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ISOLATION AND IDENTIFICATION OF ACTINOMYCETES FROM GARDEN SOIL AT MIRAJ, IN SANGLI DISTRICT

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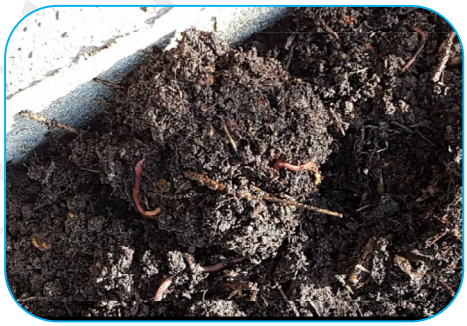
ABSTRACT:



A total of 50 isolates of actinomycetes were isolated from 5 soil samples collected from Nursery Garden land at Miraj, in Sangli district. All 50 isolates exhibited a range of different colony colours like dark grey, grey, brownish, brown, milky white and whitish. All the isolates were later purified and subjected to a few enzymatic screening. Results indicate that, 38, 36 and 31 isolates showed the ability to secrete cellulase, lipase and protease. All 50 isolates were then subjected to antimicrobial test using selected phytopathogens as test strains and it was observed that 5, 19, 23 and 29 of the isolates showed antagonistic reaction with Fusarium palmivora, Bacillus subtilis, Pantoae dipersa and Ralstonia solanacearum. Six of the most promising isolates were selected and identified using their 16S rRNA sequence. Further these six isolates identified using their 16S rRNA sequence and were identified as Streptomycetes spp. Further it can be studied and actinomycetes can be utilize in agriculture industry these potential microbes for sustainable agriculture.

KEYWORDS: : *Garden soil, isolation, Sangli District.*

INTRODUCTION

Actinomycetes is a nontaxonomic term for a group of common soil microorganisms sometimes called "thread or ray bacteria." Actinomycetes are simple prokaryotic organisms that are gram positive; they are predominant in dry alkaline soil. Actinomycetes DNA are rich in G+C content with the GC% of 57-75. They are considered highly valuable as they can produce various antibiotics and other therapeutically useful compounds with diverse biological activities [1]. The actinomycetes are important not just to the pharmaceutical industries but also the agriculture [2] also reported that the bacteria that cause firelight to apple and *agrobacterium tumefaciens* a casual agent of Crown Gall disease. Identification of actinomycetes using microscopic techniques alone was not enough to ensure certainty. Therefore biochemical methods would be the best method to identify actinomycetes to their species level. With the advancement of technology in molecular study, primers had been developed by

researchers to target specifically the 16S rRNA sequence of the actinomycetes[3,4] Identification of actionmycetes to genus level was made possible in a right way. In this study, actinomycetes from the garden soil of miraj, sangli district are isolated. The isolates were later tested for their bioactive compounds and identified using primers targeting their 16S rRNA sequence of the potent isolates.

METHODOLOGY

Collection soil samples

Soil samples were collected about 20 cm below the surface of the ground. All the soil samples were collected randomly from non agriculture land of Miraj at Sangli district. . Soil sample were then packed in a plastic bag.

Microbes' isolation and enumeration

Soil samples were air dried for 7 days prior isolation. This helps in reducing the number of gram negative bacteria. According to researcher[2] soil suspension method was used, where 1 g of the soil sample were taken and mix with 100 ml of sterile distilled water. The soil suspension was shaken vigorously on an orbital shaker at 200 rpm for 1 h. 200 ml of the soil suspension used on Starch Casein Agar at pH 7. A series of dilution of the suspension from 10^{-3} to 10^{-6} were done with duplicates. All the plates were incubated at 30°C for 14 days. Emerging actionmycetes were picked and streaked onto fresh Starch Casein Agar plates and incubated at 30°C for 7 days. Colony forming unit per one gram of soil was determined for all the samples collected.

Enzymatic Activity

All the isolates were screened for their cellulose producing ability using soluble Azo-CM-Cellulose as substrate (Peptone, 1.0 g; yeast extract, 1.0 g; MgSO4.7H2O, 0.5 g; KH2PO4, 0.5 g; (NH4)2, 1.0 g; substrate Megazyme, 1.0 g; agar, 15.0 g and distilled water, 1000 ml) at pH 7. According to researcher [5] gelatin hydrolysis and screening of protease activity was used. For Lipase activity researcher [6] method was determined for esterastic activity by using Tween 20. Formation of halo zone measured after completion on second day of incubation.

Antimicrobial screening

Well diffusion method for the antimicrobial testing against *Fusarium palmivora*, *Bacillus subtilis*, *Ralstonia solanacearum* and *Pantoae dispersa*. Isolates of actinomycetes were removed from their agar using a sterile cork bore of 4 mm in diameter and placed onto agar plate lawn with the pathogenic microbes. Halo zones were measured after completion of 48 hours.

Genomic DNA extraction

Genomic DNA was extracted from a few selected isolates using *BACTOZOL KIT* from Molecular Research Center, Inc. Isolation protocols are according to the manufacturer instruction.

