Research Article

Optimization And Production Of Bioactive Metabolite From *Streptomyces malaysiensis TMS1a*

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ABSTRACT

Actinomycetes are a group of prokaryotic organisms belonging to Gram-positive bacteria and play an important role in recycling substances in the nature. The objective of this study was to isolate and identify actinomycetes from Termite mound soil against bacterial organisms. The perception prevails that the discovery of novel antibiotics is a very rare event. To discover novel secondary metabolites, our approach is to investigate from unexplored regions of the world with the aim of isolating bioactive secondary metabolites from the termite mound soil samples. Screening was performed for different termite soils collected in and around Visakhapatnam. Out of 20 actinomycetes, six showed antimicrobial activity. Out of these 6, TMS 1a showed highest antimicrobial activity. Hence this strain was considered for further studies for antibiotic production, characterized and studied for antibacterial and antifungal profile. The medium was optimized for maximum antibiotic production. Bioautogaphy was performed for the crude compound. For maximum metabolite production different Carbon, temperature, Incubation period, Agitation rates, pH were optimized. Broth showed activity against bacteria and fungi like *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Penicillinium chrysogenum, Aspergillus niger*. Further antimicrobial study was carried for Gram-positive and Gram- negative bacteria. The morphological feature of TMS 1a strain was observed under Trinocular microscope. *Under* microscopy it was observed as fragmented network of mycelium having spores. The phenotypic characters and the phylogenetic tree were revealed by the IMTECH Chandigarh that the strain is identified as *Streptomyces malaysiensis.*

Keywords: *Actinomycetes,* Antibiotics, Antimicrobial, Bioautography, Optimization, Termite soil.

INTRODUCTION

Termites are a group of social insects and one of the important factors in ecosystem by playing an important role in the carbon and nitrogen cycles.[1] They play an important role in breaking down of organic material occurring in the soil. Though the overall contribution of termites to soil productivity is generally less than that of earthworms but their digestive system is generally more efficient. Microorganisms such as bacteria and protozoa occur in the gut of the termites. Their bacteria play an essential role in the metabolism of organic matter such as carbon and nitrogen from termite food sources.[2]

Actinomycetes are a group of Gram-positive bacteria with its DNA rich in mol G+C content.[3] They are outstanding source for bioactive compound production. Actinomycetes produce large number of important secondary metabolites such as antibiotic compounds including Streptomycin, actinomycin, and tetracycline.[4] Most antibiotics from actinomycetes have been reported by many researchers.[5,6] Among actinomycetes predominantly *Streptomycetes* sp. has been recognized as major producer of bioactive metabolites with broad spectrum of activities such as antibacterial and antifungal agents.[7-9] The isolates of *Nocardia levis* from soils[5], *Streptomyces* sp. from desert soil[10] and actinomycetes from mangrove sediments, soil against Gram-negative and Gram-positive pathogenic bacteria were reported.[11-13]

The genus *Streptomyces* was proposed by Waksman and Henrici.[14] Actinomycete isolated from terrestrial environment has potential use in the production of both primary and secondary metabolites. Members of this genus are now known to share many phenotypic characteristics and to continue a distinct phyletic line, and they are currently assigned to over 450 validity described species.[15-17] It is also clear that minimal standards for description of *Streptomyces* species need to be based on a judicious selection of genotypic and phenotypic properties.[18-20] *Streptomyces,* soil-dwelling filamentous bacteria are profile producers of wide range of antimicrobial agents.[21] *Streptomyces* continue to be a rich source of vitamins, carbon, nitrogen, amino acids utilization and enzymes.

There are no reports in the current literature dealing with the production of bioactive metabolites from *Streptomyces malaysiensis* which was isolated from termite soil. With in this context, the purpose of present study were to isolate and identify the actinomycetes obtained from termite mound soil and to carry out the fermentation studies for the production of bioactive metabolite. Furthermore the bioactive compound obtained was used to determine the antagonistic activity against Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Isolation of actinomycetes

Termite mound soil samples were collected in and around Visakhapatnam in 2011 by using sterile spatula aseptically. A soil sample of 5gms was collected into a self sealed cover by taking at different spots on the termite mound. The medium used for screening of soil sample was starch casein agar medium. Antibiotic solutions of each (50ug/ml) rifampicin and cycloheximide and 1ml of serially diluted termite mound soil sample were added to the sterilized starch-casein agar medium. The plates were incubated at 28°C for 10 days. After 10 days, the actinomycetes colonies grown on petriplates were counted at regular intervals.20 isolates of actinomycetes were obtained; out of which 6 are having antimicrobial activity.

