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EXTRACTION AND PURIFICATION OF ANTIBACTERIAL METABOLITES FROM ACTINOMYCETES SPP. ISOLATED FROM SOIL SAMPLE

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ABSTRACT

In the present investigation four actinomycetes isolates found to be positive during primary screening tentatively named as MJA1105, MJA1106, MA1107 and MJA1108. All the four isolates were found to be gram positive rods. All were subjected to secondary screening and the isolate MJA1105 was found to be most effective during secondary screening. The isolates growth parameters were studied and it was found to be growing well at 28 °C and pH 5. Production of antibacterial metabolite was carried out by submerged fermentation procedure and extracellular metabolites were extracted by solvents (chloroform and ethyl acetate as well as by salt precipitation followed by dialysis and intracellular antibacterial metabolites were extracted by methanol. Metabolites extracted by chloroform (extracellular), methanol (intracellular) and salt precipitation (extracellular) gave a zone of inhibition of 12- 13mm, 22-24 mm and 15-19 mm respectively.

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Key Words
Actinomycetes, Antibacterial properties, Solvent extraction, Salt precipitation, Antibacterial sensitivity assay.

INTRODUCTION

The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA and found mainly in soil. The name 'Actinomycetes' has been derived from Greek words 'aktis' (a ray) and 'mykes' (fungus). The actinomycetes have been reported to be the most common antibiotic producing microorganisms found in soil. They have provided about two-third (more than 4,000) of naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycoside, anthracyclines, peptides, polyenes, polyketides, actinomycines, chloramphenicol, β-lactams, macrolides and tetracyclines **[1-2]**. Actinomycetes are important for the pharmaceutical applications but it has an equal application in agriculture sector also previous studies have reported the use of actinomycetes in inhibiting various plant pathogens **[3].**

Various solvents (ethyl acetate, chloroform, ethanol and petroleum) have been reported **[4-7]** to be helpful in the extraction of antimicrobial metabolites but the present investigation is one of the few reports on optimization of the use of salt precipitation method for the extraction of proteinaceous antimicrobial metabolites. Present investigation was carried out to screen the soil actinomycetes for production of antibacterial metabolites and to optimize production by submerged fermentation procedure, optimize solvent and salt extraction procedure for maximum yield of antibacterial metabolites.

METHODOLOGY

Soil Sample

Soil sample was collected from plots with decaying leaves in Vibuti Khand, Gomtinagar Lucknow. Soil sample was collected in sterile polybags from 2-3 inches below the ground level and transferred to the Laboratory.

Test Pathogens

Three pathogenic microorganisms namely ***Staphylococcus aureus, Pseudomonas aeruginosa*** and ***Escherichia coli*** available at **MRD LifeSciences (P) Ltd., Lucknow** were sub cultured and used throughout the study. **MRD LifeSciences (P) Ltd., Lucknow** availed these pathogens from **IMTECH, Chandigarh.**

Isolation of Actinomycetes

Actinomycetes were isolated by serial dilution agar plating method in AIA (actinomycetes isolation agar) media in which 0.5gm of soil sample was diluted upto 10^{-4} dilutions, and the diluted sample was inoculated in sterile AIA plates. Plates were incubated at 28 °C for 48-120 hours.

Primary Screening for Antibacterial Nature

The plates incubated in the above step were observed after 48 hours for presence of zone of inhibition around the growing isolates, plates were further observed upto 120 hours for the presence of zone of inhibitions. The cultures showing zone of inhibition were said to be positive in primary screening and were used for further study.

Sub culturing of Isolates Positive in Primary Screening

Four isolates showing zone of inhibition around themselves were named as MJA1105, MJA1106, MJA1107, and MJA1108 and sub cultured using quadrant streaking.

