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Strain Improvement of Fresh Water Actinomycetes for Increased Antibacterial Metabolite Production

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A total of twenty four actinomycetes strains were isolated from three fresh water systems of Karimnagar, Andhra Pradesh and their antibacterial activities were tested against ten test bacteria namely *Bacillus subtilis* (MTCC 441), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424), *Enterobacter aerogenes* (MTCC 111), *Salmonella typhi* (MTCC 733), *Escherichia coli* (MTCC 40), *Sarcina lutea* (MTCC 1541), *Shigella flexneri* (MTCC 1457) *and Klebsiella pneumonia* (MTCC 7162). Among, eight strains showed good antibacterial activity. Two of these eight isolates (LAM1 and LAM2) were found to produce wide spectrum of antibacterial substances which were selected for mutation study using physical (U.V radiation and X-rays) and chemical (EtBr and NTG) mutagens. After the mutations, antibacterial activity of the strains, LAM1, LAM2 (U.V treated), LAM1 (X-ray treated) and LAM1, LAM2 (EtBr and NTG treated) was increased but the LAM2 (X-ray treated) strain showed decreased antibacterial activity.

Key words: Actinomycetes, Fresh water, Bacteria, Mutation.

Actinomycetes are the group of Gram positive filamentous bacteria which are widely distributed in different terrestrial and aquatic habitats[1-2]. They are a group of morphologically diverse, Gram positive bacteria having in common DNA with high GC content in the range of 63-78%[3-6]. Diversity and bioprospecting studies on actinomycetes are mainly pertaining to terrestrial and marine ecosystems and less importantly from fresh water systems[7]. There is an increasing realization of the potential for fresh water systems as sources of actinomycetes that produce useful bioactive compounds. A critical survey was made on occurrence, growth and role of actinomycetes in aquatic habitats[8]. Actinomycetes of fresh water origin produce novel and useful bioactive metabolites[9]. The risk of antibiotic resistant pathogenic strains dictates an increasing need for the survey of unexplored and underexplored habitats for novel antibiotic producing actinobacterial strains[10-11]. The focus is increasing towards fresh water systems for novel bioactive strains especially actinobacteria[12].

The ability of actinobacterial cultures to form antibacterial compounds is not a fixed property[13-14] but can be enhanced or minimized by using different techniques. Actinomycetes show a moderate antibacterial activity. These strains were mutated by using standard physical (UV radiation and X-Rays) and chemical (EtBr and NTG) mutations.

Strain improvement is a vital part of process development in fermentation for the production of antibiotics on commercial scale. The major motivation for industrial strain development is economic, since the metabolite concentration produced by wild strains is usually too low for the economical processes[15]. Yield increase can usually be achieved through strain development program[16].

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Baltz[17] suggested that the mutagenesis is effective method of strain improvement. A variety of chemical and physical mutagens like ethane methane sulphonate (EMS), nitrous acid, ethidium bromide (EtBr), N-methyl-N-nitro-N-Nitrasoguanidine (MNNG or NTG), ultraviolet, gamma rays and X-rays are commonly used for antibiotic yield improvement in *Streptomyces*[18]. These mutagens induce modification of the base sequence of DNA that results in base pair substitutions, frame shift mutations, or large deletions that go unrepaired[19]. The derivatives or mutants obtained are then subjected to screening and selection to get the strains whose characteristics are more specifically suited to the industrial fermentation process[20].

The present study was undertaken to isolate antibacterial actinomycetes from water and sediment samples collected from three fresh water systems of Karimnagar, Andhra Pradesh, India and checked their antibacterial metabolite production efficiency by mutations.

MATERIAL AND METHODS

A total of 144 water and sediment samples were collected from three freshwater systems of Karimnagar, Andhra Pradesh, India viz; Lower Manair Dam, Manakondur Pond and Kothapally Pond with monthly interval during July 2006 to June 2008. Water samples were collected in a sterile one liter conical flask and brought to the laboratory by closing with sterile cotton plug. Sediment samples were collected in a sterile petri dish by using sterile spatula.

Actinomycetes from these collected samples were isolated by Double Agar Layer (DAL) method on actinomycetes isolation agar containing cycloheximide (50µg/ml) to minimize fungal contamination[21] .All plates were incubated at 28°C for 1-2 weeks. The actinomycetes colonies that appeared on petri plates were counted from 5th day onwards up to 14th day.

