by measuring the optical density at 260 nm and ratio at 260/280 nm and by gel electrophoresis. Isolated DNA was stored in TE buffer at -20°C till further use.

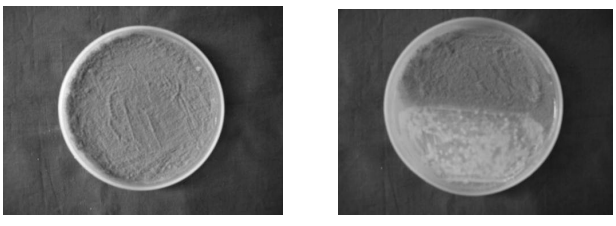
PCR amplification of rRNA gene: Isolated genomic DNA was amplified using sequence specific primers for 16S rRNA gene [forward Pf (GTAACCTGCCTGTAAGACTG) and reverse Pr (GCCCTTTGTTCTGTCCATTG)]. Amplification was performed in 20 mL mix having 5 mL of DNA (500 ng), 9 mL nuclease free water + 2 mL (pico moles) of primer mix (Pf+Pr) and 1 mL of dNTPs, 2 mL buffer. DNA amplification was performed on thermal cycler (Roche model LC 480). The temperature profile of the cycles was as follows: 1 cycle for denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, and final extension at 72°C for 1 min. PCR product was visualized on 1% agarose gel using ethidium bromide staining and photographed on UV transilluminator chamber.

Sequencing and phylogenetic analysis: The amplified product for rRNA gene was sequenced using ABI PRISM 3130 XL, and a partial sequence of about 700 bases was obtained using forward primer Pf (GTAACCTGCCTGTAAGACTG). The sequence was compared with those available in the genebank using BLAST searches to obtain the phylogenetic relationship of the selected bacteria.

RESULTS

Large number of bacterial cells were isolated from rhizosphere of groundnut crop and tested for their antagonistic activity against *Aspergillus* by PDA plate dual culture assay. Out of all the isolated bacteria 14 isolates produced inhibition zones (data not shown) and the strain MAK603 gave the best antagonistic effect against *Aspergillus* (Fig. 1). The strain was consequently selected for further studies.

Mycelial inhibition assay: Mycelial inhibition at a scale of 4 (no growth of fungus *Aspergillus*) as compared with control (scale of 1) around bacterial colony confirms the antagonistic efficacy of bacterial isolate MAK603 as observed after proper incubation of plates for 6 days. Control plate exhibits the growth at the scale of 1 (PDA plate completely covered by *Aspergillus* mycelia) while test plate having antagonistic bacteria exhibits the inhibition at scale of 4 (no growth of fungal mycelia around the edges of visible bacterial colony). Similarly PDA plate with centrally challenged



(A) (B)

Fig. 1: PDA plate displaying the (A) Control plate completely covered by fungus *Aspergillus* while (B) shows the antagonism by bacterial strain MAK 603.

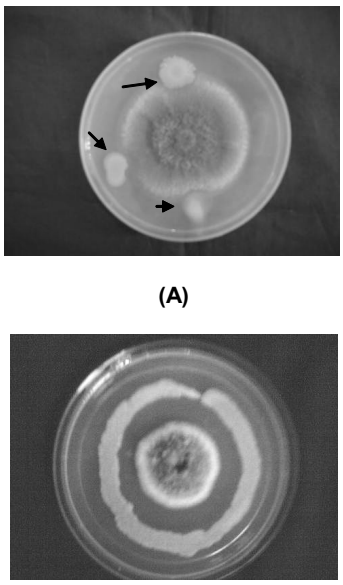


Fig. 2: PDA plate displaying the (i) Mycelial Inhibition Assay (shown by arrow) as centrally placed fungal mycelial plug being inhibited around the edges of bacterial inoculation. (ii) Mycelial inhibition assay carried out by streaking the freshly prepared bacterial culture around centrally placed mycelial plug of fungus *Aspergillus*.

