Isolation and Identification of Actinomycetes From Municipal Waste Soil and Their Screening for Antagonastic Activity

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Abstract- *In the present study, the soil samples were selected from municipal waste of Bilaspur city, chattisgarh. Municipal solid waste (MSW) is a rich source of lingo-cellulosic materials thus providing an intense environmental for the growth of bacteria. The actinomycetes were screened out and isolated from the pre-treated soil samples by serial dilution method on starch casein agar medium and AIA on spread plate technique. After 72 h, whitish pin-point colonies characteristics of actinomycetes. In sample 1 plate 10-1 103, 10-3 1, and 10-5 82 colonies sample 2 plate 10-1 98, 10-3 105 colonies obtained. The total 10 different isolates were selected found to be gram positive and filamentous. All isolates were later purified and subjected to biochemical tests. The purified colonies were performed in biochemical tests such as, IMViC test, catalase, oxidase, motility test, carbohydrates fermentation test, TSI test. All the isolates were subjected to a few enzymatic screening like cellulose, starch, urease and casein hydrolysis test. All the isolates were screen antagonistic activity by modified cross streak method on MHA and starch casein agar medium. The antagonistic activities were tested against E.coli ATCC strain (gram negative) and S.aureus (gram positive). All colonies show positive result except C-1 for S.aureus and all colonies show negative result for E.coli except C-3.*

Keywords- Municipal waste, actinomycete, biochemical test, antagonistic activity

I. INTRODUCTION

During 1914 to 1939, Selman A. Waksman had been consistently systematically screening soil bacteria and fungi to find an antibiotic for tuberculosis. In 1939, he discovers the effects of certain fungi specially actinomycetes on bacterial growth. In 1940, he was able to isolate an effective T.B. antibiotic, acctinomycin and for this he got success in 1944, with the discovery of spectromycin. For all this work in 1952, he got the Noble price in physiological and medicine.

About 43% of the world's municipal waste is generated annually by asian countries while the north American countries and European union contributes to 28% of it. Several measures have been taken to tackle this problem. Among the various options available, the most modern and appropriate one is the recycling of this municipal waste in a natural way. MSW (municipal solid waste) is rich in lingocellulosic material mainly composed of cellulose and lignin with a small ratio of hemicelluloses. Lignocellulosic materials can be turned into a worthwhile and effective asset by utilizing microorganisms which can use it as a sole carbon source and results in the production of valuables substance such as different organic acids and antibiotics. But unfortunately most of this waste is burnt off for disposal throughout the world.

Actinomycetes are Gram-positive bacteria belonging to the order Actinomycetales characterized by the formation of substrate and aerial mycelium on solid media, presence of spores and a high GC content of the DNA (60-70 mol %). The majority of soil actinomycetes form a very important class of bacteria since they produce numerous natural products such as antibiotics and enzymes. More than 50% of the known natural antibiotics produced are from actinomycetes. These bacteria can be separated into different genera on the basis of morphological, physical and chemical criteria. The name actinomycete was derived from the Greek "actys" (ray) and "mykes" (fungus) and the actinomycetes were initially regarded as minute fungi because of their mycelium-like growth. The soil actinomycetes produce a volatile compound called geosmin, which literally translates to "earth smell". This organic substance for contribution of odour that occurs in the air when rain falls after a dry spell of weather. In natural habitats, Streptomyces are common and are usually a major component of the total actinomycetes population. Some actinomycete genera such as Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora, Microbispora, Micromonospora, Nonomuraea, which are often very difficult to isolate and cultivate due to their slow growth, are called rare actinomycetes.

II. MATERIALS AND METHODS

Soil sample collection -: For each sample, soil was first dug out with clean shovel up to 3 to 4 cm depth using disposable and sterile wooden plough. All the soil samples after collection was properly sealed labelled and send to laboratory where they were kept at 4˚C.

Enrichment and pre-treatment of soil samples -: All the collected soil samples were dried at room temperature for one week into the aseptic condition and after that crushed the soil sample and sieved it and finally given pre-treatment of heat 45˚C for 1 hour. Heat treatments of sample were preferred to select actinomycetes groups. Mild heat at 45˚C for 1-2 hours has been used to eliminate contamination in the form of lower bacteria, fungal spores mites etc.