Secondary Screening for Antibacterial Nature

For secondary screening all the six isolates were inoculated in 7 ml of media containing (glucose 30 g/l, KNO_3 6 g/l, NaH_2PO_4 1 g/l, KCl 5 g/l, $MgSO_4$ 0.04 g/l, $FeSO_4$ 0.02 g/l, Peptone 10 g/l, Beef Extract 6 g/l, pH 6.8) **[8]** and incubated for 48 hours at 28°C at 120 rpm. In order to extract the crude metabolite 1.5 ml of broth was taken in sterile eppendorf tube and spun a 5000 rpm for 5 minutes and supernatant was collected and used for antibacterial testing. For antibacterial testing agar well diffusion method of **[9]** was used with slight modifications wherein sterile NA plates were prepared and spreaded with 25 µl of test pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*). Four wells of 8 mm diameter were dug by the help of sterile borer, first two wells were loaded with 50 µl of crude antimicrobial extract, third with 50 µl of standard antibiotic tetracycline (50 µg/ml) and the fourth with 50 µl of sterile D/W as control. Similar procedure was carried out for all the four extracts. Plates were incubated at 37 °C for 24 hours and observed for zone of inhibition.

Identification of the Isolate MJA1105

Basic identification of the isolate belonging to the actinomycetes family was done by gram staining characteristics and the growth potential of the isolate in selective media i.e. Actinomycetes isolation agar.

Study of Growth Parameters of MJA1105

Study of Growth Curve

In order to have an idea of the growth pattern of the isolate MA1105 before going for fermentation growth curve was prepared so that the day on which the culture reaches its stationary phase (phase giving maximum yield of secondary metabolites) is well known. For plotting the growth curve 100ml of actinomycetes growth media containing the following (Beef Extract 10 g/l, Tryptose 10 g/l, casein enzyme hydrolysate 4 g/l, Yeast extract 5 g/l, Dextrose 5 g/l, L-cystein hydrochloride 1 g/l, Starch 1 g/l, NaCl 5 g/l, KH_2PO_4 15 g/l, Ammonium sulphate 1 g/l, CaCl 0.02 g/l, MgSO4 0.20 g/l pH 7.2) (**Himedia Laboratories**) was prepared and divided into two flasks one containing 80 ml of media and another containing 20 ml of media. Both the flasks were autoclaved and cooled to room temperature. The flask containing 80 ml media was inoculated with 1 ml of 24 hour old grown broth culture of the isolate MJA1105 and incubated at 28 °C/ 120 rpm. The flask containing 20 ml of media was stored as blank. After every 24 hour absorbance of the inoculated flask was read at 600 nm against the uninoculated blank. A graph was plotted between time in days and absorbance at 600 nm.

Effect of Temperature on Growth of MJA1105

For getting the optimum temperature for growth of isolate MJA1105 it was streaked on AIA plates and the plates were incubated at different temperatures (22 °C, 28 °C, 37 °C, 50 °C). Growth of the isolate was tracked by visual assessment.

Effect of pH on Growth of MJA1105

In order to get an idea about the optimum pH for the growth of isolate MJA1105 it was grown at different pH (5, 7, 9, 11) and the growth was tracked by reading the absorbance at 600 nm.

Fermentation Process

For production of antimicrobial metabolites 50ml of production media containing (glucose 30g/l, KNO_3 6g/l, NaH_2PO_4 1 g/l, KCl 5g/l, $MgSO_4$ 0.04g/l, $FeSO_4$ 0.02g/l, Peptone 10g/l, Beef Extract 6g/l, pH6.8) was prepared in two sets and was inoculated with 0.5ml of 24 hour old grown broth of the isolate MJA1105. Flask was incubated in a shaking incubator at 28°C/120 rpm for four days.

Extraction and Purification of Antibacterial Metabolites Extracellular

Fermented broth was transferred into centrifuge tubes and spun at 5000 rpm for 5 minutes. Supernatant was treated as crude extracellular extract and used for solvent extraction and salt precipitation. These two procedures were used as the nature of metabolite was uncertain.

Solvent Extraction

Two solvents (Chloroform and Ethyl acetate) were used for extraction of antimicrobial metabolites. 500 µl of the crude extracellular extract was taken in eppendorf tubes and 500 µl of the respective solvent was added. Gentle mixing was done for 1 hour and the tubes were spun at 10000 rpm for 10 minutes, chloroform / ethyl acetate phase (upper) containing dissolved metabolites was collected in a weighed petriplate and the plate was kept in hot air oven (50 °C) for drying of chloroform/ ethyl acetate. The petriplate was once again weighed and amount of metabolite extracted was calculated by subtracting the weight of empty pertiplate from the weight of petriplate after drying. The metabolite was dissolved in 500 µl of sterile distilled water. The final concentration of metabolite can be seen from the **Table 1** below.