Screening of actinomycetes isolates for antibacterial activity

A total of twenty four different actinomycetes were isolated viz; eleven from Lower Manair Dam (LAM1 to LAM11) seven from Manakondur Pond (MAM1 to MAM7) and six from Kothapally Pond (KAM1 to KAM6). All isolates were, sub cultured and maintained on agar slants. The isolated actinomycetes strains were tested for their antibacterial activity against ten test bacteria namely *Bacillus subtilis* (MTCC 441), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424), *Enterobacter aerogenes* (MTCC 111), *Salmonella typhi* (MTCC 733), *Escherichia coli (*MTCC 40), *Sarcina lutea* (MTCC 1541), *Shigella flexneri* (MTCC 1457) *and Klebsiella pneumonia* (MTCC 7162). Among, eight isolates showed good antagonistic activity against test bacteria. Two of these eight isolates (LAM1 and LAM2) were showed very potent antagonistic activities which were selected and identified. The characterization of LAM1 and LAM2 was done by following the guide lines adopted by the International *Streptomyces* Project[22]. Colors were assessed on the scale adopted by Kornerup and Wanscher[23].

Effect of mutation on antibacterial activity:

The strains (LAM1 and LAM2) which showed efficient antibacterial activity were further selected to study the effect of mutations on their antibiotic production.

U.V treatment

The selected strains (LAM1 and LAM2) were cultured in the tubes containing 9 ml of starch nitrate broth. The tubes were inoculated with one loop full of the strain and incubated on rotary shaker at 30°C for 96 hrs. After incubation the tubes were removed from the shaker and 3 ml of each culture was exposed to U.V irradiation at a distance of 30 cm for different time periods (60 sec to 360 sec). One ml of exposed culture was transferred to 9 ml of starch nitrate broth and tubes were incubated in dark for 96 hrs on a shaker at 30°C. After incubation the tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 min and the supernatant was used to examine post mutation effect on the strains for antibacterial activity.

X-ray treatment

Culture suspensions of LAM1 and LAM2 were X-rayed at radiology department of OSVEN Super Specialty Diagnostic centre, Karimnagar. The X-rays were produced at 180 Kv. The rays were unfiltered, and had a H.V.L of 0.19 mm Cu. The intensity as measured in air was 2,000 roentgens per minute. The selected strains (LAM1 and LAM2) were cultured in the tubes containing 9 ml

of starch nitrate broth. The tubes were inoculated with one loop full of the strain and incubated on rotary shaker at 30°C for 96 hrs. After incubation the tubes were removed from the shaker and 3 ml of each culture was exposed to X-rays. One ml of exposed culture was transferred to 9 ml of starch nitrate broth and tubes were incubated in dark for 96 hrs on a shaker at 30°C. After incubation the tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 min and the supernatant was used to examine post mutation effect on the strains for antibacterial activity.

Ethidium bromide (EtBr) and N-methyl-N'- nitro-N-Nitrasoguanidine (*NTG*) treatment

The selected strains (LAM1 and LAM2) were cultured in the tubes containing 9ml of starch nitrate broth. The tubes were inoculated with one loopful growth of each strain and incubated in a shaker at 30°C for 96 hrs. The culture broth was centrifuged at 3000 rpm for 10 min and the pellets were collected. The pellets were suspended with 2 ml of Tris buffer (pH 7.2) in the test tubes with different concentrations of ethidium bromide and NTG (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml). The tubes were incubated at 30°C for 30 minutes. After incubation, 1 ml of EtBr and NTG treated culture transferred in to 9 ml of starch nitrate broth and the tubes with culture were incubated for 96 hrs on shaker at 30°C. The tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 min. the supernatant was used to examine the post mutational effect on the strains for antibacterial activity.

RESULTS

The isolated actinomycetes were screened to generate bioactive compounds. The most potent strains, LAM1, LAM2 were selected and identified. The characterization LAM1 and LAM2 was done by following the guide lines adopted by the International *Streptomyces* Project[22]. Two of twenty four actinomycetes cultures LAM1, LAM2 were found to exhibit various degrees of activities against Gram-positive and Gram-negative bacteria [Table 1].

The most potent antagonistic actinomycetes strains were selected to irradiate with U.V, X-Rays and treated with Ethidium bromide and NTG for strain improvement.

Effect of mutation on antibacterial activity

The strains, LAM1 and LAM2 showed good antibacterial activity against tested bacteria were treated with physical and chemical mutagens to study the effect of mutation on their antibacterial activity. Mutated strains were checked for antibacterial activity against two test bacteria namely *Bacillus subtils* (MTCC 431) and *Escherichia coli (*MTCC 40).