Microorganism maintenance and propagation

The stock culture was maintained on Bennett's medium and Yeast-extract-malt extract medium.[22]

Fermentation Process

Culture medium

Laboratory fermentations were run in 250ml Erlenmeyer flasks containing 50ml of medium for seed development. Seed medium contains of the following soybean meal 2.5%(w/v), glucose 1.5%(w/v), glycerol 0.25%(w/v), sodium nitrate 0.4%(w/v), di-potassium hydrogen phosphate 0.5%(w/v), sodium chloride 0.25%(w/v), zinc sulphate 0.004%(w/v), calcium carbonate 0.04%(w/v) and distilled water upto-50ml, pH 7.0±2. Seed flasks were incubated on rotary shaker for 2 days to obtain good growth at temperature 26°c.

All experiments, with two replicates flasks for each condition, were independently carried out three times. In each flask, antimicrobial activity was performed in triplicate. The data were reported as the average of three replicates.

Production of secondary metabolite from the actinomycetes

Production medium was same as that of the seed medium. Flasks for production were inoculated with 5% of the mature seed broth and kept on rotary shaker at 28°C for 5 days. After 5 days bioactive metabolite was extracted by solvent extraction method.

Extraction of Secondary metabolite by solvent extraction Method

After the production of secondary metabolite the broth was aseptically transferred to sterile centrifuge tubes and centrifuged at 5000rpm for 30 mins. The supernatant was collected in sterile conical flask and the pellet was discarded. The supernatant was transferred to the separating funnel and mixed with ethyl acetate in the ratio 1:3 (supernatant: ethyl acetate). After 15 mins, separating funnel was shaked and kept undisturbed for 10-15 mins for separation of two layers. Both organic and aqueous phases were tested for antimicrobial activity. Organic phase was concentrated by rotary evaporation at 40°C.This crude extract was also tested for its antimicrobial activity by agar diffusion method.

Determination of antimicrobial activity

The antimicrobial activity was determined by agar overlay and agar well method by using the concentrated crude extract and the inhibition zone diameter were measured against Gram–positive and Gram-negative bacteria[23].

Biological Characterization by Bio autography

The procedure in bio autographic methods is similar to the one used in agar diffusion methods. The difference is that the tested compounds diffuse into inoculated agar medium from the chromatographic layer, which is adsorbent or paper.

The TLC plates were dipped in a chamber which contains solvent system (i.e. Hexane and Ethyl acetate). The solvent system was run up to 3/4th of the TLC plates and then TLC plates were dried. Now, sterile Petri plates were taken and add nutrient agar (which was previously inoculated with Gram positive and Gram negative bacteria, separately) and kept aside for 15 minutes to solidify. Place the TLC plates on the inoculated nutrient agar and keep it aside for minutes to hours in freezer for diffusion. Now, the TLC plate is removed and the plates are incubated for 24 hours at 37°C. The zone of inhibition appears in the places, where the antimicrobial compound was in contact with the agar layer.

Optimization of nutritional and cultural conditions

Production of secondary metabolites by microorganisms is often connected with and influenced by primary metabolism. The composition and concentrations of the media are closely linked with the metabolic capacities of the producing organism and greatly influence the biosynthesis of the bioactive molecules. The effect of other factors including medium, temperature, initial pH of the medium and incubation period on secondary metabolites were by one factor-one level method.

Nutritional Conditions

Five carbohydrate sources (Dextrose, Fructose, Maltose, Sucrose and Lactose) were studied individually as sole carbon source at 1.5% (w/v) in the above production medium by keeping all other ingredients constant.

Effect of Physical parameters (Temperature, pH, Incubation period and Agitation Rate)

The effect of culture conditions, namely incubation temperature (22-37°c), initial pH (5-9), incubation period (2-8) days and agitation rate (160-200rpm) on growth and secondary metabolite production having antimicrobial activity were studied.

RESULTS AND DISCUSSION

This is the first report on optimization & production of bioactive metabolite from *Streptomyces malaysiensis* TMS1a, which was isolated from Termite mound soil. *Streptomyces malaysiensis* previously described as an interesting secondary metabolite producer against pathogenic fungus of chilli Anthracnose.[24] A total of 20 actinomycetes isolates were obtained from termite soil, out of which 6 isolates showed antimicrobial activity. Among them, TMS1a isolate showed highest antimicrobial activity by agar overlay method as shown in Figure 1. Screening medium used for isolation of TMS 1a was starch casein agar medium. Similar medium was used in the isolation of antibiotic producing actinomycetes.[25] Culture medium used in the production of bioactive metabolite by TMS 1a was same as the medium used as in the case of optimization of fermentation conditions for antibiotic production by actinomycetes strain against *Sclerotinia sclerotiorum.*[26]

