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Optimization of Conditions for the Production Antibiotics by a UV Mutant Strain of *Streptomyces griseus*

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Actinobacteria were isolated from a marine sediment using starch casein agar medium. Out of the 10 strains tested, two, the A-3 and A-3 mutant strains were evaluated for maximum antibiotic production; these were was tentatively identified as *Streptomyces griseus*. Several fermentation parameters, such as incubation period, temperature, pH, DO2 and carbon and nitrogen sources were also optimized for highest production of antibiotic from both the A-3 and A-3 mutant strain. The most marked antibiotic production occurred after 72 h. of incubation at 29°C, pH 7, DO2 60%, and in the presence of lactose and peptone as carbon and nitrogen sources, respectively. Data from the present study points to the possibility of using this mutant strain of *Streptomyces griseus* in the large scale production of the antibiotic.

Key words: Actinobacteria, Fermentation process, Antibiotic production, Optimization conditions, *Streptomyces griseus*.

The successful search for microbial metabolites for use as antimicrobial compounds (antibiotics) is critical to combating human, animal and plant diseases. Microorganisms constitute an inexhaustible reservoir of compounds which possess pharmacological, physiological, medical or agricultural applications. Marine actinobacteria appear to be a particularly promising source of such novel bioactive metabolites. These organisms make up about 10% of bacteria which colonize marine aggregates and they can be isolated from marine sediments including those obtained from the deepest part of the Mariana Trench, at depths of 10,898 m. At present, the immense biotechnological promise of actinobacteria has led to exhaustive screening of terrestrial habitats for their presence, and an associated increase in the numbers of known compounds which have been isolated from them. Marine actinobacteria, in contrast, have been less intensively screened for antibiotics and other novel metabolites. Within the actinobacteria, the *Streptomycetes* have the widest distribution and occur in all soil types, while non -*Streptomycete* actinobacteria, which comprise some 100 genera, exhibit great diversity in morphology, phylogeny and ecology and are worthy of further investigation in relation to their ability to produce antibiotics and other metabolites [1].

For more than 50 years, soil-derived actinobacteria of terrestrial origin have provided the main major pharmaceutical resource for the

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discovery of antibiotics and related bioactive compounds; marine actinobacteria, in contrast, have received very recent attention. By the late 1990s, some 40 new bioactive microbial products have been isolated from marine organisms, and nearly half of them have been isolated from actinobacteria[2]. Actinobacteria are, of course, well known for their ability to produce antibiotics and with the exception of a small number of antibiotics produced by bacteria and fungi, all of the widely used, medically antibiotics are synthesized by actinobacteria[3, 4].

MATERIALS AND METHODS

Isolation of actinobacteria

The sediment samples were collected from the sea off Dammam, Kingdom of Saudi Arabia, using a sterile spatula. The samples were transferred to sterile polythene bags and transported immediately to the laboratory, where they were air-dried aseptically for one week. Air-dried sediment samples were then incubated at 55°C for 5 min[5] and then a10-fold serial dilutions was prepared using filtered and sterilized sea water (50%). Duplicate serially diluted samples were next transferred to starch casein agar medium in petri dishes. To minimize bacterial and fungal contamination, all the agar plates were supplemented with 20 mg/l of nystatin and 100 mg/l of cycloheximide[6]. Actinobacteria colonies appearing on the petriplates were counted from 5th day onwards, upto 28th day using a colony counter. All of the colonies growing on the medium were separately picked off and streaked onto actinomycete agar medium; they were then sub-cultured, tested for their axenicity and then maintained on actinomycetes agar medium slants. The isolates were identified using standard methods[7, 8,9,10].

Preliminary screening of crude antibiotic produced (Agar streak method)

A series of culture tubes containing 9 ml of sterile water were taken. From the stock culture, 1 ml of suspension was transferred aseptically to the 1st tube (10^{-1}), and mixed thoroughly. From the 1st tube, a 1 ml of suspension was transferred into the 2nd tube (10^{-2}), and mixed. Similarly, dilutions up to 10^{-5} were made. An aliquot of the suspension (0.1 ml) from each culture tube was then spread on to sterile soybean casein digest medium (SBCD), actinomycetes isolation agar (AIA) medium and starch casein agar medium; the transfers were done plates aseptically in a laminar air flow cabinet. The plates were incubated at 27°C (± 2°C) for 84 hrs and observed intermittently during incubation.

