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PRODUCTION AND PURIFICATION OF ANTIMICROBIAL COMPONENTS FROM MARINE ACTINOMYCETES SPECIES MJG1210

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ABSTRACT

In the present study, seven Actinomycetes isolates were isolated from Marina Beach, Chennai and Colva Beach, Goa marine samples (Sediments and Water). Among the seven isolated strains of Actinomycetes (MJG1208, MJG1209, MJG1210, MJG1211, MJG1212, MJG1213 & MJG1214) isolate MJG1210 actinomycetes strain was found to be most effective in screening tested against three pathogen namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus.* Optimum conditions for growth of MJG1210 were at pH 5 and 37°C. Production of metabolite from MJG 1210 was done by shake flask fermentation. The crude antimicrobial component was extracted from fermented broth by centrifugation. Crude metabolite was purified for extracellular antimicrobial component by solvent extraction method. Agar well diffusion was performed to evaluate the antimicrobial activity. Out of the five solvents Chloroform, ethyl acetate and Acetone extracts were effective most effective being ethyl acetate extract showing zone of inhibition of 17 mm against *P. aeruginosa*.

INTRODUCTION

Antimicrobial is an agent that kills microorganisms or suppresses their multiplication or growth. The history of antimicrobials begins with the observations of **Pasteur** and **Joubert**, who discovered that one type of bacteria could prevent the growth of another. The first antimicrobial agent in the world was salvarsan, a remedy for syphilis that was synthesized by **Ehrlich** in 1910. In 1935, sulfonamides were developed by **Domagk** and other researchers. These drugs were synthetic compounds and had limitations

in terms of safety and efficacy. Antimicrobial agents are classified functionally according to the manner in which they adversely affect a microorganism. Some interfere with the synthesis of the bacterial cell wall. This results in cell lysis because the contents of the bacterial cell are hypertonic and therefore under high osmotic pressure. A weakening of the cell wall causes the cell to rupture, spill its contents, and be destroyed. The penicillins, cephalosporins and bacitracin are examples of the first group of antimicrobials.

A second group of antimicrobial agents interfere with the synthesis of nucleic acids. Without DNA and RNA synthesis a microorganism cannot replicate or translate genetic information. Examples of antimicrobials that exert this kind of action are griseofulvin, fluoroquinolones and rifampicin.

A third group of antimicrobial agents change the permeability of the cell membrane, causing a leakage of metabolic substrates essential to the life of the microorganism. Their action can be either bacteriostatic or bactericidal. Examples include amphotericin B and polymyxin B.

A fourth group of antimicrobial agents interfere with metabolic processes within the microorganism. They are structurally similar to natural metabolic substrates, but since they do not function normally, they interrupt metabolic processes. Most of these agents are bacteriostatic. Examples include the sulfonamides, aminosalicylic acid (PAS) and isoniazid (INH).

A fifth group interfere with translation of proteins by the ribosome. This action may be bacteriocidal, if errors in translation are induced (aminoglycosides) or bacteriostatic, if translation is inhibited (macrolides, tetracyclines, chloramphenicol) **[1]**.

Antimicrobials have been be classified as:

- Antibiotics
- Antivirals
- Antifungals
- Antiparasitics
- Non-pharmaceutical antimicrobials
    - Essential oils
    - Cations and elements

Marine Microflora

Marine microbiology focuses primarily on prokaryotic organism mainly bacteria, actinomycetes, algae, viruses etc.

The marine microorganism produce novel chemicals to withstand extreme variations in pressure, salinity, temperature and so forth prevailing in their environment and the chemicals produced are unique in diversity, structural and functional features.

There is tremendous diversity and novelty among the marine bacteria and actinomycetes present in marine environments. These marine actinomycetes produce different types of new secondary metabolites.

Many of these metabolities possess biological activities and have the potential to be developed as therapeutic agents. They are taxonomically diverse, large productive biologically active and chemically unique offering a great scope for discovery of anticancer drugs, antioxidant, immune stimulatory, antitumour activities, antibiotics and bioactive compounds, production of vitamin B12 and many enzymes from marine microflora.

Marine invertebrates have developed highly specific relationship with numerous associated microorganisms and these association are of recognized ecological and biological importance.

Marine bacteria and actinomycetes have been used by various researchers some of them being **[2-4]**.

Looking at the importance and previous interests **[2, 5-7]** on the antimicrobial production from bacteria and actinomycetes the present study was also aimed at the isolation of antimicrobial producing marine bacteria and marine actinomycetes species, and was carried out with the following objectives in mind:

- **Isolation and purification of marine actinomycetes species from marine samples.**
- **Screening of the obtained cultures for production of antimicrobial components.**
- **Identification of the isolate showing positive result in screening.**
- **Production and purification of antimicrobials from the isolate most effective in screening by solvent extraction**
- **Antibiogram analysis of purified antimicrobials.**

MATERIALS AND METHODS

Collection of Sample

The marine samples (water and sediments) were collected from two sites Marina beach in Chennai and Colva beach in Goa.