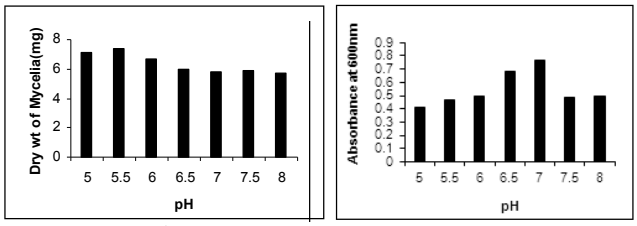


Fig. 3: Effect of different pH (5-8) on the growth of (A) fungus *Aspergillus* and (B) bacterial strain MAK 603.

fungal mycelial plug was streaked around by freshly prepared bacterial culture followed by incubation at 30°C for 5-7 days. Plates were then observed for zone of inhibition.

Optimization conditions for growth at different temperature, pH and salt (NaCl) concentration: Optimization of both fungus *Aspergillus* and bacterial isolate MAK603 for growth conditions at different physiological parameters like temperature, pH and salt (NaCl) concentration was carried out to observe the proper growth condition at different physiological parameters. Bacterial isolate MAK603 exhibited the best growth at the pH and different temperature range at which fungus *Aspergillus* showed the optimum growth (Fig. 2).

Effect of co-inoculation on dry weight of mycelia: Co-inoculation of M1 media with freshly prepared fungal spores (1×10^6 spores) and bacterial isolate MAK603 resulted in significant reduction in dry weight of mycelia as observed after proper incubation of M1 media at 28°C± 2°C (Fig. 3). Decrease in dry weight of mycelia from the flask containing both bacterial and fungal culture at an equal concentration explains the suppression of growth of fungal mycelia in presence of bacteria as compared to control, which contains only the spores of fungus *Aspergillus*. Weight of properly dried mycelia was found to be 10.55 mg/10 mL of M1

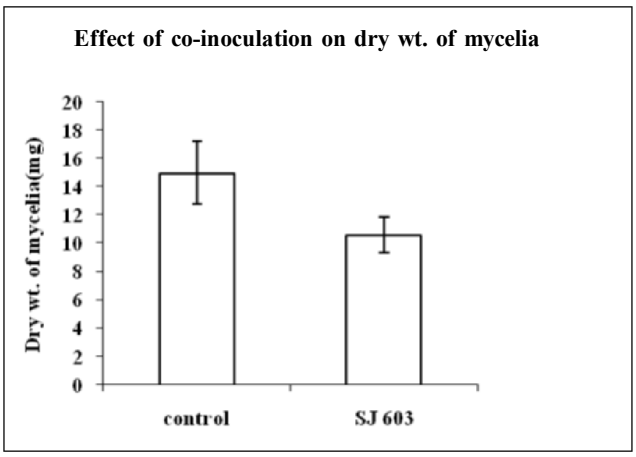


Fig. 4: Effect of co-inoculation of MAK 603 with fungal spores in M1 media. Control consisiting of media being inoculated by fungal spores only. Bars representing the SE of mean as the experiment being carried out in triplicate.

