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Research Article

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Antimicrobial Activity and Strain Development of Actinomycetes Isolated from Deccan Region of India Soil Samples

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ABSTRACT

The study is aimed at process of isolation, purification and characterization of actinomycetes species from soil which exhibits antimicrobial property versus 8 chosen pathogenic strains. The soil samples collected from different habitats present in Secunderabad, India. The collected samples were subjected to serial dilution and are plated on agar media containing actinomycetes isolates. Screening, purification and storage of potential colonies in glycerol stock solution is carried out followed by characterization of isolates biochemically and morphologically is carried out. The extraction process of isolates is carried out to produce antibacterial compound and evaluation process is conducted by Minimum inhibitory concentration method. A total of 6 isolates of actinomycete species were tested for antagonistic property vs 8 pathogenic microbes. S-4 among isolates found to be very active and activity of the isolate is further increased by the influence of UV mutation. The strain AM2 exhibited maximum antibiotic producing property which is 4.9 mg/ml and it is referred to as S-4 mutant strain. S-4 mutant strain is further used to carry out fermentation related studies.

Keywords: Actinomycetes; Antibacterial activity; UV mutation

INTRODUCTION

Most of the therapeutically important products and synthesis of microorganisms are isolated and identified from soil resources. One such order is actinomycetales that is comprised of 80 genera present in terrestrial soils primarily as saprophytes [1]. The genera are also present in water and plants and exhibit diversity both chemically and morphologically considering process of evolution [2]. Actinomycetes are gram positive in nature and contain high percentage of Guanine-Cytosine content (55%) and found to grow slowly as filaments on branches [3]. Actinomycetes commonly grow on bacteria rich media include nutrient agar, blood agar and trypticase soy agar media [4,5]. The dominant type is Streptomyces and most of the actinomycetes genera produce either aerial or substrate mycelium [6]. Secondary metabolites which are produced from actinomycetes species accounts for 75% from more than two decades [7]. Actinomycetes serves as a source for many bioactive compounds that exhibit clinical importance for example drugs that are administered into humans [8-12]. Nearly one third among thousands of naturally producing antibiotics was obtained from actinomycetes [13]. The main objective is to screen actinomycetes from various habitats to determine antimicrobial property and act as antagonist to pathogens that are drug resistant.

EXPERIMENTAL SECTION

Soil Sampling and Pre-treatment

The soil samples collected from different habitats present in Secunderabad, India. All soil samples were collected from a depth of 11-15 cm from surface of the ground and are placed inside poly bags by maintaining sterile condition and are transferred to laboratory for the purpose of isolation procedures. Aseptically soil samples were subjected to heating for a period of one hour at 45°C. 1% $CaCO_3$ is added to inhibit the unwanted fungal and bacterial growth and this enables to increase actinomycetes population in the soil.

Isolation of Pure Culture of Actinomycetes

Actinomycetes were isolated from soil samples; with isolation media as Starch-casein agar media, Soybean-Casein Digest Medium (SBCD) and Actinomycetes Isolation Agar (AIA) and are subjected to crowded plating method which pinpoints the colonies with inhibition zone. Actinomycetes isolated from the media are subjected to multiple streaking procedures in agar media to get pure actinomycetes culture from 9 strains. Each strain was preserved in agar slants containing actinomycetes at 4°C temperature. Purified isolates of actinomycetes were maintained in International Streptomyces Project (ISP) 1 (containing Tryptone yeast extract broth) at 4°C and glycerol stocks containing 25% v/v preserved at 40°C for a long period of time [8,14].

Isolates Characterization

A smear was prepared using broth culture by heat drying process on a glass slide and it is covered using crystal violet for 30 seconds and washed with water. To this added Gram's iodine and after 30 seconds added alcohol which serves as decolorizing agent for a period of 60 seconds. In final step safranine is added to smear which acts a counter stain for 2 minutes. Then after thorough washing and drying process slides were viewed using phase contrast microscope with 100x lens [8,15]. Based on preliminary studies conducted it is found that the isolates were remained positive and are further subjected to biochemical studies [16] .

Test for Microbial Sensitivity

The isolated strains of actinomycetes were tested for microbial Sensitivity against three fungal strains and five bacterial strains using streak method with agar medium. The Table 1 below shows S-4 is a broad spectrum with better score compared to other strains.

