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Research Article

STUDIES ON THE ISOLATION AND IDENTIFICATION OF ACTINOMYCETES FROM LIGNITE MINE ECOSYSTEM AND THEIR ANTIMICROBIAL ACTIVITY

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A B S T R A C T

In India, larger amount of lignite are being excavated from NLC, Neyveli, Tamilnadu. A study was conducted to isolate Actinomyctes from this mine soil. A total of eight isolates were isolated and they were identified based on their morphological and biochemical characterization. The identified actinomycetes isolates were *Streptomyces vastus, Streptomyces mirabilis, Actinomycetes longiporus, Actinomycetes aureocirculatus, Streptomyces roseole, Streptomyces platensis, Actinomycetes janthinus* and *Actinomycetes malachitorectus*. Among them, many isolates had the inhibition activity against some bacterial pathogens. Gram positive bacteria were highly sensitive to secondary metabolites produced by actinomycetes, than the gram negative bacteria.

INTRODUCTION

Mining is one of the oldest human activities in the world. Increased energy demand leads to the use of more fossil and nuclear fuels. World's population is expected to reach over 8 billion by 2030 and consequently global energy demand will grow by almost 60 percent by 2030 and rise to 16.5 billion tonnes of oil equivalent per year. Fossil fuels and in particular coal will meet up this challenge in future. Coal-based electricity is set to double in the first three decades of 21st century, from 16,074 TWh in 2002 to 31,657 TWh in 2030 and will continue to play a key role in electricity generation in world. Around the world, coal production is about 3.5×10^9 tonnes per year, among this 38 % used for generating electricity (AUA, 2007).

Coals of variable rank are still one of the most important energy sources/heat sources in the world due to it their abundant world's reserves (Franco and Diaz, 2009). Lignite as the youngest type of coal is one of the abundant natural resources. The lignite is being mined by underground and open cast method. Nowadays, about 95 to 98 % of lignite is mined by the open cast method and a few percent by the underground method.

Coal mine ecosystem is one of the ecosystems which abound in microbial diversity. Microbial diversity at mine sites may also be greater in previously thought.

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concentration of heavy metals can survive. Microbial communities consisting of bacteria, actinomycetes, fungi, yeast and algae occupy important niches in mining ecosystems. These organisms play key roles in the earth's biogeochemical cycle (Dave and Natarajan, 1987; Dave *et al*., 2002).

MATERIALS AND METHODS

Enumeration of total microorganisms in lignite mine ecosystem

The collected effluent and soil samples were serially diluted upto 10^{-6} dilution to determine the population of bacteria, fungi, actinomycetes and algae. 0.1 mL from the 10^{-6} dilutions were plated on sterile Petri plates containing Nutrient Agar (NA) medium and incubated at 28 ± 2°C for two days for enumerating the bacterial population, 10^{-4} dilutions were plated on sterile Petri plates containing Rose Bengal Agar medium (RBA) and incubated at 28 ± 2°C for 3 days for enumerating fungal colonies and 10^{-5} dilutions were plated on sterile Petri plates containing Kenknight's Agar medium (KKA) and incubated at 30 ± 2°C for 5 to 7 days for enumerating actinomycetes colonies. From 10^{-2} dilution, 0.1 mL was added into the plate containing Fogg's medium for enumerating the algal population. After incubation, the number of bacterial, fungal, actinomycetes and algal colonies in the respective plates were counted and the population was expressed in terms of cfu/g or mL.

Isolation and identification of actinomycetes from lignite mine soil sample

Ten gram of soil sample collected from lignite mine was transferred to 100 mL of sterile distilled water in a 250 mL Erlenmeyer flask and incubated on a rotator shaker (100 rpm) for 30 minutes at 28° C. The well mixed suspension was then diluted appropriately upto 10^{-6} dilution. One mL of suspension from 10^{-5} dilution was aseptically transferred to sterile Petri plates and 10-20 mL of selective Starch Casein Agar medium was added and incubated at 28ºC for 7 days. The antifungal (50µg/mL cyclohexamide) and antibacterial (20µg/mL of tetracycline) were added to medium after sterilization. The individual colony further streaked on the same medium. The isolated actinomycetes strains were identified on the basis of their phenotypic, physiological and biochemical characterization.