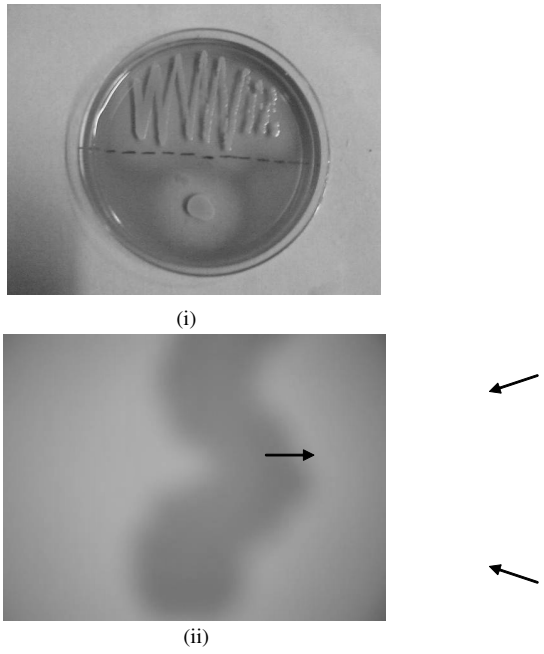


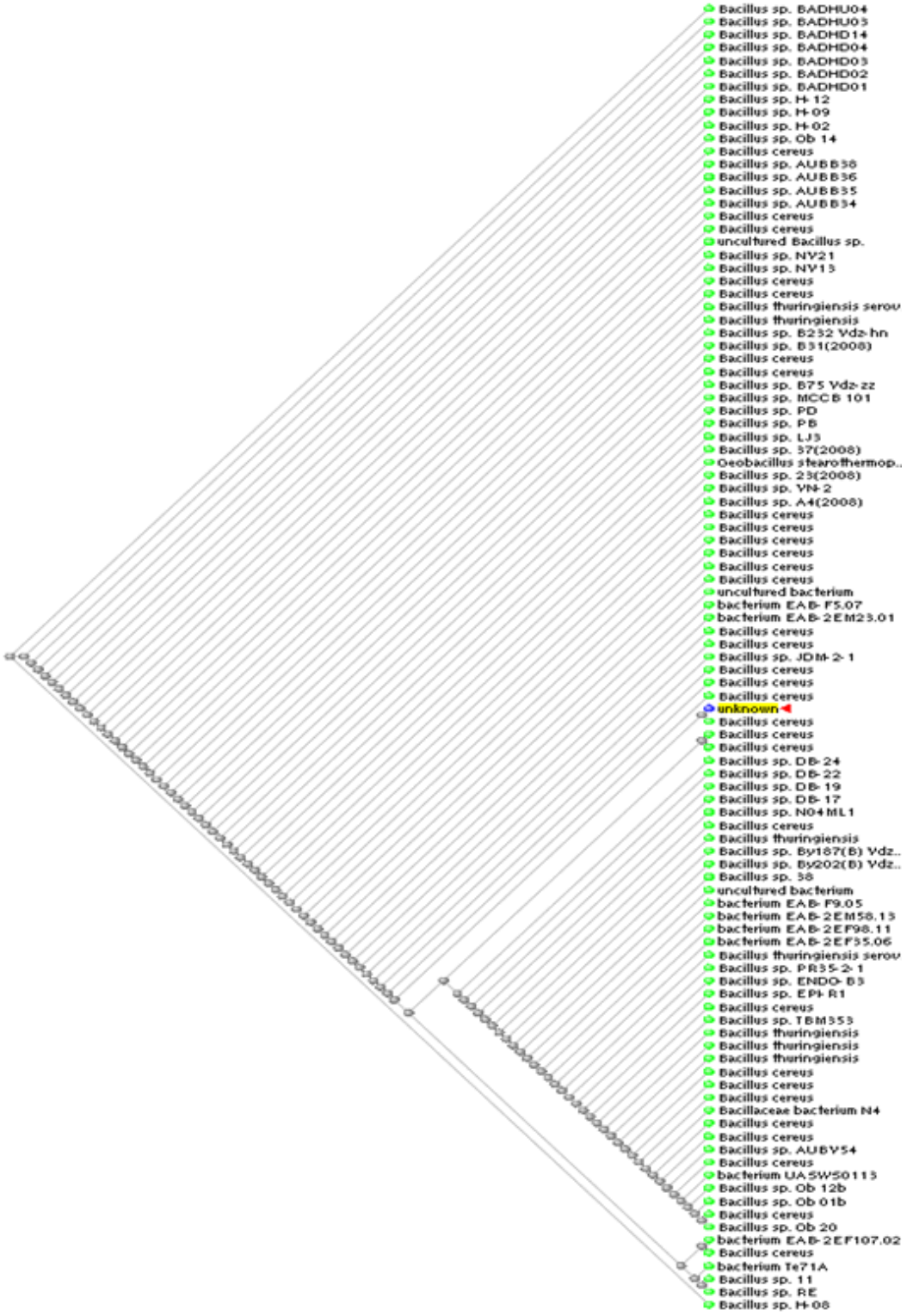
Fig. 5 (i): Figure displaying the hydrolysis of DNA around streaked and inoculated area (shown by arrow) on to DNase agar plate. (ii): Picture displaying area of hydrolysis by MAK 603 on to DNase agar plate in a closer view (indicated by arrow).