Isolation and Purification of Actinomycetes Species

Marine actinomycetes were isolated from water and sediment samples by serial dilution agar plate method on specific media actinomycetes isolation agar **(Titan Biotech)** and incubation the plates at 28 °C for 48 hours. Mixed colonies obtained after incubation were purified by quadrant

streaking on AIA plates. Purity of cultures was checked by gram's staining procedure.

Screening of Purified Actinomycetes Isolates for Production of Antimicrobial Components

The purified plates were screened for production of antimicrobial components by inoculating them in 5 ml of sterile production media g/l (Glucose 30, KNO_3 6, KCl 5, NaH_2PO_4 1, $MgSO_4$ 0.04, $FeSO_4$ 0.02, Peptone 10, Beef Extract 6, pH 6.8). The antimicrobial components were extracted by centrifuging the fermented broth at 5000 rpm for 5 minutes. Supernatant was collected and used for antibiogram analysis. For antibiogram analysis agar well diffusion method was used and in the plates spreaded with bacterial (*Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) wells were dug by the help of sterile cork borer and loaded with the 50 µl of crude metabolite, tetracycline and distilled water.

Confirmation of Marine Nature of Isolate Showing Best Zone of Inhibition During Screening

Marine nature of the isolates MJG1210 positive in screening was confirmed by streaking in the specific media for marine bacteria the Kuster's agar containing the following in g/l (Glycerol 10, Casein 0.3, KNO_3 3, K_2HPO_4 2, NaCl 2, $MgSO_4$ 0.05, $CaCO_3$ 0.02, $FeSO_4$ 0.01 Agar 16, pH 7) and incubation at 28°C for 48 hours.

Study of Growth Parameters of MJG1210

a) Growth Curve

For growth curve, the isolate MJG1210 was inoculated in 100 ml of sterile actinomycetes isolation broth and incubated at 28 °C/ 120 rpm in shaker incubator. OD was read at 600 nm after every 24 hours against uninoculated media.

b) Effect of pH on Growth

For studying the optimum pH for the growth of MJG1210 it was inoculated in sterile actinomycetes isolation broth and incubated at 28 °C/ 120 rpm in shaker incubator. OD was read at 600 nm after every 48 hours against uninoculated media.

c) Effect of Temperature on Growth

For studying the effect of temperature the isolate was streaked on sterile AIA plates and incubated at 28 °C for 48 hours. Growth was observed after incubation.

Gram Staining of MJG1210

Gram's staining of isolate was performed in order to study is gram characteristics and cellular morphology.

Production of Antimicrobial Component by Shake Flask Fermentation

Producion of metabolite was carried out in production media containing the following in g/l (Glucose 30, KNO_3 6, KCl 5, NaH_2PO_4 1, $MgSO_4$ 0.04, $FeSO_4$ 0.02, Peptone 10, Beef Extract 6, pH 5), {pH 5 was taken as growth of the isolate was maximum at pH 5} by shake flask fermentation. Inoculated broth was incubated at 28 °C/ 120 rpm for 4 days as stationary phase was reached after 4^{th} day of incubation during growth kinetics study.

Purification of Antimicrobial Components by Solvent Extraction Method

Crude antimicrobial components were extracted from the fermented broth by centrifuging the broth at 5000 rpm for 5 minutes. Crude antimicrobial metabolite was subjected to purification by solvent extraction procedure by various solvents namely Chloroform, Ethyl Acetate, Acetone, Petroleum Ether and Hexane. 500 µl of the antimicrobial metabolite 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, ethyl acetate phase/petroleum ether/hexane (upper), chloroform (lower) and acetone (mixed) containing dissolved metabolites were collected in a weighed eppendorf tubes. The tubes were kept in incubator (37 °C) for drying of solvents. The tubes were once again weighed and amount of metabolite extracted was calculated by subtracting the weight of empty tubes from the weight of tubes after drying. The metabolite was dissolved in double volume of sterile DMSO.

Antibiogram Analysis of Purified Metabolites

Purified metabolites were assessed for their antimicrobial properties by agar well diffusion method as explained earlier, difference being the control that was sterile DMSO here.

RESULTS AND DISCUSSION

Isolation and Purification of Actinomycetes from collected Sediment and water samples

Seven different isolates were selected from mixed culture plates obtained after serial dilution, differentiated on the basis of colony morphology and named as MJG1208, MJG1209, MJG1210, MJG1211, MJG1212, MJG1213 and MJG1214. All the seven were streaked on AIA plates by quadrant streaking method.

Screening of Purified Actinomycetes Isolates for Production of Antimicrobial Components All the seven isolates were screened for production of antimicrobial components by agar well diffusion method, results of the same can be seen below in

Table 1. The isolate MJG1210 showing maximum zone of inhibition was stained with gram's staining procedure and was found to be gram positive rod in chain.

