Screening of Antibiotic Producing Actinomycetes from the Sediments of Undisturbed Forest Areas and Its Hyper Activity after Mutation

P.Ashok Kumar and P.Karpagam

***Abstract*—** Wide and uncontrolled usage of antibiotics has made the pathogens to become resistant to currently used antibiotics. There is an urgent need for development of a new drug or a highly active molecule for controlling antibiotic resistant strains. In this study 32 strains of Actinomycetes were isolated and subjected to primary screening by giant colony method against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aeromonas hydrophilia*. The secondary screening was carried out by fermentation process. Antibacterial activity was evaluated by well plate method. The extract of isolates was subjected to well plate method against pathogenic bacteria. *B. subtilis* and *E. coli* were highly inhibited by AK1 isolate. Other isolates showed limited inhibition of bacteria. The AK1 isolate was mutated by UV irradiation. The mutants differed from the wild parent in reduced growth rates, changes in the shape and size of the colony, sporulation level, antibiotic activity and variation in the color of the mycelium. The zone of inhibition was higher with Actinomycetes AK1 inoculated plates. The secondary metabolite production was enhanced by UV mutagenesis when compared to wild type. Actinomycetes may produce different molecule that can inhibit different types of pathogens, however efforts like strain development can be done to produce new bioactive components against multidrug resistant bacteria.

***Index Terms*—** *Aeromonas hydrophilia*, Antibiotic producing Actinomycetes, Multi drug resistant bacteria, *Pseudomonas aeruginosa*.

I. Introduction

The history of the discovery of new drugs shows it comes from natural sources (Bevan *et al*., 1995). Agro industrial residues are composed of complex polysaccharides that support the microbial growth for the production of useful products (enzymes, organic acids, drugs, etc.) (Javed *et al*., 2013). The antimicrobial resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat the pathogens (Freire-Moran *et al*. 2011). The development of resistance to multiple drugs is a major problem in the treatment of infections by pathogenic microorganisms (Boucher *et al*., 2009). New strain development for antibiotic production has been an essential prerequisite for scale up of antibiotic production and also for search of new antibiotics (physical and chemical mutagenesis). In general, strain improvement is considered as one of the main factors involved in the achievement of higher titers of industrial metabolites (Hu and Ochi. 2001). Random mutagenesis and fermentation screening have been reported as an effective way to improve the productivity of industrial microbial cultures (Medema *et al*., 2011). Antibiotics, because of their industrial importance, are the best known products of Actinomycetes (Nanjwade *et al*., 2010). Improvement of microbial strains for over production of industrial product has been the hallmark of all commercial fermentation processes.

The prevalence of bacterial pathogens resistant to the available antibiotics has been increasing over the past several decades (Kumar *et al*., 2012). To prevent exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, periodic replacement for existing antibiotics is necessary (Ilic *et al*., 2007). Actinomycetes have been and remain the most fruitful source of microorganisms for all types of bioactive metabolites, including agro active type. Over one thousand secondary metabolites from Actinomycetes were discovered during 1988-1992 (Doumbou *et al., 2001*). Secondary metabolites are primarily aimed at discovering novel secondary metabolites, which can counteract resistance from pathogens and circumvent side effects that are typically associated with most indispensable therapeutic drugs (Bapela *et al*., 2008). About 70% of all known drugs have been isolated from Actinomycetes bacteria of which 75% and 65% are used in medicine and agriculture, respectively (Tanaka and Omura, 1993). Industrial development practice includes natural selection, mutation and protoplast fusion (Smith, 1986). The UV radiation is the most convenient of all mutagens to use, and it is also very easy to take effective safety precautions against it. It gives a high proposition of pyrimidine dimmers and includes all types of base pair substitutions (Rani *et al*., 2012). This work, describes the isolation of Actinomycetes strains having antimicrobial activities from the soil samples of Asella town, Ethiopia and the mutational analysis using UV treatment, to enhance the production of secondary metabolites from screened Actinomycetes.