Selective isolation -: soil samples were weighing for 1 gram and were serially diluted by 5 fold dilution using distilled water. The dilution was thoroughly mixed for a minute. With the help of micropipettes aliquots of 0.1ml from L- shaped each dilution were spread evenly with sterile onto starch casein agar (SCA) (Hi media) and actinomycetes isolation agar (AIA) (hi media). To minimize fungal and bacterial contamination all agar plates were supplemented with 50µg/ml of nystatin and Ampicillin. All plates were incubates at 30˚C for 1 week

Purification and preservation method -: The isolates subculture repeatedly onto starch casein agar medium until they formed single same colonies. Pure culture were maintained on the starch casein agar plate or agar slant and preserved at 4˚C.

Morphological characteristics -: Morphological characteristics of isolates colonies and the isolate where studied following the standard microbiological methods -:

Colony characters of the isolated : The isolates were streaked on actinomycetes agar plates incubated for five days at 30'C. The shape, size, colour, margin and opacity were recorded from isolate colonies.

Gram staining - A loopful of culture was taken in a clean glass slide and heated gently over a flame. The smear was covered with a thin film of crystal violet for 1 min and washed gently in slow running tap water. Grams iodine solution was flooded over the smear for 1 min and washed with tap water. Alcohol was used to decolorize the smear until the violet colour ceased to flow away. The slide was washed with water and counter stain saffranin was flooded over the smear for 2 min, then the slide was washed, drained, air dried, and viewed under microscope. The culture retaining the violet colour indicated that it was Gram-positive organism.

III. ENZYME UTILIZATION TEST

Starch hydrolysis test - Capacity of the organisms to hydrolyze starch into simple substances like dextrin, glucose, maltose etc. by amylase enzymes was detected by spot inoculating the bacterial cultures on NA plates containing 1% soluble starch. After incubation for 96 hrs at 30 degree C, all the plates were then exposed to iodine vapour for 5 to 10 minutes. Starch hydrolysis was noted from a clear zone formed around the colonies. Reddish-brown area around the colonies indicated partial hydrolysis of starch.

Cellulase degradation test – Cellulose degradation was determined by spot inoculating Actinomycetes on Czepak mineral salt medium and incubated at 30°C for 2-3 days. For detection of cellulose degradation plates were flooded with 1% Congo red solution. After 5 minutes excess dye was drained. 1M NaCl was then added repeatedly until color disappeared. Clear zone of hydrolysis around the cellulose producing organism was observed due to hydrolysis of cellulose.

Casein hydrolysis - Casein hydrolyzing activity of the bacteria was recorded from liquefaction of casein by skim milk agar were prepared. All the colonies were streak on the plates & incubated at 30 degree C for 24 to 48 hours. Formation of a clear zone adjacent to the bacterial growth, after inoculation and incubation of agar plate cultures Formation of a clear zone adjacent to the bacterial growth, after inoculation and incubation of agar plate cultures.

IV. BIOCHEMICAL TEST

Indole production test – The test is used to check ability of the organisms to form indole from tryptophan or to detect the presence of enzyme tryptophanase which converts tryptophan to indole. The test was performed by inoculating the bacterial cultures into indole disc.

Methyl red test - The test is used to detect acid production from glucose. Production of acid lowers the pH of the medium below 4.2 which is detected by the pH indicator methyl red. Actinomycetes were inoculated into tubes containing methyl red-Voges Proskauer (MRVP) broth and incubated at 30°C for 96 h. After incubation alcoholic methyl red indicator was added. Positive reaction was indicated by change of color of medium to red.

Voges-Proskauer test - The isolates were tested on MR-VP broth medium to detect their ability to produce neutral products like acetoin (acetyl methyl carbinol) during metabolism of glucose present in the medium. After incubation, 0.6 ml of 5% naphthol was added followed by 0.2 ml of 40% KOH to about 1 ml of broth culture. The solution was allowed to stand for 30 minutes. A change in colour of the medium to wine red was as positive reaction while copper colour indicates a negative result.