Study of Growth Parameters of MJG1210

Growth parameters were studied in order to have an idea of the optimum temperature; pH and growth curve of the isolate so that the same culture conditions could be provided during production of the metabolite.

a) Growth Curve

Figure 1 below shows the growth curve of the isolate MJG1210, it can be seen that the stationary phase was reached between 2nd to 3rd day after inoculation.

b) Effect of pH on Growth

Optimum pH for the growth of MJG1210 was determined and it was found that the isolate grows maximally at pH 5, thus the production media was maintained at the same pH. Table 2 below shows results of effect of pH.

c) Effect of Temperature on Growth

For studying the best suitable temperature for the growth of the isolate, it was streaked on AIA plates and growth was quantified based on growth in the plates. **Table 3** below shows the results of the same.

Antibiogram Analysis of Purified Metabolites

Antimicrobial components purified by solvent extraction procedure were assessed for their antibacterial properties by agar well diffusion method, results of the same can be seen in the **Table 4** below.

Table 1: Screening of Antimicrobial Metabolites for Antibacterial Properties

PATHOGENS **SAMPLES**	***S. aureus***	***P. aeruginosa***	***E. coli***
	ZONE OF INHIBITION (In mm)		
MJG1208	**00**	**00**	**00**
MJG1209	**00**	**16**	**00**
MJG1210	**15**	**18**	**15**
MJG1211	**00**	**00**	**00**
MJG1212	**00**	**00**	**00**
MJG1213	**00**	**00**	**00**
MJG1214	**00**	**00**	**00**
TETRACYCLINE	**27**	**25**	**31**

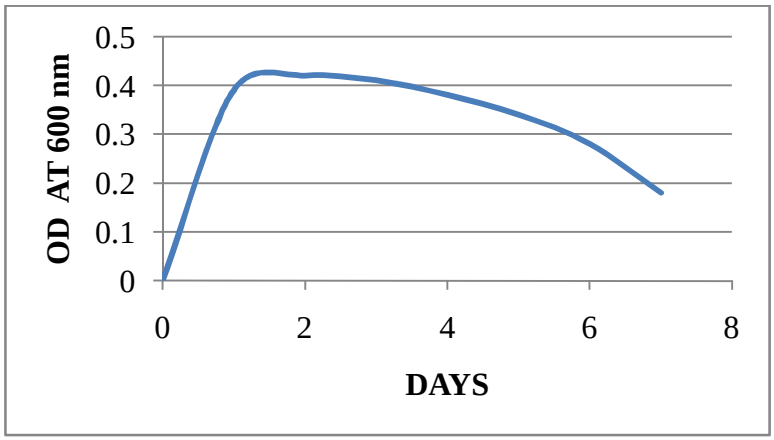

Figure 1: Growth Curve of MJG1210 (Graphical Representation)

Table 2: Effect of pH on Growth of MJG1210

S. No	pH	Absorbance at 600nm
1	5	0.93
2	7	0.39
3	9	0.68
4	11	0.25

Table 3: Effect of Temperature on Growth of MJG1210

S. No	Temperature (° C)	Result
1	22	+
2	28	++
3	37	+++
4	50	-

Table 4: Antibiogram Analysis of Purified Antimicrobial Metabolites Against Bacterial Pathogens

PATHOGENS / SAMPLES	*S. aureus*	*P. aeruginosa*	*E. coli*
	ZONE OF INHIBITION (In mm)		
CHLOROFORM	00	00	12
ETHYL ACETATE	16.5	17	16.5
ACETONE	13	12	12
HEXANE	00	00	00
PETROLEUM ETHER	00	00	00
TETRACYCLINE (50 µg/ml)	27	25	31

DISCUSSION

Actinomycetes species were isolated and purified from the marine sample on actinomycetes isolation agar and later on Kuster Agar media (a selective media for marine actinomycetes) as done earlier by **[7].**

Screening of actinomycetes species isolated from marine samples was performed by agar well diffusion method of **Kirby Buer** against pathogens *E.coli*, *S.aureus* and *P.aeruginosa* as done previously by **[8].**

Production of antimicrobial components was done by using shake flask fermentation as done previously by **[9].**

Antibiogram analysis of the crude antimicrobial extracts was performed by agar well diffusion method and zone of inhibition of MJG1210 was found to be 15.0mm against *E.coli and S. aureus*, 18.0mm against *P.aeruginosa*. Earlier, it was found that zone of inhibition against *E.coli* was 20.0mm by KUV2, 19.0mm against *S.aureus* by KUV05 **[8]**.

Crude antimicrobial metabolite was purified by solvent extraction procedure using solvents like Chloroform, ethyl acetate, acetone, hexane and petroleum ether, Nearly all the solvents used resulted in inhibition of bacterial and fungal pathogens, best being ethyl acetate extract showing a zone of 17 mm against *P. aeruginosa*. Earlier solvent extraction has been used by **[9, 10]**.

CONCLUSION

Finally based on the present study it can be concluded that the marine actinomycetes species **MJG1210** can be a potent antimicrobial producer, which can be effective against pathogens.

Future work of the present study includes further purification of the antimicrobial components by sophisticated techniques. Optimization of production media for further enhancement of the antimicrobial properties. and also the antimicrobial purified can be used in combination of pre-existing antibiotics, in order to enhance their effects.

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