Strains exposed to Ultra violet radiation showed variation in antibacterial activity against two test bacteria namely *Bacillus subtilis* (MTCC 441), *Escherichia coli (*MTCC 40). As compared to wild strain, U.V mutated strain LAM1 showed an increase in the inhibition zone against *Bacillus subtilis* (+3mm) and *Escherichia coli* (+2mm) at an exposure time of 180 Sec and LAM2 showed increased zone of inhibition against *Bacillus subtilis* (+2mm) and *Escherichia coli* (+2mm) at an exposure time of 240 Sec (Fig.1)

The LAM1 and LAM2 strains are subjected to antibacterial activity testing after exposure to X-rays. The strains LAM1 showed increased antibacterial activity against test bacteria after exposure to X-rays and there is a decrease in antibacterial activity in X-ray treated LAM2 (Fig. 2).

Table 1. Antibacterial activity of LAM1 and LAM2 strains

Isolates	Test Organisms (inhibition zone in mm)									
	B.s.	S.a.	S.l	E.c.	K.p.	P.v.	P.a.	S.t.	S.f	E.a
LAM 1	20	18	17	12	13	15	15	17	9	14
LAM 2	15	12	13	12	11	7	13	5	8	7

B.s - *Bacillus subtilis*
P.a.-*Pseudomonas aeruginosa*
E.c- *Escherichia coli*
E.a- *Enterobacter aerogenes*
P.v-*Proteus vulgaris*
S.l- *Sarcina lutea*
S.f- *Shigella flexneri*
S.a- *Staphylococcus aureus*
S.t- *Salmonella typhi*
K.p- *Klebsiella pneumonia*

Table 2. Antibacterial activity of EtBr treated LAM1 and LAM2

S. No.	Concentration of NTG in µg/ml	Test organisms (Zone of inhibition in mm)			
		Bacillus subtilis		*Escherichia coli*	
		LAM1	LAM2	LAM1	LAM2
1	0	20	15	12	12
2	20	20	15	11	12
3	40	19	17	12	14
4	60	22	14	14	10
5	80	20	16	11	14

Table 3. Antibacterial activity of NTG treated LAM1 and LAM2

S. No.	Concentration of NTG in µg/ml	Test organisms (Zone of inhibition in mm)			
		Bacillus subtilis		*Escherichia coli*	
		LAM1	LAM2	LAM1	LAM2
1	0	20	15	12	12
2	20	20	15	11	12
3	40	19	16	12	11
4	60	18	14	12	10
5	80	23	18	13	13

The chemical mutated strains of LAM1 showed variation in zone of inhibitions against two test bacteria. As compared to wild strain, the EtBr mutated strain of LAM1 showed an increase in inhibition zone against *Bacillus subtilis* (+2mm) and *Escherichia coli* (+2mm) at a concentration of 60µg/ml and [Table 2] EtBr treated LAM2 showed increased antibacterial activity at a concentration of 40 µg/ml.

The NTG treated strain of LAM1 showed improved antibacterial activity against test bacteria at 80 µg /ml concentration of mutagen [Table 3] and LAM2 showed increased antibacterial activity testing at NTG concentration of 80 µg/ml.

The improved antibacterial activity of physical and chemical induced mutants of LAM1 and LAM2 may be due to the mutation in partial activity genes of these strains which are responsible for the production of antibacterial metabolite.

DISCUSSION

Actinomycetes constitute a diverse group of microorganisms that are widely distributed in terrestrial, fresh water and marine environments[24]. They play vital role in decomposition of organic matter and thereby replenish the supply of nutrients