Citrate Utilization test - The capability of the isolates to utilize citrate as the sole shores of carbon & energy was studied on Simmons citrate agar medium. Colour change of the slant from green to royal blue was considered as positive result while no change in colour was taken as negative.

Catalase test - Presence of the enzyme catalase which catalyses breakdown of hydrogen peroxide into water and oxygen was studied on culture plates (NA) flooded with hydrogen peroxide solution. Positive reaction was indicated from effervescence of oxygen from the plate.

Oxidase test - The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzyme. To detect presence of the enzyme oxidase in the bacteria which catalyses transport of electrons between bacteria and the redox dye e.g. N-tetramethyl-p-phenylene diamine dihydrochloride or dimethyl-p-phenylene diamine or methylene blue (oxidase reagents) and causes intense purple colouration was observed.

Urease test - To test for the presence of the enzyme urease in the isolates which split urea into ammonia and CO2 was studied using Christensen's urea agar medium. Colour change of the slant from yellow to pink was considered as positive result while no change in colour was considered as negative.

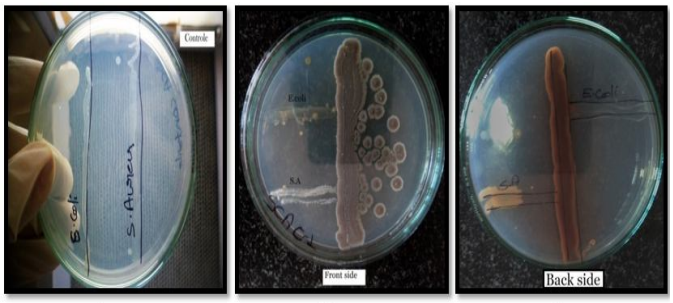
Carbohydrates fermentation test - In the carbohydrate fermentation test, the test bacteria is grown in a carbohydrates broth medium containing one of the sugars or sugar derivatives and bromocresol purple. An inverted Durham tube is kept submerged in it. If the bacteria have the ability to ferment the sugar or sugar derivative, the colour of the broth changes from purple to yellow. If gas accumulation is seen as a bubble in the Durham tube, it is an acrogenic bacteria, while if no gas accumulation is seen, it is an anaerogenic bacteria.

TSI (triple sugar iron) - Triple sugar iron agar test is used to determine whether organisms utilize glucose and lactose or sucrose fermentative and produce hydrogen sulfide (H2S). The isolates which use glucose, lactose and sucrose were studied using TSI agar medium. Colour changes of the slants represent different results of utilization.

Screening of antimicrobial activity - There are many techniques for detecting antimicrobial activity; most of them are based on methods involving diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms. Determination of antimicrobial activities of 8 pure actinobacterial cultures was performed by modified cross-streak method (MCSM), muller- Hinton agar (MHA himedia) and starch casein agar (HI media) were prepared and inoculated with isolated cultures by a single streak in the centre of petri dish and incubated at 30 degree C for 7 days. The plates were seeded with test organisms by streaking perpendicular to the line of actinomycetes growth. The plates containing active organisms were kept for 6-7 days to determine any further growth towards the master streak, or whether they remain stationary or whether lyses of the test pathogens occurred.

V. RESULTS

Actinomycetes have been intensively studied in several underexplored environments, niche and extreme habitats in various parts of the world (including India) in the last few years. Yet there is no report regarding isolation of actinomycetes from bilaspur region (C.G.). Total ten bacterial isolates were isolated from the four different soil samples. All the isolates were designated as shown in (Table no. 1) the isolated strains were filamentous, Gram positive, and aerobic in nature. All the isolates had different colonies and in gram staining all shows filamentous structures (table 2). There were done some biochemical test like IMViC test, oxidase, catalase test, carbohydrates fermentation test, TCI test, and urease test. Most of the actinomycetes isolates showed positive results for catalase test, while the isolates showed negative results for MR and Vp test and indole test also except C8 and C10. The biochemical properties of actinomycetes isolated were recorded (table 3). There were also performed some enzymetic degradation from actinomycetes isolates (table 4). The isolates were screened for their inhibitory activity against two pathogenic bacteria. The results show that all the isolates except C1 and C10 were able to inhibit growth of the test organisms. Although C2exhibited the highest activity against E.coli and S.aureus respectively.