Table 1: Sensitivity of different microorganisms towards the soil isolates by agar streak method

Soil isolates	*B.Subtilis*	*S.aureus*	*E.coli*	*K.Pneumoniae*	*P.aeruginosa*	*A.niger*	*A.terreus*	*C.albicans*
S1	++	+	+	-	++	-	-	+
S2	+	++	+	-	++	-	-	+
S3	-	-	++	-	++	-	+	++
S4	++	++	+++	++	+	+	-	++
S5	+	-	+	+	+	-	-	-
S6	+	-	+	-	-	-	-	-
S7	++	-	+	+	+	+	-	+
S8	-	+	+	+	-	+	+	-
S9	-	+	-	+	-	-	-	-

+++ = Better inhibition; ++ = Good inhibition; + = Moderate inhibition; - = No inhibition

Test Organism

Test organisms were obtained from IMTECH, Chandigarh. The microorganisms selected for this study include *Bacillus subtilis* BSCC 499, *Staphylococcus aureus* SACC 600789, *Klebsiella pneumoniae* KPCC 2405, *Escherichia coli* ATCC11105, *Pseudomonas aeruginosa* ATCC25619, *Aspergillus niger* ATCC6275, *Aspergillus terreus* DMSRDE945 and *Candida albicans* ATCC18804.

S-4 Strain by UV Mutation

Genetic alteration of S-4 strain using UV irradiation and drug resistance procedures was carried out. A 5 ml sample suspension containing spores of S-4 cultured strain is transferred to actinomycetes agar plates in aseptic condition followed by producing duplicates. UV irradiation experiment is conducted by subjecting S-4 strain to UV rays which is at 35 cm distance and 324 nm at regular intervals of time. The plates were placed in BOD incubator for 5 days. Mutant strains which are isolated from plates were subjected to UV rays and are used for production of antibiotics by assay procedure involving inoculation of mutant strain onto a medium comprised of NaCl (0.5%),

$CaCO_3$ (0.35%), lactose (5%) and Yeast extract (0.3%). Then it is transferred to 250 ml flask and after a period of 4 days it is placed in orbital rotator shaker and subjected to rotation at 150 rpm. From seed culture a portion of 2 ml is used for inoculation of 150 ml medium containing strain for a period of 2 days (Table 2).

Table 2: Antibiotic productivity by UV mutant strains

S.No	Microbial Source	Colonies (Before UVmutation)	Time of exposure to UV (sec)	Colonies (After UV mutation)	Mutants produced by antibiotic compound
1	A 5 ml spore suspension is plated on each agar plate containing Actinomycetes	70	0	--	--
2		75	5	60	4
3		80	10	55	2
4		85	15	52	5
5		95	30	30	4
6		100	60	34	5
7		75	90	42	7
8		70	120	35	2
9		84	150	21	8

Drug Resistance

Drug resistance is determined by inoculation procedure involving 2 ml portion from seed culture onto a medium comprised of NaCl (005%), $CaCO_3$ (0.35%), lactose (5%) and yeast extract (0.3%). Then it is transferred to 250 ml flask and after a period of 4 days it is placed in orbital rotator shaker and subjected to rotation at 150 rpm and finally solvent extraction procedure using ethyl acetate is conducted.

RESULTS AND DISCUSSIONS

Characterization of Isolates

All the six strains namely S-1, S-2, S-3, S-4, S-5 and S-6 showed positive results in starch hydrolysis and nitrate reduction tests shown in Table 3. Strain S-1, S-4 and S-6 showed positive results in pigment production test in Waksman medium (Brown pigment, light in colour) and S-2, S-3, S-5 showed negative results (limited to growth) in melanoid formation test but they produced good growth in above said test medium. Totally 6 strains showed positive when tested with gelatine liquefaction and H_2S production test and all these strains produce acid when tested in acid production test. All strains exhibited proteolytic property except strains S-5 and S-6. Out of all strains S-4 exhibits effective antibiotic productivity and it is considered for further analysis with reference to fermentation media optimization and antibiotic production on large scale basis.

Table 3: Taxonomical characterization of soil isolates

Soil Isolates	Proteolysis	Assimilation of Carbon	Formation of Melanoid	Reduction of Nitrate	Liquefication of Gelatin	Hydrolysis of Starch	Production of acid	Production of Hydrogen sulphide (H_2S)
S-1	Transparent acid reaction	Glucose	Brown pigment, light in color	+	+	+	+	-
S-2	Transparent acid reaction	Glucose	limited to growth	+	+	+	+	-
S-3	Alkaline reaction, not clear	Fructose	Limited to growth	+	+	+	+	+
S-4	Acidic reaction is not clear	Lactose	Brown pigment, light in color	+	+	+	+	+
S-5	-	Lactose	Limited to growth	+	+	+	+	-
S-6	-	Maltose	Brown pigment, light in color	+	+	+	+	-

'+' Positive reaction; **'-'**Negative reaction

Embody of S-4-strain

S-4 strain belongs to genus actinomycetes after careful study of cultural, morphological and taxonomical features. S-4 strain in ISP-5 and ISP-7 medium exhibited filamentous growth and in ISP-6 medium showed pigmentation. Gram staining procedure is conducted to study morphological characters of S-4 strain and it is represented in the table below (Table 4).