Table 1: Extraction of metabolite by solvents

SOLVENT	WEIGHT OF EMPTY PETRIPLATE (g)	WEIGHT OF PERTIPLATE AFTER DRYING (g)	WEIGHT OF METABOLITE (g)	AMOUNT OF D/W USED (ml)	CONC. OF METABOLITE (g/ml)
CHLOROFORM	28.711	28.730	0.019	0.5	0.038
ETHYL ACETATE	28.778	28.788	0.01	0.5	0.020

Extraction Using Salt Precipitation

For extraction of extracellular metabolite with salt precipitation method crude extracellular extract as described earlier was extracted and ammonium sulphate precipitation upto 70% saturation was carried out in order to precipitate the proteinaceous metabolite wherein ammonium sulphate was added pinch by pinch. Later dialysis was carried out in order to remove the salts if present. Dialyzed sample was transferred to a weighed petriplate and left in hot air oven (50 °C) for drying. Petriplate was again weighed and amount of metabolite extracted was determined by subtracting the weight of empty plate from weight of plate after drying. The metabolite was dissolved in 500 µl of sterile distilled water. The final concentration of metabolite can be seen from the **Table 2** below.

Table 2: Extraction of metabolite by salt precipitation

SAMPLE	WEIGHT OF EMPTY PETRIPLATE (g)	WEIGHT OF PERTIPLATE AFTER DRYING (g)	WEIGHT OF METABOLITE (g)	AMOUNT OF D/W USED (ml)	CONC. OF METABOLITE (g/ml)
AFTER DIALYSIS	32.345	32.420	0.075	0.5	0.15

Intracellular

After collecting the supernatant during crude extracellular metabolite extraction the eppendorf tube containing cells was used for extraction of crude intracellular metabolite. Pellet of cell in eppendorf tube (24) were resuspended in 467µl of TE buffer (Tris 200mm; EDTA 50mm), 30 µl of 10% SDS, and 3µl of Proteinase K were added. Tubes were incubated at 37 °C for 1 hour and spun at 10000 rpm for 10 minutes. Supernatant was collected and treated as crude intracellular metabolite.

Solvent Extraction

For solvent extraction 500 µl of crude intracellular metabolite was taken in eppendorf tube (24) and 500 µl of methanol was added to all the tubes, gentle mixing was done for 1 hour and the tubes were spun at 10000 rpm for 10 minutes. Methanolic phase containing dissolved metabolites was transferred to a weighed petriplate and kept in hot air oven (50 °C) for drying. Petriplate was again weighed and amount of metabolite extracted was determined by subtracting the weight of empty plate from weight of plate after drying. The metabolite was dissolved in double volume of sterile

distilled water thus making a final concentration of 0.5 g/ml.

Antibacterial sensitivity Assay of Purified Antibacterial Metabolites

For antibacterial sensitivity assay agar well diffusion method of **[9]** was used with slight modifications wherein sterile NA plates were prepared and spread with 25 µl of pathogens namely *Staphylococcus aureus, pseudomonas aeruginosa, Escherichia coli,* wells of 8mm diameter were dug by the help of a sterile borer. Wells were loaded with extract, standard antibiotic and distilled water as blank. Plates were incubated at 37 °C for 24 hours and plates were observed for zone of inhibition after incubation period. All the experiments were performed in triplicates.

Table 3: Antibacterial sensitivity assay of crude metabolites

TEST PATHOGENS	ZONE OF INHIBITION BY MJA1105 (mm)	ZONE OF INHIBITION BY MJA1106 (mm)	ZONE OF INHIBITION BY TETRACYCLLINE (50 µg/ml)
Staphylococcus aureus	00	18	24
Pseudomonas aeruginosa	15	19	23
Escherichia coli	15	14	22

NOTE: well diameter = 8mm

Table 4: Antibacterial sensitivity assay of crude metabolites

TEST PATHOGENS	ZONE OF INHIBITION BY MJA1107 (mm)	ZONE OF INHIBITION BY MJA1108 (mm)	ZONE OF INHIBITION BY TETRACYCLLINE (50 µg/ml)
Staphylococcus aureus	00	00	24
Pseudomonas aeruginosa	00	14	23
Escherichia coli	00	00	22

NOTE: well diameter = 8mm

Identification of Isolate MJA1105

As all the isolation procedure was done in a selective media (Actinomycetes isolation agar) for the isolation of actinomycetes it was said that the isolate belongs to actinomycetes family and the gram staining characteristics (gram positive rods) also gave an indication of the isolate belonging to actinomycetes family.