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II. Materials and Methods

A. Isolation of Actinomycetes

In this investigation, samples were collected from the superficial layer of soil at a depth not exceeding 3–5 cm with a plastic spoon in sterile polyethylene bags, from the undisturbed forest area of Asella town, Ethiopia. Actinomycetes were isolated by using Starch Casein Nitrate (SCN) agar. Cyclohexamide and nystatin (50µg/ml) were added into the medium as antifungal agent (Porter *et al*., 1960; Phillips and Hanel, 1950). The plates were incubated at 28^{o}C for 5-10 days. Plates were checked for the growth of typical Actinomycetes colonies up to 10 days. Individual colonies with characteristics of Actinomycetes morphology were identified based on the methods described by Williams and Cross (1971). Isolated colonies were pure cultured by repeated streaking on SCN agar plates and the pure strains were stored at 4°C in SCN agar slants. The potent Actinomycetes were characterized by morphological and biochemical methods (Gogoi *et al.*, 2005).

B. Giant Colony Technique for Screening of Actinomycetes for Antimicrobial Activity

All colonies of Actinomycetes isolates were streaked in a narrow band across the centers of Muller Hinton agar plates (Lazzarini *et al*., 2002). The plates were incubated at room temperature for 7-10 days. The test organisms were streaked from the edges of the plate to the Giant colony but not touching the colony. Again the Muller Hinton Agar plates were incubated at room temperature for 2 to 3 days. After incubation the length of growth line was measured in millimeter from the edge of the Giant colony to the tip of the growth of the test organism. The highly active isolates were inoculated into the Muller Hinton broth with 0.02 % Tween 80 and 1% Glycerol and incubated at room temperature for 48 hours.

C. Antibiotic production

Shake flask fermentation was carried out with 250 ml of Antibiotic production medium (soluble starch 25 g, glucose 10g, yeast extract 2 g, $CaCO_3$ 3 g, Trace salts solution 1ml, distilled water 1 liter, pH 7.5±0.2 [Trace salt solution – $FeSO_4.7H_2O$ – 0.5 g; $CuSO_4.5H_2O$ – 0.5 g; $ZnSO_4.7H_2O$ – 0.5 g; $MnCl_2.4H_2O$ – 0.5 g in 100 ml of distilled water]. The flasks were shaken at 28^{o}C and 160 rpm for 5-7 days on the rotator shaker and finally the filtrate was used to test the antimicrobial activity.

Solvent extraction was performed with selective Actinomycetes spent medium using Ethyl acetate as solvent. The spent medium and the solvents were thoroughly mixed. The upper solvent layer was concentrated *in vacuo* to get extracted substances (Gogoi *et al.*, 2005). The antimicrobial activity was determined by agar well plate method (Thakur *et al*., 2007). Antibacterial activities were tested against different bacteria like *Aeromonas hydrophilia, Bacillus subtilis, E. coli*, and *Pseudomonas aureginosa*.

D. Mutational Analysis

Well sporulated agar plate was selected for collecting spores. Required amount of sterile water with wetting agent like Tween 80 was added to plate. The surface of growth was gently scraped with an inoculating loop to liberate spores in the water. The suspension was poured into a sterile centrifuge tube and vigorously mixed for one to two min. The suspension was filtered through nonabsorbent cotton wool using a filter tube, to remove mycelia and agar pieces. The spore suspension was centrifuged at low speed about 5000 rpm for 20 min. The supernatant was discarded immediately after the centrifugation. This will prevent the detachment of pellet from the wall of tubes. The pellet was suspended with 1ml of sterile water.