Table 4: Morphological and cultural characterization of the strain

S.NO	Medium	S-4
1	Tryptone Yeast extract broth	Formation of pellicle indicates growth
2	ISP-2	White creamy colonies with clear zone is observed
3	ISP-3(Oatmeal agar)	Thick creamy black colored colonies is observed
4	ISP-4(Inorganic Salt-Starch agar)	Thick black and brownish colored colonies were observed and surface was convex and waxy in appearance.
5	ISP-5(Glycerol asparagines agar)	Thin white colored colonies with striated surface are observed and growth of filament is indicated.
6	ISP-6(Peptone yeast extract iron agar base medium)	Black colored pigments which are thin, clear and soluble is observed and indicates no filamentous growth.
7	ISP-7(Tyrosine agar base medium)	Cream, convex and lobe shaped mycelium is observed
8	ISP-9(Carbon consumption agar medium)	Thin golden yellow colored colonies is observed which indicates very less growth of mycelium

Improvement of A-4 Strain by UV Mutation

The strains which survived were tested for antibiotic production and were exposed to UV radiation for 2 minutes and showed improvement of strain. The genetic modification is considered for better production of antibiotic in larger quantities. The auxotrophs were chosen based on each strain resulted in amount of antibiotic compound produced. The highest antibiotic potency observed from the Table 5 shows that 3.5 mg/L in AM8 mutant at 180 sec for UV exposing, whereas lowest antibiotic potency observed from the Table 5 shows that 1.5 mg/L in AM2 mutant at 5 sec for UV exposing.

Table 5: Comparison of UV mutant strains for the antibiotic production

S.No	Mutants (Auxotrophic type)		Time of exposure to UV (sec)	Potency (mg/L)	Mutant selected (Auxotrophic type)
1	2	AM1	5	1.5	AM2
		AM2		1.9	
2	2	AM1.1	10	1.7	AM2.2
		AM1.2		1.9	
3	4	AM1.1.1	15	1.4	AM1.1.4
		AM1.1.2		1.3	
		AM1.1.3		1.2	
		AM1.1.4		1.8	
4	1	AM2.1	30	2	AM2.1
5	3	AM3	60	1.8	AM4
		AM4		2.2	
		AM5		1.6	
6	2	AM3.1	90	2.2	AM3.2
		AM3.2		2.3	
7	1	AM4.1	120	2.6	AM4.1
8	2	AM5.1	150	2.6	AM5.1
		AM5.2		2.5	
9	3	AM6	180	2.8	AM8
		AM7		3.1	
		AM8		3.5	

Drug Resistance

Auxotrophic strains selected and screened for drug resistance studies include AM2, AM2.2, A M1.1.4, AM2.1, AM4, AM3.2, AM4.1 and AM5.1. The differentiation based on physiological characteristics in microbes is observed when cells fight against adverse environmental conditions and for drug resistance studies. It showed that concentration of antibiotics up to 2-8 mg/ml (i.e. Streptomycin and Rifampcin) is enough to produce mutants that exhibit resistance and it is spontaneous. The antibiotic production is further increased in comparison with UV mutant strains in which is nearly 2 to 7 fold higher and UV mutants that are studied for drug resistance are listed below in Table 6. AM2 strain produced maximum antibiotic property which is 4.9 mg/ml and it is then designated as S-4 mutant strain. S-4 mutant strain is used for the further fermentation studies.

Table 6: Effect of antibiotic compounds and their productivity

S.NO	Strain	Streptomycin concentration used for streptomycin mutants(g/ml)	Rifampcin concentration used for selection of Rifampcin mutants(g/ml)	Frequency(Percentage of mutants producing antibiotic in increased concentrations)	Detection of highest productivity(mg/ml)
1	AM2	4	4	36(36/100)	3
2	AM2	6	6	28(28/100)	4.9
3	AM2.2	4	4	2(2/100)	2.6
4	AM2.2	6	6	31(31/100)	1.5
5	AM1.1.1	4	4	7(7/100)	2.7
6	AM1.1.1	6	6	10(10/100)	3.2
7	AM2.1	4	4	6(6/100)	3
8	AM2.1	6	6	8(8/100)	3.1
9	AM4	4	4	40(40/100)	3.5

CONCLUSION

Actinomycetes species are used widely for production of antibiotics. By using crowded plate method actinomycetes strains were isolated from soil and from this technique totally 6 strains were isolated and are tested for antibiotic production. A total of 3 fungal, 5 bacteria and one strain were selected and all these strains exhibited antimicrobial property were used for further bioprocess studies. S-4 strain is selected after studying taxonomical features on ISP medium and it is designated as actinomycete. S-4 strain was exposed to UV irradiation and drug resistant process to get mutated. Antibiotic compounds Rifampcin and Streptomycin were used for drug resistant process. Results indicated that evolution rate of mutant strains is very less in comparison with drug resistance studies. Mutants were selected by random screening procedure and were tested to know whether exhibit antibiotic production property. Out of all mutants screened, one mutant strain showed ability for highest antibiotic production and it is named as S-4 mutant strain.

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