TTCCCTTATGGTGAGTGTGAGATTGACCGGACTGTTGACACTTGCTGCTGCTCTGCCTCC
GTCAACGCGGAGATAACCTTTTGTTTTTACTATGGATGTAATCATTTTATTGGCTTTACTTT
CTACTTGACGCTCCATAATACGATCCATCATATGATTAACCGTGACATTCGTCCCGCGTTA
CTTGAGCCAATATGCAGCTTTTGGTGGGTCTGTATACTCATGATTACCACATTATTTTTCTT
GACGGTACAGTCTTCGGGTAGTTACGTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGC
GTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTA
GCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTG
TAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGGGCCGCAAGGCTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGGAAGCAACGGCGAGGAA
TCTTTACCAGGTCTTGACATCCTTCTGAACAACCCTAGAAGATAGGGGCTTCCTCTTCGGA
AGCAGAGGTGACCAGGTGGGTGCATGGGTTGTCGTCCAGCTCCGTGGTCCTGAGAATGT
TGGGTTAAGGTCCCGCAAAGCGAGGGCGCAACCCCTGGATTCTTTAGTTGCCATTCATTT
TATGTTTGGCTACTTCCTAAGGGGTGACTGGCCGGGTGAACAAACCGTGAGGGAAGGGT
GGGGTATATACATTCTAAATCCTTATTGTCCCCTTATTAGACCTGGGGGGTTCACCCCACG
GGTCTAACATGTGGAAGAAAACAAGGAGGAGTGAACATGGTCTTCCTCG

Fig. 6: Sequence of MAK 603 using forward primer.

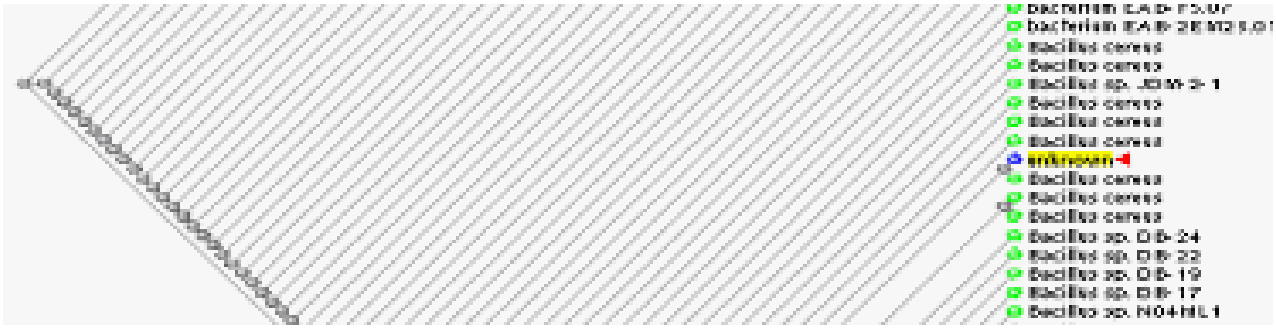
TCGTATTAGCGAGTTTATACGCGGTGATTTTCATAAACGCCCGGGCCTCGTTCAATGCTG
CGATAAACACGTCTTCCATCGCCTTCCTCCAGCGCTCATGAAGTCAAGGTGCTGACAACT
CCCTTTGTTCATCTGTTGCATCCCGCTCCGGCCAGGAGGCTCCCAGCTCGTCTATGGCCT
GTTTCAGCAATTCATTATTCAGTTCCCGCTCTTTACAGACCGCCTGACCGTTGCTGATCAC
CTGAGTTAGCTCACGCTCCAGGCTCCTGAGTTCATTGTCTGCATTATGTTTTTCTAATCCC
GCCAGCAGCTGTTCCTGCCGTGGCTGAGTCGACGTACCCCGCAAAACATCTGATTCGTCT
GTCAGCCGTTGATAACGATCCAACCATGTCTTCGATATCCTCCCCAGCGAAAAGAGATTA
ACCACGAAGACCGGGTTTATTTTTTTCATGCCGGCGGATGCGCGGATCGAGTTCGTCCGG
TTCTCCAGTAGACGCACAAAAGGAACAGTGTTCCTCCATATCTCTACGCATTTCACCGCT
ACACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCA
CGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCC
AATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCAACTAGCACTTGTTCTTCC
CTACACAGAGTTTTACGACCCGAAAGCTTCATCACTCACGCGGCGTTGCTCGTCAGACTT
TCGTCCATTGCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGCGTGTCTCAGTCC
CAGTGTGGCGATCACCCTCTCAGGTCGCTACGCATCGTTGCCTGGTGAGCGTAACTCACA
CTAGCTATGCGACGCGGGTCATCATAGTGACAGCCGAAGCCGCTTCCAGTTCGACCATG
CTTTCAATGTATCGATAAGCCCGGCTCCGACTATCCAGTTCAGGCAGGTTCACAGT