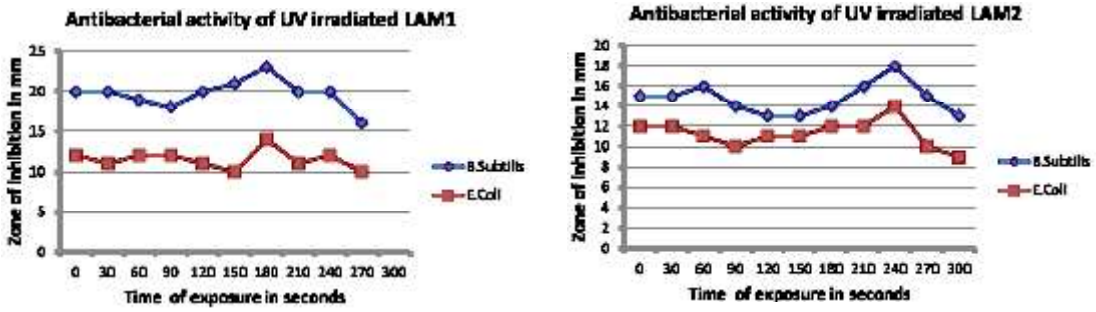

Fig.1. Effect of U V irradiation on antibacterial activity of LAM1 and LAM2

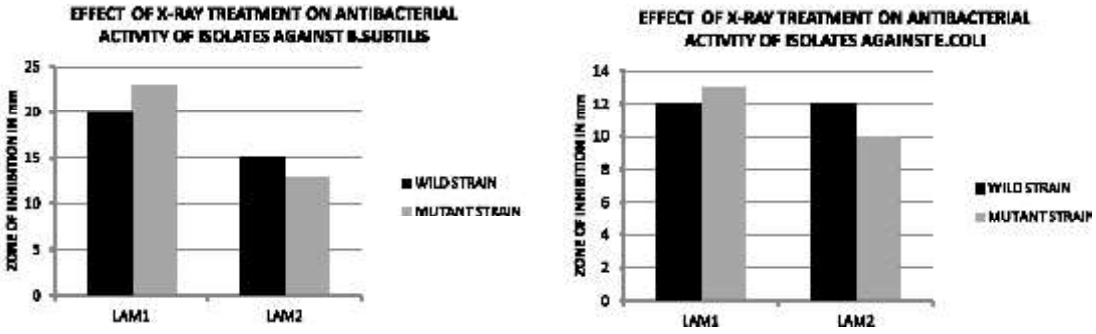

Fig. 2. Effect of X-ray treatment on antibacterial activity of LAM1 and LAM2

in fresh water systems[25]. Several members of actinomycetes from different soil and water samples are a virtually unlimited source of natural secondary metabolites many kinds of which are used as pharmaceutical and agrochemical products[26-28] and they have a wide variety of chemical structures, including tetracyclines, macrollides, quinocyclines and meroparamycins. These antibiotics showed antagonistic activity against both Gram-positive and Gram-negative bacteria[29-32]. Natural strains of actinomycetes showed moderate antibacterial activity. Mutagenesis has been a very important strategy for strain improvement; it has been extensively applied to enhance the product yield[33-34]. The results presented here show the effect of commonly used chemical and physical mutagens on the antimicrobial activity of wild strains of fresh water actinomycetes. The mutagenic treatments of actinomycetes with chemical and physical mutagens exhibited significant variation in antibacterial activity of the mutants against Gram positive and Gram negative bacteria. Lee and Rho[35] reported that the yield of tylosin was enhanced 14 fold by treating the actinomycetes by U.V. and in the present work similar enhanced results were shown. The enhancement of antibacterial activity was observed in U.V treated LAM1 and LAM2 at an exposure time of 180 seconds and 240 seconds respectively. The most important products of UV action are dimmers, thymine-thymine, thymine-cytocine, cytocine-cytocine dimmers, formed between the pyrimidines of complimentary strands and results in cross linking. U.V radiation also induces transitions of GC-AT, transversions, frameshift mutations and deletions[36]. Among the ionizing radiations, X-rays and gamma rays has been effectively used for obtaining mutants with significant increase in antibiotic production[37-38] . From the results, it is evident that X-rays are very good mutagens for enhancement of antibacterial metabolite production. Shaw and Piwowarski[39] suggested that Ethedium bromide treatment enhances antibiotic production in actinomycetes. NTG also proved to be the most potent mutagen when used under appropriate conditions[19]. Significant variation in the antibacterial activity of LAM1 and LAM2 were observed in all the treatments with two chemical mutagenic agents (EtBr and NTG). In treatments with different concentrations of EtBr (20µg/ml, 40µg/ml, 60 µg/ml, 80 µg/ml, 100 µg/ml), altered antibacterial activity was observed in both the strains. LAM1 showed increased antibacterial activity at a concentration of 60 µg/ml EtBr and 80 µg/ ml NTG. LAM2 showed enhanced antibacterial activity at 40 µg/ml of EtBr and 80 µg/ml of NTG. The results of present research depicts that the mutagenic agents like U.V at 254 nm and X-rays have almost similar impact on the activity of actinomycetes. In all the cases it is still needed to find out the genetic variations in the selected mutants as compared to the wild strains. On the whole the present study reveals that the fresh water systems are promising source of inimitable bioactive actinomycetes, which should be continuously isolated, characterized and investigated for their antibacterial activity.

CONCLUSION

From the results of the present work, it can be concluded that the fresh water actinomycetes are potential source of interesting antibacterial metabolites. Their continuous exploration and mutagenesis yield over production

of novel metabolites which can be helpful to combat the increasing problem of antibiotic resistance among the pathogenic bacteria and reduce the cost of the production process. Effectiveness of physical and chemical mutagen treatments in strain improvement for enhanced antimicrobial activity was demonstrated in the present investigation. It is hoped that the high yielding antagonistic mutant strains of actinomycetes can be exploited commercially for large scale industrial production of antibacterial metabolites.

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