Phenotypic characterization

The classification of actinomycetes was originally based largely upon the morphological observations. So, morphology is still an important characteristic for the description of taxa and it is not adequate in itself to differentiate between many genera. In fact, it was the only characteristic which was used in many early descriptions, particularly of Bergey's manual. These observations are best made by the variety of standard cultivation media. Several of the media suggested for the International *Streptomyces* Project by Pridham *et al.* (1957) have proven to be useful in our hands for the characterization of strains accessioned into the ARS Actiomycetales culture collection. It includes some basic tests *viz.,* Aerial mass color, Reverse side pigment, Melanoid pigments, Spore chain morphology and Spore morphology (Shirling and Gottileb, 1966).

Species affiliation - physiological and biochemical characteristics

Assimilation of carbon sources

The ability of different actinomycetes strains in utilizing various carbon compounds as the source of energy was studied by following the method recommended in International *Streptomyces* Project. Stock solution of sugars i.e; xylose, inositol, sucrose, raffinose, fructose, mannitol and rhamnose having concentration of 10x was prepared in autoclaved water and sterilized by filtering through 0.22 µm pore size membrane filters and stored at 4°C. Growth of actinomycetes strain was checked by taking 1% carbon source in ISP2 media. Plates were streaked by inoculation loop by flame sterilization technique and incubated at 28°C for 7 to 10 days. Growth was observed by comparing them with positive and negative control (Pridham and Gottlieb, 1948).

Assimilation of nitrogen sources

The ability of different actinomycetes strains in utilizing various nitrogen compouns as source of energy was studied by following the method recommended in International *Streptomyces* Project. Stock solution of nitrogen compounds namely L-Aspergine, L-Argine, L-Citrulline, L-Histidine, Glycine, L-Lysine and L-Proline having concentration of 10x was prepared in autoclaved water and sterilized by filtering through 0.22 µm pore size membrane filters and stored at 4ºC. Growth of actinomycetes strain was checked by taking 1% nitrogen sources in ISP2 medium. Plates were streaked by inoculation loop by flame sterilization technique and incubated at 28ºC for 7 to 10 days. Growth was observed by comparing them with positive and negative control (Pridham and Gottlieb, 1948).

Indole test

Peptone broth medium was prepared and distributed to sterile test tubes. The broth was sterilized at 121ºC in 15 lbs pressure for 10 minutes. Then, the tubes were inoculated with isolates and incubated for 3 to 4 days at 30ºC. After the incubation period Kovac's reagent was added. Cherry red color was formed at the top after the addition of Kovac's reagent is positive result. Absence of red color ring formation considered as negative result.

Methyl red test

MR broth was prepared and sterilized at 121ºC in 15 lbs pressure for 10 minutes and poured into sterile test tubes. Then, the tubes were inoculated with isolates and incubated at 30ºC for 2 to 4 days. After incubation, 5 drops of methyl red was added in test tubes. Red color formation indicates the positive result. If it remains yellow, it is said to be negative.

Citrate utilization test

Simmon's citrate agar slants were prepared by adopting aseptic techniques. Then, the isolates were inoculated into the agar slants and incubated at 30ºC for 2 to 4 days. Appearance of royal green to royal blue color indicates the positive result. Otherwise it is said to be negative.

Hydrogen-sulphide production

The inoculated Tryptone-Yeast extract agar slants were incubated for 7 days for this test. Observations on the presence of the characteristic greenish-brown, brown, bluish-black or black color of the substrate, indicative of H_2S production were recorded on 7^{th}, 10^{th} and 15^{th} days. The incubated tubes were compared with uninoculated controls.

Hydrolysis of Gelatin

The medium consists of nutrient supplemented with 12% gelatin, this high gelatin concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Gelatin liquefaction was studied by sub-culturing the strain on gelatin agar medium and inoculated them at 28°C. Observation was made after 7 days. The extent of liquefaction was recorded after keeping the tubes in cold conditions (5-10°C) for 1 hour. Cultures that remained liquefied were indicative of slow gelatin hydrolysis.

Hydrolysis of starch

Starch hydrolysis was studied by sub-culturing the strains on Starch Agar medium and incubated at 28°C. Observation was made after 7 days. The development of a clear zone around the culture streaks when the plates were flooded with Lugol's iodine solution should be recorded as the hydrolysis of starch.