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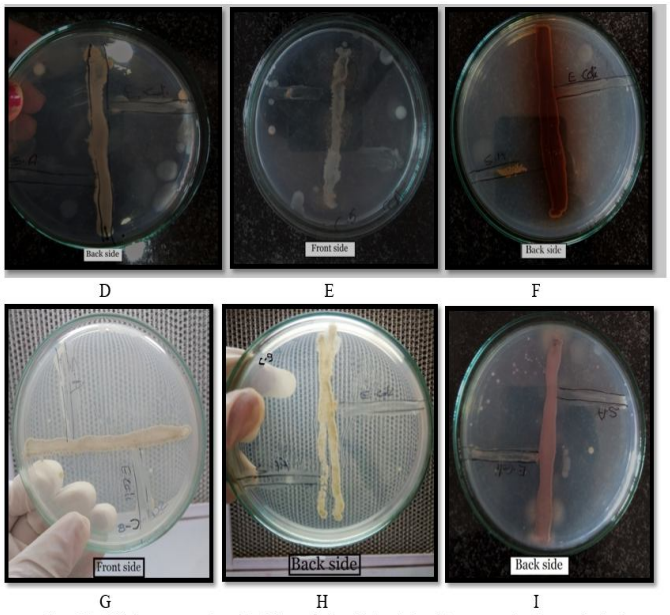

Fig:- Modified cross streak method for antimicrobial activity (A = control, others colonies)

Table - 1.Occurrences and Distribution of Actinomycetes from Municipal waste soil of different site

Serial no	Samples	Places	Soil depth	Serial dilutions	No of isolates
1.	**Sample 1**	Near torwa chowk	4cm	**10^{-1}**	**103**
2.	**Sample 1**	Near torwa chowk	4cm	**10^{-3}**	**1**
3.	**Sample 1**	Near torwa chowk	**4cm**	**10^{-5}**	**82**
4.	**Sample 2**	Vyapar vihar	**5cm**	**10^{-1}**	**98**
5.	**Sample 2**	Vyapar vihar	**5cm**	**10^{-3}**	**105**
6.	**Sample 2**	Vyapar vihar	**5cm**	**10^{-5}**	**Nil**

Table - 2. Morphological characteristics of *Actinomycetes*

S. no.	colonies	Nature of colony	Colour of Myceliu m	Gram stain
1.	**C_1**	Raised wrinkled, powdery	Dark grey	+
2.	**C_2**	Leathery form	Greyish	+
3.	**C_3**	Leathery, powdery, raised wrinkled	Yellowish	+
4.	**C_4**	Sticky, smooth	Off white	+
5.	**C_5**	Smooth colony	Off white	+
6.	**C_6**	Powdery	Blackish	+
7.	**C_7**	Granular	Dark brown	+
8.	**C_8**	Hairy like	Whitish	+
9.	**C_9**	Powdery	Yellowish	+
10.	**C_{10}**	Powdery concentric colony	Pale yellow	+

Table - 3. Biochemical tests for isolated colonies.

S. no.	Colonies	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease
1.	**C_1**	-ve	-ve	-ve	+ve	+ve	-ve	+ve
2.	**C_2**	-ve	-ve	-ve	+ve	+ve	-ve	Nd
3.	**C_3**	-ve	-ve	-ve	-ve	+ve	+ve	+ve
4.	**C_4**	-ve	-ve	-ve	+ve	-ve	-ve	-ve
5.	**C_5**	-ve	-v	+v	-ve	+ve	+ve	Nd

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6.	C_6	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7.	C_7	-ve	-ve	-ve	+ve	+ve	-ve	Nd
8.	C_8	+ve	-ve	-ve	+ve	+ve	-ve	-ve
9.	C_9	-ve	-ve	Nd	-ve	+ve	+ve	+ve
10.	C_{10}	+ve	-ve	-ve	-ve	+ve	-ve	-ve