Secondary metabolites were extracted after 5days by solvent extraction method. For extraction (supernatant: ethyl acetate) were used in the ratio of 1:3 for TMS 1a as described earlier. For extraction of secondary metabolites in marine sponge**,** by solvent extraction method the supernatant was transferred to the separating funnel (supernatant: ethyl acetate) were used in the ratio of 1:1. Then the mixture in the separating funnel was shaked continuously for 15mins. After 15mins, keep the separating funnel undisturbed for 10-15mins. After 15mins, three layers were formed and the middle layer was removed in a sterile petriplate. The petriplate containing secondary metabolites in the suspension was allowed to dry in the air. This dried sample contains the secondary metabolites.[27] The zone of inhibition in bio autography indicates that the concentrate of the crude compound is having antibacterial activity. Solvents used are hexane and ethyl acetate. In 75:25 concentrations the antibacterial activity is more for the crude compound on both gram positive and gram negative bacteria. It is immediately apparent from these data that the bioactive metabolite as a group inhibits a wide variety of Gram-positive and Gram-negative bacteria.[28, 29] The strain TMS 1a showed highest antimicrobial activity, this strain was selected for further optimization studies as shown in Figure 2. The maximum antimicrobial activity was observed when lactose was used as sole carbon source as shown in Table 1. *Streptomyces sp*. TMS 1a showed a narrow range of incubation temperature for good growth and bioactive metabolite production. The optimum temperature was 26^{o}C.The highest antimicrobial activities against five tested microorganisms were obtained at an initial pH 7 of the culture medium, concerning incubation period, antimicrobial activities appeared to be pronounced after 2nd day of growth with a maximum at 5th day of incubation. This activity remained stable between 5-7 days and then decreased after 8th day of incubation. For TMS 1A species agitation rates of 160 and 200rpm gave low antimicrobial activity, while best result was noticed at 180 rpm as shown in (Table 2 & 3). Nutritional and physical parameters were optimized by method given by Fourati.[30]

Whereas *Streptomyces* sp.TN$_2$6$_2$ strain, a new strain from Terrestrial soil showed a narrow range of incubation temperature 30^{o}c, pH 7 and incubation period at 72 hrs was reported for maximum activity.[31] Microscopic observation indicates that the isolated organism has Fragmented network of Aerial and substrate mycelia with spore orientation, when viewed under Trinocular microscope as seen in Figure 4. The phylogenetic analysis of TMS1a given by IMTECH CHANDIGARH revealed 100% that the strain is *Streptomyces malaysiensis* as shown in Figure 3.

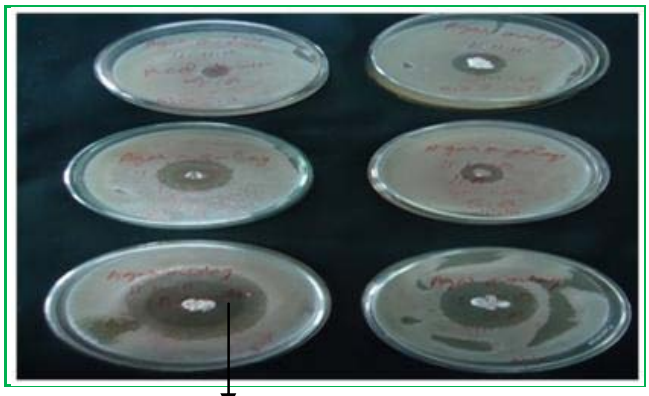

Figure 1: TMS 1A (Agar overlay method)