E. UV Irradiation - Mutagenesis

The spores of highly active isolate were collected and stored in distilled water. The spores in 1 ml of water were counted by viable plate counting method. Ultraviolet (UV) mutagenesis was carried out at a distance of 30 cm for 20, 40, 60, 80, 100, 120,140 and 160 seconds (Zhang *et al*., 1993). Nitrosoguanidine (NTG) mutagenesis was performed according to Holmalahti *et al*., (1993) for 0, 15, 30, 45, 60, 75 and 90 seconds. In all mutagenesis techniques, photo-reactivation was avoided by keeping the isolated colonies overnight in the dark by wrapping the plates in aluminum foil (Held and Kutzner, 1991) and a survival rate of not more than 1% was considered. The mutated colonies were identified by the physical features of the colonies. The mutant colonies were sub-cultured (7 days, 28˚C) again to express and stabilize the mutants. The mutant colonies were designated as AK 1 to 5.

F. Bioassay of UV Mutated Strains of AK1 isolate

After incubation, colonies with different morphology were selected for analysis. Selected mutants survivors are patched over the surface of the antibiotic production medium and incubated for 7-9 days at room temperature in dark. To test for the antibacterial activity, *Staphylococcus aureus* ATCC 25923 was used as a test organism. Mueller-Hinton agar medium was used as an assay medium. The agar medium at 45ºC was mixed with 0.1 ml bacterial suspension containing approximately10^5 cfu/ml. The mixture was poured into 9 cm Petri dish and allowed to solidify. Sterile paper discs (6 mm) were placed on the dried surface of the medium. Each disc received 20 µl of the culture filtrate. Petri dishes were incubated at 37ºC for 18 hours. The inhibition zone, if any, was measured in mm diameter (Amade *et al*., 1994).

III. Results

32 strains of Actinomycetes were isolated and subjected to primary screening by giant colony method against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Aeromonas hydrophilia*. The antibacterial activity was evaluated by well plate method. The zone of inhibition was higher with Actinomycetes AK1 inoculated plates. In the case of *Aeromonas hydrophilia,* all the isolates (spent medium and

mycelial extract) showed limited inhibition. *Bacillus subtilis* and *E. coli* were highly inhibited by AK1 isolate. Other bacteria were inhibited by both spent and mycelial extract. In some cases, mycelial extract showed no activity against bacteria (Table I).

TABLE I
SCREENING OF ANTIBACTERIAL ACTIVITIES BY WELL PLATE METHOD (MM)
(S - SALINE: EA - ETHYL ACETATE)

Organisms	AK1		AK2		AK3		AK4		AK5	
	EA	S	EA	S	EA	S	EA	S	EA	S
A.hydrophilia	28	21	-	-	9	-	13	-	-	-
B.subtilis	24	18	11	23	15	-	11	-	14	-
E.coli	30	25	11	13	19	10	10	-	14	18
P.aeruginosa	15	-	10	-	14	20	10	-	11	-
S.aureus	19	20	11	-	11	-	11	-	11	-

A. Mutagenesis of AK1 isolate- UV Mutagenesis:

The AK1 isolate was mutated by UV irradiation. The rate of mutation was parallel with the increasing exposure and was followed later by a decline. The mutants differed from the wild parent in reduced growth rates, changes in the shape and size of the colony, sporulation level, antibiotic activity and variation in the colour of the mycelium. The survival count of AK1 UV mutation was tabulated (Table. II).

TABLE II
SURVIVAL COUNT OF AK1 ISOLATE AFTER UV IRRADIATION

Time duration (min)	**CFC/ml**
0	1.86×10^7
20	1.056×10^5
40	7.26×10^4
60	6.43×10^4
80	5.78×10^4
100	4.50×10^4
120	2.22×10^4
140	9.60×10^3
160	7.4×10^3

B. Bioassay UV Mutated Strains of AK1 isolate:

The UV mutated AK1 colonies were subcultured and bioassayed by agar gel disc diffusion method against *S.aureus*. The antimicrobial activity of mutated strain patch numbered as 24 and 23 showed higher activities and number 69 showed lower activity (Fig 1).