Polymerase chain reaction amplifications

Amplifications were performed in a 25.0 µl mixture containing 16.3 ml of distilled water, 2.5 ml of 10X PCR buffer (Promega), 1.5 ml of 25 mM MgCl2 (Promega), 0.5 ml of 10 mM dNTP's (Promega), 0.2 ml of *Taq* polymerase (Promega), 1.0 ml for both 0.05 mM of Com1 (5'CAGCAGCCGCGGTAATAC3') and 0.05mM of Com2 (5'CCGTCAATTCCTTTGAGTTT3') primer [3] respectively, and 2.0 ml of genomic DNA. The reaction tube was then put into Thermal cycler, which had been programmed to preheat at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and elongation at 72°C for 45 sec before a final extension of 72°C for 10 min. Product size estimated was 408bp. Sterile distilled water which substitute template DNA was used as negative control. PCR products were purified using Invitrogen kit according to its protocol.

Sequencing of PCR products

Sequencing of purified PCR products was done by DNA sequencer. The obtained 16S rRNA sequences were compared to sequences in the NCBI genebank database with the Basic Alignment Search Tool (BLAST) [7].

RESULTS

Microbes' isolation and enumeration

Colony forming unit per gram of soil, showed the density of actinomycetes isolated from the soil was highest at 8.6 X 10^7 from the Miraj soil and the lowest was from the garden soil with just 9.4 x 10^6. Appearance of colony details of 50 isolates of actinomycetes was isolated as shown in table no. 1 each of the isolates were later categorized accordingly their morphology on colour as in figure 1.

Enzymatic screening

Enzymatic tests were conducted basically to determine the ability of actinomycetes to act as a degrader of organic compounds when applied for agricultural usage. Cellulose comprises the highest of the carbon sources in plants apart from lignin while lipid and protein are both present mostly in animal manure or other organic agrowaste but are least in plants. The study showed that 48, 46 and 41 isolates of actinomycetes produced cellulase, lipase and protease respectively.

Antimicrobial testing

Three strains of pathogenic microbes (*Fusarium palmivora*, *Bacillus subtilis*, *Ralstonia solanacearum* and *Pantoae dispersa*) were chosen as the test strains for the study. All the strains were chosen due to the reason that these microbes exhibited pathogenic effect towards certain commodity plants. Antimicrobial tests conducted showed that 5, 19, 23 and 29 isolates of actinomycetes produces antagonistic reaction for *F. palmivora*, *B. sub tilis*, *R. solanacearum* and *P. dispersa* respectively. Results obtained from the sequencing of purified PCR products showed that all the actinomycetes isolates belongs to the genus Streptomyces.

Table 1. Shows appearance of soil samples

Soil samples Nursery Garden	Isolate number	Colony colour	cfu/g
Vijay nagar Sangli	1,2,3, 4,5,6,7,,8 9,10,11,12	Grey Brownish Whitish	4.0 x 10^7
Ganesh Nursery Sangli	13,14,15,16,17, 18,19, 20 21,22,	Dark grey Whitish Grey	1.5 x 10^7
Ganga Nursery Kupwad	22,23,24,25,26, 27,28,29,30,	Whitish Grey	9.4 x 10^6
Tarai Nursery Sangli Islampur road	31,32,33, 34,35,36, 37, 38, 39, 40,41,42	Brownish Grey Milky white	8.6 x 10^7
Mayur Nursery Inam Dhamni	43,44,45,46, 47,48,49, 50	Whitish Grey Brown	2.3 x 10^7

Figure 1. Colony colour on Starch Casein Agar.

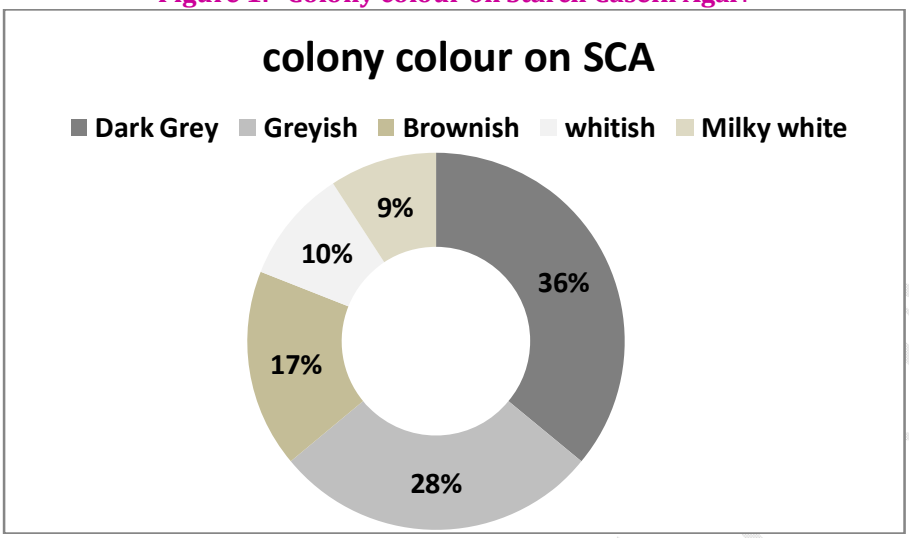


Table no.2 Bioactivities produce by potential actinomycetes for Enzymatic activity