Study of Growth Parameters of MJA1105

Growth parameters (phases of growth, incubation temperature, pH of media) were studied in order to optimize the optimum conditions required for maximum growth of the isolate MJA1105.

Growth Curve

As the maximum yield of secondary metabolites have been reported to be in stationary phase of the microbial growth, growth curve was plotted in order to have an idea of the stationary phase of growth of the isolate MJA1105. The results of the same can be seen in **Table 5** and **Figure 1** below.

Table 5 Growth curve

S. No.	TIME (In Days)	OD AT 600nm
1	0	0.00
2	1	0.09
3	2	0.20
4	3	0.26
5	4	0.26
6	5	0.24
7	6	0.22
8	7	0.20

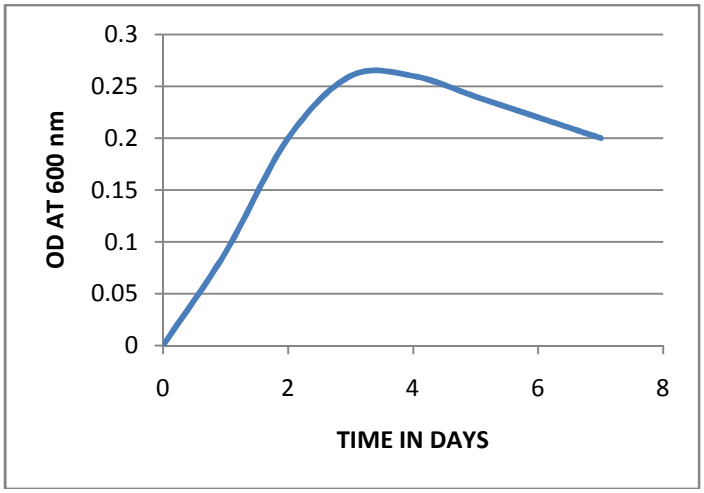

Figure 1: Growth kinetics

Effect of Temperature on Growth

To optimize the temperature for optimum growth of isolate MJA1105 it was streaked on AIA plates and incubated at different temperatures. **Table 6** below shows that optimum temperature for the growth of isolate is 28 °C.

Table 6: Effect of temperature on growth

S. No.	INCUBATION TEMPERATURE	REMARKS (Growth)
1	**22 °C**	**-**
2	**28 °C**	**+++**
3	**37 °C**	**++**
4	**50 °C**	**-**

Effect of pH on Growth

To optimize the pH for optimum growth of isolate MJA1105 it was grown in media with varied pH and it can be seen from **Table 7** that pH 5 was the optimum pH for growth of isolate MJA1105.

Table 7: Effect of pH on growth

S. No.	pH OF MEDIA	OD AT 600nm
1	5	0.95
2	7	0.87
3	9	0.89
4	11	0.00

Antibacterial Sensitivity Assay of Purified Antibacterial Metabolites

Antibacterial sensitivity assay of antibacterial metabolites (extracellular and intracellular) extracted by solvent and salt precipitation methods was carried out by agar well diffusion method and the results of the same can be seen in **Table 8 - 10** below.

Antibacterial Sensitivity Assay of Chloroform and Ethyl acetate Extracts (Extracellular)

Table 8: Antibacterial sensitivity assay of chloroform and ethyl acetate extracts

TEST PATHOGENS	ZONE OF INHIBITION BY CHLOROFORM EXTRACT (mm)	ZONE OF INHIBITION BY ETHYL ACETATE EXTRACT (mm)	ZONE OF INHIBITION BY TETRACYCLLINE (50 µg/ml)
Staphylococcus aureus	12	00	20
Pseudomonas aeruginosa	00	00	22
Escherichia coli	13	00	19

NOTE: well diameter = 8mm

Antibacterial Sensitivity Assay of Salt Precipitated Metabolite

Table 9: Antibacterial sensitivity assay of salt precipitated metabolite

TEST PATHOGENS	ZONE OF INHIBITION SALT PRECIPITATED EXTRACT (mm)	ZONE OF INHIBITION BY TETRACYCLLINE (50 µg/ml)	ZONE OF INHIBITION BY STERILE D/W (mm)
Staphylococcus aureus	19	24	00
Pseudomonas aeruginosa	15	17	00
Escherichia coli	18	21	00