Hydrolysis of casein

Milk coagulation was studied with Skimmed milk agar medium. The skimmed milk tubes were inoculated with the isolates and incubated at 37ºC. The extent of coagulation was

recorded on the 7th and 10th of incubation. The importance of milk as a culture medium for the study of bacteria has long been recognized by workers in bacteriology. Although, milk is a very complex medium, it is more or less standard in composition and the reactions produced upon it by microorganisms are so characteristic, that it has found general acceptance. The plates were incubated for the 7 to 10 days after the inoculation.

Determination of Antimicrobial activity the isolates

Antimicrobial activity of the secondary metabolites produced by actinomycetes was assessed by the Kirby-Bauer disk diffusion method. One week old broth cultures of the isolated actinomycetes were used with sterile paper disk soaked in the cultures for 30 minutes were used to inoculate actinomycetes on media seeded with pathogenic bacteria. The results were observed after 24 hrs by incubated the plates at 37 °C.

RESULTS AND DISCUSSION

Enumeration of total microorganisms from lignite mine ecosystem

The enumeration of microorganisms from lignite mine soil, lignite mine effluent and effluent irrigated soil were analyzed and the results were presented in Table-1. The effluent irrigated soil sample recorded the highest microbial population (58.1 x 10^6 cfu/g of bacterial population, 27.84 × 10^4 cfu/g of fungal population, 34.1 × 10^5 cfu/g of actinomycetes and 9.6 × 10^2 cfu/g of algae) and the lignite mine soil contains lowest microbial population (2.1 x 10^6 cfu/g of bacterial population, 5.2 × 10^4 cfu/g of fungal population and 13.9 × 10^5 cfu/g of actinomycetes and Nil algal population).

Table 1 Enumeration of microorganisms from lignite mine soil, lignite mine effluent and effluent irrigated soil

S. No.	Microorganism	Population		
		Lignite mine soil	Lignite mine effluent	Effluent irrigated soil
1.	Bacteria (10^6 cfu/mL or g)	2.1	17.24	58.1
2.	Fungi (10^4 cfu/mL or g)	5.2	7.4	27.84
3.	Actinomycetes (10^5cfu/mL or g)	13.9	14.5	34.1
4.	Algae (10^2 cfu/mL or g)	–	1.5	9.6

Isolation and identification of Actinomycetes from lignite mine soil

The lignite mine soil sample was serially diluted and plated on Starch Casein agar medium. Eight species of actinomycetes were isolated and designated as, LMA1, LMA2, LMA3, LMA4, LMA5, LMA6, LMA7 and LMA8 (LMA - Lignite Mine Actinomycete). They were identified based on phenotypic and biochemical characterization and the results are given in Table-2. The identified actinomycetes isolates were Streptomyces vastus, Streptomyces mirabilis, Actinomycetes longiporus, Actinomycetes aureocirculatus, Streptomyces roseole, Streptomyces platensis, Actinomycetes janthinus and Actinomycetes malachitorectus.

Several researchers have reported that the presence of different Species of actinomycetes in diverse environment, such as coal mines (Gopinath *et al.,* 2013; Gopinath and Charya, 2013), salt mines (Aftab *et al.,* 2015) and iron mine soil (Ashok Kumar *et al.,* 2012).

Determination of Antimicrobial activity the isolates

The antimicrobial activities of the isolated actinomycetes species, against nine different pathogenic bacteria, were determined and the results were shown in Table-3. Formation of clear halo zones around the discs indicated that bacterial growth inhibition ability. This showed that the actinomycetes produced secondary metabolites that were active against the tested bacteria. Absence of zones indicated that the test organisms was resistant against metabolites.

The zone of inhibition indicates susceptibility of the test bacteria to antimicrobial substance (Jorgensen and Ferraro, 2009; Whaet, 2011). The size of the halo zones varied with different isolates. This could be due to differences in the diffusion rates of these metabolites through the agar. The rate of diffusion was dependent on the diffusion against solubility properties of the metabolites. Faster diffusing metabolites formed larger halo zone compared to slow diffusing metabolites (Cheesbrough, 2006).

Molecular weight of the metabolite might be important reason for variation in size of zone.