Isolate No.	Actinomycetes spp	Enzymatic activity (mm)		
		Cellulase	Lipase	Protease
5	*Streptomycetes* spp.	24.0	19.5	0
8	*Streptomyces aureofaciens*	20.5	23.5	27.0
13	*Streptomycetes* spp.	25.5	20.0	26.5
16	*Streptomycetes* spp.	16.0	18.0	0
17	*Streptomycetes* spp.	0	13.5	20.5
21	*Streptomycetes* spp.	27.0	21.5	30.0

Table no.3 Bioactivities produce by potential actinomycetes for Antimicrobial activity

Isolate No.	Actinomycetes spp	Antimicrobial activity (mm)			
		F. palmivora	*B. subtilis*	*R. solanacearum*	*P. dispersa*
5	*Streptomycetes* spp.	0	12.0	31.0	14.0
8	*Streptomyces aureofaciens*	0	18.0	28.5	24.0
13	*Streptomycetes* spp.	0	0	28.5	29.0
16	*Streptomycetes* spp.	0	28.0	26.0	32.0
17	*Streptomycetes* spp.	0	27.0	24.0	28.0
21	*Streptomycetes* spp.	0	34.0	30.0	36.0

DISCUSSION

From the 5 soil samples collected from Garden land at Miraj, in Sangli district it was garden observed that the colony forming unit per gram of soil produce by each of the soil samples collected in this study were higher than the colony forming unit per gram of soil obtained from researcher study conducted byresearcher[8], they observed that the highest colony forming unit per gram of soil for actinomycetes isolated from soils planted with ornamental plants were $1.5^{7} \times 10^{3}$ which was very much lower than the count obtained in this present study 0.99 - 8.6 x 10^{7}. According to researcher[9], showed that colony forming unit per gram of soil for both Chesapeake Bay soil 1.8 x 10^{2} - 1.4 x 10^{5} and Korean vegetative garden soil 1.17 - 4.20 x 10^{6} respectively, were also lower than the results obtained in this study. One reason may be the other media, which influence the growth rate of the microorganisms and also the environment of the soil such as the humidity and other reason pH which are noted to influence the microorganisms' growth rate according to researcher[2]. Approximately 96% that is 48 isolates of the isolate produced one or more enzymatic activity. From the total isolates 20.4% produced only one enzyme, while 49.4% produces two types of enzymes and 26.2% produces all the tested enzymes. This indicates that actinomycetes possess the potential to secrete broad range of enzymes, which may be nature's selection of the microorganisms in order to survive in a often changeable environment. A total of 4.25% of the actinomycetes produced antibacterial substances towards only gram positive bacteria, 34.3% towards only Gram negative bacteria and 36.2% against both gram positive and negative bacteria. The potent six isolates of the potential producers of antibacterial and enzymes that were selected based on their activity, clear zones produced by isolates 5, 8, 13, 16, 17 and 21. The most prominent secondary metabolite producer's is isolate number 21 which produces clear zone measuring 27.0 mm for cellulase, 21.0 mm for lipase, 30.0 mm for protease, 34.0 mm for *B. subtilis*, 30.0 mm for *R. solanacearum* and 36.0 mm for *P. dispersa* shown in Table 2. In this study it's observed that identification of actinomycetes by 16S rRNA confirms not a single actinomycetes produces both the antibacterial and antifungal activity. May be due to the cell wall of Gram negative bacteria is very cushy to break than those of the Gram positive and fungi can be one of the reason. This finding is not agreed by various researchers, according to them antagonistic reaction against the Gram positive bacteria were much higher than the Gram negative [10]. PH also plays an important role may be one of the reason for the optimum production of antibiotics from actynomycetes, [11]showed that for the optimum production of antibiotics certain carbon and nitrogen sources are also required. At the same time [12] suggest that use of media, pH, salanity and carbon and nitrogen affect the growth and antibiotic production by actionmycetes. To find potent metabolites it requires a large number of isolates. It was observed that different plants produce different chemical metabolite which may be useful for the microbes. In order to withstand present variable conditions of the environment microbes need to adapt the environment.

CONCLUSION

Actinomycetes isolated from the garden soil collected Miraj, in Sangli district. Three isolates 38, 36 and 31 showed enzymatic activity towards cellulase, lipase and protease while for the antimicrobial activity of actinomycetes isolates 5, 8, 13, 16, 17, and 21 showed antagonistic activity against *F.* palmivora, *B. subtilis*, *R. solanacearum* and *P. dispersa*. More intensive study should be conducted on the isolated actinomycetes to utilize potential actinomycetes either as biocontrol or bioremediation agents.

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