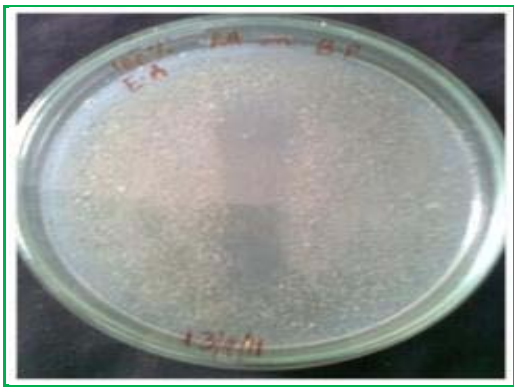

Figure 2: Zone of inhibition in the bio autography plate

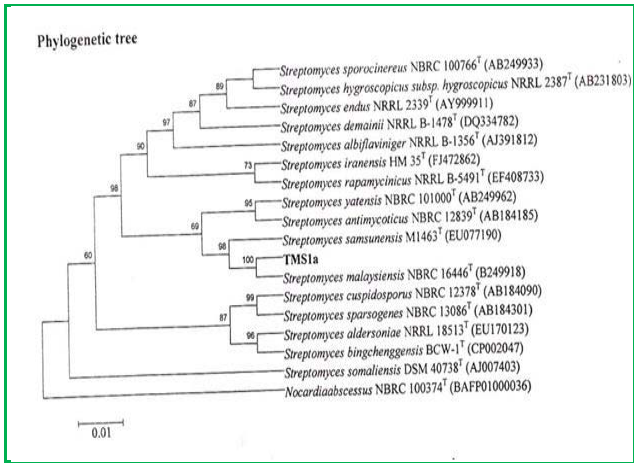

Figure 3: The phylogenetic analysis of TMS1a

Table 1: TMS 1a, when the medium was optimized with different carbon sources

Optimization parameter (Carbon source)	Mean values of Inhibition zone diameter (mm)				
	S.aureus	*B.subtilis*	*B.pumilus*	*P.aeruginosa*	*E.coli*
Lactose	**18 ± 1**	**19.5 ± 0.5**	**19.5 ± 0.5**	**19.5 ± 0.5**	**19.5 ± 0.5**
Dextrose	11 ± 1	12.5 ± 0.5	13 ± 0.5	11.5 ± 0.5	13.5 ± 0.5
Maltose	8.5 ± 0.5	10.8 ± 0.76	9.5 ± 0.5	10.8 ± 0.76	11.5 ± 0.5
Fructose	11 ± 0.76	11.5 ± 0.5	9.5 ± 0.5	8.5 ± 0.5	9.8 ± 0.76
Sucrose	11 ± 0.76	10.8 ± 0.76	8.5 ± 0.5	11.5 ± 0.5	12.5 ± 0.5

Table 2: TMS1a when the medium was optimized with parameters (temperature and rpm)

Optimization parameter	Mean values of Inhibition zone diameter (mm)				
	S.aureus	*B.subtilis*	*B.pumilus*	*P.aeruginosa*	*E.coli*
22°C	13.8 ± 0.76	13 ± 0.5	12.16 ± 0.28	11.16 ± 0.28	10.16 ± 0.28
26°C	15.8 ± 0.76	14.5 ± 0.5	13.1 ± 0.28	14.5 ± 0.5	13.16 ± 0.28
28°C	14.5 ± 0.5	13.5 ± 0.5	13.1 ± 0.28	11.8 ± 0.76	11.16 ± 0.28
32°C	12.8 ± 0.76	10.5 ± 0.5	8.8 ± 0.76	9.16 ± 0.28	10.5 ± 0.5
37°C	8.5 ± 0.5	8.8 ± 0.76	0	0	0
Rpm					
160	10.5 ± 0.5	9.8 ± 0.76	9.8 ± 0.76	12.16 ± 0.28	11.16 ± 0.28
180	16.5 ± 0.5	13.1 ± 0.28	14.5 ± 0.5	16.8 ± 0.28	13.8 ± 0.28
200	10 ± 0.5	9.16 ± 0.28	11.8 ± 0.76	10.8 ± 0.76	9.5 ± 0.5

Table 3: TMS1A, when the medium was optimized with parameters (incubation period and pH)

Optimization parameter	Mean values of inhibition zone diameter (mm) for				
	S.aureus	*B.subtilis*	*B.pumilus*	*P.aeruginosa*	*E.coli*
Incubation period (days)					
2	13.8 ± 0.76	13.8 ± 0.76	14.5 ± 0.5	O	12.8 ± 0.76
4	14.5 ± 0.5	12.8 ± 0.76	11.16 ± 0.28	0	11.16 ± 0.28
5	16.5 ± 0.5	18.6 ± 0.52	16.5 ± 0.5	0	18.6 ± 0.52
6	14.5 ± 0.5	14.5 ± 0.5	14.5 ± 0.5	0	15.5 ± 0.5
7	13.8 ± 0.76	13.8 ± 0.76	12.8 ± 0.76	0	12.8 ± 0.76
8	12.8 ± 0.76	11.16 ± 0.28	11.16 ± 0.28	0	12.8 ± 0.76
pH					
5	10 ± 0.5	0	9.8 ± 0.76	0	11.16 ± 0.28
6	0	0	0	0	0
7	15.5 ± 0.5	13.8 ± 0.76	18.6 ± 0.52	0	18.6 ± 0.52
8	13.8 ± 0.76	11.16 ± 0.28	12.8 ± 0.76	0	11.16 ± 0.28
9	12.8 ± 0.76	0	10.5 ± 0.5	0	10 ± 0.5

CONCLUSION

This is the first report on optimization & production of bioactive metabolite from *Streptomyces malaysiensis* TMS1a, which was isolated from Termite mound soil. This strain showed antimicrobial activity against Gram-positive and Gram-negative bacteria. Maintaining the above conditions in the medium containing lactose, 72 hr incubation, 26°C temperature, 180 rpm agitation yielded the highest antimicrobial activity. Further study needs to be undertaken to analyze the mechanism for the antimicrobial activity of this bioactive compound. It may be considered as a potential source of drug production. Based on the results of the cultural characteristic studies and phylogenetic analysis, this isolate has been assigned as *Streptomyces malaysiensis*.

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