NOTE: well diameter = 8mm

Antibacterial Activity of Methanol Extracts (Intracellular)

Table 10: Antibacterial sensitivity assay of methanol extracts (Intracellular)

TEST PATHOGENS	ZONE OF INHIBITION BY METHANOL EXTRACT (mm)	ZONE OF INHIBITION BY TETRACYCLLINE (50 µg/ml)	ZONE OF INHIBITION BY STERILE D/W (mm)
Staphylococcus aureus	**24**	**23**	**00**
Pseudomonas aeruginosa	**22**	**20**	**00**
Escherichia coli	**25**	**22**	**00**

NOTE: well diameter = 8mm

DISCUSSION

Actinomycetes isolates were isolated from soil by serial dilution agar plate method as done earlier by **[4].** Actinomycetes isolates were screened for antibacterial properties by primary and secondary screening by crowded plate technique and agar well diffusion method. **[6].** The isolate showing maximum zone of inhibition during secondary screening was found to be gram positive rod and the physical factors like incubation temperature and pH were optimized and it was found that 28 °C and pH 5 are the best temperature and pH for the optimum growth of MJA1105.

Submerged fermentation was carried out in order to get antibacterial metabolite as done earlier by **[4; 10].** For extraction of extracellular and intracellular antibacterial metabolites solvents such as ethyl acetate, chloroform and methanol were used as reported earlier by **[4; 6; 10; 11].** Extraction of extracellular antibacterial metabolite was also performed by salt precipitation method this is one of the few reports on the use of salt precipitation method for the extraction of extracellular antibacterial metabolite.

Antibacterial sensitivity assay was carried out in order to check the antibacterial properties of the extracted metabolites by agar well diffusion method of **[9]** with slight modification. Results of the same suggest that chloroform is a better solvent than for the extraction of extracellular antibacterial metabolite from the isolate MJA1105 studied in the present investigation Salt precipitation method lead to extraction of very effective metabolite as during antibacterial sensitivity assay zone of inhibition of 15- 19 mm were observed against the used pathogenic strains which were a bit comparable to the zone of inhibition of 17- 21 mm shown by the standard antibiotic tetracycline. Intracellular antibacterial metabolites extracted by methanol were most effective and they gave a zone of inhibition of 22 – 24 mm against the used pathogenic strains which were even more than the zone of inhibition shown by the standard antibiotic tetracycline. Chloroform and ethyl acetate extracts (1 mg/ml) of **Marine Actinomycete, *Nocardiopsis* sp. VITSVK 5 (FJ973467)** have been reported to form a zone of inhibition ranging between 6-20 mm against gram positive and negative bacteria by **[4].** Antimicrobial metabolites extracted by ethyl acetate from six actinomycetes spp. have been reported to show a zone of inhibition ranging between 5- 23 mm by **[11].** The results obtained in the present investigation are not only comparable but better in some cases.

CONCLUSION

Based on the above study it can be said that actinomycetes spp. isolated in these study can be a good source of antibacterial metabolites. The metabolites extracted here can be used as a full proof drug after proper pharmacological evaluation.

Future work of present investigation includes identification of the isolate, Phytochemical screening of the antibacterial metabolite, evaluation of antifungal properties, determination of MIC (minimum inhibitory concentration).

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RESULTS

Primary Screening for Antibacterial Nature

Four different actinomycetes isolates growing on AIA media showing zone of inhibition around themselves were called to be positive in primary screening and were named as MJA1105, MJA1106, MJA1107 and MJA1108.

Secondary Screening for Antibacterial Nature

All the four isolates found to be positive in primary screening were sub cultured and crude antimicrobial extracts were subjected to secondary screening by agar well diffusion method of **[9].** Results of the same can be seen in **Table 3-4** below. Results of **Table 3** suggested to go for further studies with culture MJA1105 and MJA1106, we went for the same but after purification antibacterial properties of MJA1105 were more effective that's why we are reporting the further studies (identification, growth parameters, extraction of metabolites) of MJA1105 only.

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