Fig 1. Antibacterial Activity Of Ak1-UV Mutated Strain (1 - Wild Type; Others Were UV Mutated Strains).

The bioassay of AK1 mutated strains were tabulated (Table III).

TABLE III: SECONDARY METABOLITE PRODUCTION INFLUENCED BY UV MUTAGENESIS

Time duration (seconds)	Patch numbers	Inhibition zone (mm)
0	1	19
60	45	19
	69	15
80	8	18
	11	20
100	20	19
	21	20
120	24	24
	23	24
	37	20
140	38	21
	30	23
	60	19
160	55	20
	54	22

C. NTG Mutagenesis:

The AK1 isolate was also mutated by NTG, a chemical mutagen. The rate of mutation was parallel with the increasing dosage and later by a decline. The survival curve showed decreased phase. The mutants differed from the wild parent in reduced growth rates, changes in the shape and size of the colony, sporulation level, antibiotic activity and variation in the colour of the mycelium. The survival ratio of AK1 NTG mutation was tabulated (Table. IV).

TABLE IV: SURVIVAL COUNT OF AK1 ISOLATE AFTER NTG TREATMENT

Time duration (min)	**CFC/ml**
0	1.86×10^7
15	4.30×10^6
30	3.56×10^6
45	2.76×10^6
60	1.27×10^6
75	6.0×10^5
90	2.6×10^5

D. NTG Mutagenesis Bioassay:

The NTG mutated AK1 colonies were subcultured and bioassayed by agar gel disc diffusion method against *S. aureus*. The antimicrobial activity of mutated strains resulted in no variations in their wild type activity. But the strain patch numbered as 24 showed lower activity (Fig 2).

Fig 2. Antibacterial Activity Of Ak1-Ntg Mutated Strain (1 - Wild Type; Others Were Ntg Mutated Strains)

The bioassay of AK1 mutated strains were tabulated (Table V).

TABLE V: SECONDARY METABOLITE PRODUCTION INFLUENCED BY NTG MUTAGENESIS

Time Duration (min)	Patch Numbers	Inhibition Zones (mm)
0	1	19
	81	19
15	82	19
	84	20
	68	21
30	69	20
	78	19
	54	20
45	59	19
	61	17
	64	18
	32	20
60	37	20
	50	20
	23	21
75	24	15
	25	20
	12	20
90	14	20
	16	21

IV. DISCUSSION

In Actinomycetes, a close relationship between secondary metabolism and cell differentiation has been established and exogenous metabolites can positively regulate the production of antibiotics (Gohar and El-Naggar, 2001). The present study explored the utility of Actinomycetes in the war against microbial infection. The pharmaceutical industries were interested in the true source of secondary metabolites from potent natural sources like Actinomycetes. Shikha *et al*., (2007), reported 1.44 fold alkaline protease productions by *Bacillus panththenticus*. However, Dutta and Bannerjee, (2006) observed a 2.5 fold increase in alkaline protease productivity by UV mutant *Pseudomonas* sp. JNGR 242.

Mutation and screening of industrially useful microorganisms are very important for the successful development of various strains which are required in the fermentation industry (Dutta and Bannerjee, 2006). Similar trend of decrease in survivability with increase in exposure time has also been reported by some other investigators (Shikha *et al*., (2007), Nadeem M, *et al*., (2010), Mukhtar and Haq, 2008). All these findings indicated that the survivability of parent strain depended on the nature of the microorganism, treatment period and the type of mutagens (Javed *et al*., 2013).

This study demonstrated that the Actinomycetes has potential microbial demulsify and could be the ever remedy for infectious diseases. As stated earlier, Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances (Nanjwade *et al*., 2010). The phenotypic relatedness could be also used to eliminate duplicate actinomycete strains in the future by a microbial screening programme. Further investigations should definitively answer the question of how secondary metabolism and aerial mycelium development are regulated in this strain (Gohar and El-Naggar, 2001).

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