Table 2 Phenotypic and biochemical characterization of Actinomycetes isolated from lignite mine soil sample

S. No	Isolates	Phenotypic characterization				Biochemical characterizatoion																				Tentatively identified as
						Assimilation of carbon sources							Assimilation of nitrogen sources														
		Aerial mass color	Melanoid pigment	Revere side Pigment	Spore chain	Inositol	Xylose	Sucrose	Raffinose	Fructose	Mannitol	Rhamnose	L. Aspergine	L.Argine	L.C itrulline	L.Histidine	Glycine	L.Lysine	L.Proline	Indole	Methyl red	Citrate utilization	H_2S Production	Hydrolysis of Gelatin	Hydrolysis of starch	Hydrolysis of casein	
1	LMA1	Gy(W)	+	+	R	+	+	+	±	+	+	+	±	±	++	±	+	-	+	-	+	-	+	-	+	+	Streptomyces vastus
2	LMA2	Gy(W)	+	+	S	+	-	-	-	-	+	+	-	++	-	-	±	±	±	-	-	-	-	-	+	+	Streptomyces mirabilis
3	LMA3	Gy(W)	+	-	S	+	+	+	±	+	+	+	±	+	±	±	±	-	-	+	+	+	+	-	+	+	Actinomycetes longiporus
4	LMA4	W	-	-	S	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	+	-	±	-	Actinomycetes aureocirculatus
5	LMA5	D	-	+	S	+	+	+	±	-	-	±	±	+	-	±	-	-	+	-	-	+	+	-	+	+	Streptomyces roseole
6	LMA6	Y	-	-	S	+	+	+	+	+	+	+	+	±	+	+	+	+	±	+	-	-	-	-	+	+	Streptomyces platensis
7	LMA7	Gy(W)	-	-	S	+	+	+	-	+	+	+	-	++	++	±	+	±	+	+	-	+	+	-	+	++	Actinomycetes janthinus
8	LMA8	Gy	+	+	RA	+	+	+	-	+	+	+	++	+	±	-	-	±	+	+	+	+	+	-	+++	++	Actinomycetes Malachitorectus

Table 3 Determination of Antimicrobial activity the isolates

Test bacteria	Actinomycetes isolates							
	S.v	***Sm***	***Al***	***Aa***	***Sr***	***Sp***	***Aj***	***Am***
S.a	+	+	+	+	+	+	+	+
B.c	+	+	+	+	+	+	+	+
B.m	+	+	+	+	+	+	+	-
P.a	-	-	-	-	+	-	-	-
E.c	+	+	+	-	+	+	-	-
K.p	-	+	-	-	-	+	-	-
S.f	-	-	+	+	-	-	-	-
S.d	+	-	+	+	-	-	-	-
S.s	+	-	-	+	-	-	-	-

Sa-Satphylococcus aureus, B.c- Bacillus cereus, B.m-Bacillus megaterium, P.a-Pseudomonas aeruginosa, E.c- Escherichia coli, K.p- Klebsiella pneumonia, S.f-Shigella flexnrri, S.d- Shigella dysentery, S.s- Shigella sonnei

Sv- Streptomyces vastus, Sm -Streptomyces mirabilis,Al- Actinomycetes longiporus, Aa -Actinomycetes aureocirculatus, Sr -Streptomyces roseole, Sp- Streptomyces platensis, Aj -Actinomycetes janthinus, Am -Actinomycetes Malachitorectus

Compounds with low molecular weights diffused rapidly when compared with compounds having higher molecular weight (Dougherty and Pucci, 2012).

Actinomycetes have been recognised as source of several secondary metabolites like antibiotics and lytic enzymes among which isolates have been shown to have antimicrobial characteristics which make them useful as antagonistic agents against pathogens (Kariminik and Baniasadi, 2010).

Many bacteria were resistant against the metabolites produced by the actinomycetes isolates. This resistance could be attributed by these bacteria by transferring the resistant genes from the neighbouring resistant bacterial cells in the previous environment (Canton, 2009; Jorgensen and Ferraro, 2009).

The results also revealed that most of the actinomycetes isolates were active against gram-positive bacteria than gram-negative bacteria. The reason for different sensitivity between gram positive and gram negative bacteria could be due to their morphological differences between these organisms. Gram-negative bacteria having an outer polysaccharide membrane, carrying the structural lipopolysaccharide compounds. This makes the cell wall impermeable to lipophilic solutes. The peptidoglycon layer could not be an effective barrier. The results of the present study was well correlated with previous studies by Pandey *et al.,* 2004 and Cwala *et al.*, 2011.

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