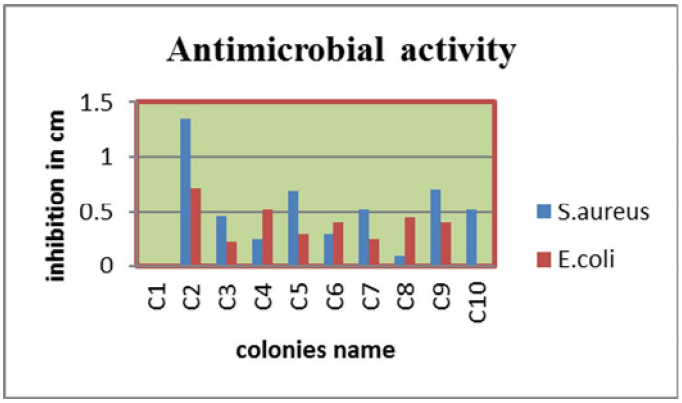
(+= positive, -= negative, Nd= not diagnosis)

Table - 4. Enzyme activities of actinomycetes strains

S. no.	Colonie	Amylase	Caseinase	Cellulose
1.	C_1	Positive	Negative	Negative
2.	C_2	Positive	Positive	Positive
3.	C_3	Positive	Negative	Positive
4.	C_4	Negative	Negative	Positive
5.	C_5	Positive	Negative	Positive
6.	C_6	Positive	Negative	Negative
7.	C_7	Positive	Positive	Negative
8.	C_8	Negative	Negative	Positive
9.	C_9	Negative	Negative	Positive
10.	C_{10}	Positive	Negative	Positive

Table – 5 Antimicrobial potency of isolated colonies against *E. Coli* and *S. aureus.*

S. No.	Isolates	Incubation period	
		E.coli	S. aureus
1.	C_1	0	0
2.	C_2	0.70	1.35
3.	C_3	0.22	0.46
4.	C_4	0.52	0.25
5.	C_5	0.30	0.69
6.	C_6	0.40	0.30
7.	C_7	0.25	0.52
8.	C_8	0.45	0.10
9.	C_9	0.40	0.71
10.	C_{10}	0	0.52



Graph: - Antimicrobial activity of isolates against S. Aureus and E.coli strain

VI. DISCUSSION

In the course of screening for novel antimicrobial substances (antibiotic) from soil samples antibiotics producing actinomycetes culture were recorded from soil sample taken in bilaspur, Chhattisgarh india. The composition of starch casein agar media (SCA) more suitable for the selective isolation of aerobic as compare to Actinomycetes isolation media (AIA) was given. Soil samples were aseptically air dried one week to avoid bacterial and fungus contamination. Pre treatment techniques aid the development of actinomycetes population in the soil. In present study ten actinomycetes selected. All the isolates were found to be gram positive and filamentous in shape and shows different colonies with different colour on selective media. The presence of relatively large population of actinomycetes in the soil samples of bilaspur municipal waste indicates that it is a suitable ecosystem that promotes the isolation of actinomycetes during screening programs. All the 10 isolates were subjected to antibacterial activity against 2 pathogenic bacteria S.aureus and E.coli by modified cross streak methods. Out of the 10 antibacterial isolates screened, the isolated colony C2 showed good inhibitory activity against both pathogens and the C1 and C10 colony not showed any inhibitory effect against pathogens. A maximum zone of inhibition of 1.35 cm was expressed against S.aureus. the isolates C3, C4, C5, C6, C7, C8, and C9 were also found to be potential inhibitors of pathogenic bacteria.

VII. CONCLUSION

From the present study it could be concluded that municipal waste soil provided a rich source of diversity of actinomycetes. The media used to differentiate this group of bacteria are SCA and AIA. Among those isolated most had the ability to produce antimicrobial compounds and enzymes. However more detailed investigation is required to demonstrate the potential of these organisms for the treatment

of pathogenic organisms which may be useful in pharmacological and medical fields in the future.

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