Fig. 7: Sequence of MAK 603 using reverse primer.



(i)

Fig.8 (i): Displaying the blast tree obtained from NCBI BLAST of the sequence obtained for MAK 603.



(ii)

Fig. 8(ii): Shows the more closer view of same displaying the resemblance of bacterial isolate MAK 603 with *Bacillus cereus*.

medium in case of test flask whereas it was 15.74 mg/10mL of M1 medium in case of control.

Effect of variable pH on the fungal and bacterial growth: Effect of variable pH on the growth of both *Aspergillus* as well as antagonist MAK603 was assessed by growing them at variable pH (5-8; at an interval of 0.5). Dry weight of fungal mycelia was taken after the proper incubation of potato dextrose broth inoculated with freshly grown spores of fungus *Aspergillus*. Maximum dry weight (7.3mg/10mL) was observed in the medium having pH of 5.5 while in other flasks with pH range of 6-8 shows the less dry weight of mycelia compared to dry weight from flask of pH 5.5 (Fig. 4). The growth of bacterial strain MAK603 was estimated by measuring the O.D. at 600nm (Fig. 4).

DISCUSSION

Natural soil-borne agents, particularly microbes living in close vicinity to disease causing pathogens particularly fungi, have been reported to be suppressive and regular attempts are being made to determine their nature of suppression (Worku 1996). *In vitro*, mycelial inhibition on solid and liquid media are useful screening techniques for assessing the potential antagonistic activity against mycotoxigenic (secreting the most potent mycotoxin, aflatoxin) fungi *Aspergillus* (Wilsonboyd 2000). Production of hydrolytic enzymes by antagonistic bacteria has been extensively studied and has been found out to play a key role in control of various plant pathogens (Weller 1988, Whipps 1997). DNase agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called deoxyribonuclease or DNase, that hydrolyses DNA. DNase agar contains nutrients for the bacteria, DNA, and methyl green as an indicator. Methyl green is a cation which binds to the negatively-charged DNA. Deoxyribonuclease allows the organisms that produce it to break down DNA into smaller fragments. When the DNA is broken down, it no longer binds to the methyl green, and a clear halo will appear around the areas where the DNase-producing organism has grown. Secretion of nucleases may be one of the mechanism involved for antagonistic effect. Secretion of DNase may play an important role in biology of the soil bacteria *Bacillus cereus*. Results from the antagonism assay clearly demonstrate that the selected strain MAK603 strongly antagonize the fungus *Aspergillus*. Sequencing of the amplified product when searched onto the database available from NCBI shows strong resemblance with *Bacilli* group and highest resemblance has been found out to be with *B. cereus* (95%). Selected strain MAK603 tested positive for gram stain. As much of the research in the area of biological control has been centralized or focused mainly on to gram-negative bacteria belonging to the genera pseudomonads and *Erwinia* (Shoda 2000, Costa 2001). Bacilli group has been proven to be quite advantageous for the development of perfect formulation as they are endospore forming and resist drought conditions and also their efficacy has been highly striking (Elizabeth 1999).

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