The antibacterial activity of the isolates was analyzed using the 'Agar streak method'. Each isolate was streaked across SBCD medium and incubated at 27°C for about 6 days (144 hours). After 6 days, a variety of test strains of bacteria were streaked at right angle, but not touching to the isolate-streak and incubated at 37°C for 24 h. The isolated actinobacteria were screened against the following test organisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Effect of UV mutation on antibacterial activity

The strains which showed efficient antibacterial activity were next exposed to UV to determine the effect of mutation on their ability to produce antibacterial agents. The selected strains were cultured in test tubes containing 9 ml starch casein broth. The tubes were inoculated with the isolate, using a loop, and incubated in a rotary shaker at 250 rpm at 30°C for 72-96 hours. After incubation, the tubes were removed from the shaker and 3 ml of each culture was exposed to UV-â mutation at a distance of 30 cm for 180 seconds. Then, 1 ml of the treated culture was transferred to 9 ml of glycerol-starch broth and the tubes were incubated for 72-96 hours on a rotary shaker at 250 rpm for 30°C. After incubation, the tubes were removed from the shaker and the broth was centrifuged at 2000 rpm for 20 minutes; the supernatant was then used to examine the post-mutation effect on the strain for antibacterial activity.

Optimization of fermentation process for antibiotic production

Experiments were conducted in Erlenmeyer flasks (250 ml) containing glycerol-glutamine broth. After sterilization of the broth by autoclaving at 121°C for 15 min, the flasks were cooled and the strains were incubated (5 mg/ml) for 7 days (except for incubation period experiment). Fermentation was carried out in triplicate at different temperatures (28°C, 29°C, 30°C, 31°C and 37°C), pH (5, 5.5, 6, 6.5 and 7), DO_2 (20%, 30%, 40%, 50% and 60%), incubation periods (24, 48, 72, 96

and 120 hrs). The carbon sources used were: (1% of glucose, maltose, mannitol, sucrose and lactose), and nitrogen sources were (1% of ammonium chloride, ammonium phosphate, soyabean meal, peptone and yeast extract). The optimum condition identified for one parameter was used to optimize the other parameters one by one.

Growth of isolates was carried out for 4 days, keeping all the optimized fermentation parameters constant. The medium was then centrifuged at 10,000 rpm for 30min. at 4°C to remove the biomass and cell debris and the supernatant was poured off (supernatant 1). The cell pellet of biomass was then triturated with sterile sand (to disrupt the cell walls), washed with tris buffer (pH 7.4) and then filtered and centrifuged. The supernatant which contained all intracellular components was separated out (supernatant 2). Both supernatants (1 and 2) were assayed for antibacterial activity. Supernatant 1 showed antibacterial activity and was purified as follows.

Solvent extraction

Each supernatant (10 ml) was transferred to a separate funnel and extracted with10 ml of the following: petroleum ether, ethyl acetate, n-butanol and methanol. The organic solvent layer was then concentrated by evaporation and was tested for antibacterial activity.

In all cases, only ethyl acetate layer showed antibiotic activity. The remaining growth medium was also extracted with ethyl acetate and the solvent layer was concentrated at vacuum at 37°C ; it was further purified by extracting with various solvents for increasing the polarity i.e., petroleum ether, n-butanol and methanol. The antibacterial activity of each solvent concentrate was then checked; since the methanol extract showed maximum antibacterial activity it was subjected to further purification. The dried active product obtained from the concentration of methanolic fraction was then dissolved in cold methanol, which resulted in two portions, an amorphous powder precipitate and a soluble component. Both portions were again tested for antibacterial activity. Only the amorphous portion showed antibacterial property; the soluble part did not show any activity.

Purification of antibacterial compounds

TLC analysis

Preparative chromatography was performed using silica gel plates (Merck Art. 5735, Kiesselgel 60F 254) for the partial purification of antibacterial fractions. Dried crude extracts of three selected actinobacteria were dissolved separately in methanol, spotted onto the chromatography plate and developed in the solvent system, n-butanol-acetic acid-water (BAW) (3:1:1, v/v). The developed TLC plates were then air dried overnight to remove all traces of solvents. The separated compounds were visualized under UV at 254 nm (absorbance) and at 365 nm (fluorescence) and the active spots were detected by bioautography[11]. The retention factors (RF) of the active spots were also measured.

HPLC analysis

High performance liquid chromatographic (HPLC) was used, for the identification of antimicrobial compounds. A Shimadzu HPLC was used with a UV-VIS Detector and LC-18 column (25 cm 4.6 mm, 5m), the eluent being n-pentane, MeOH and H_2O (1000:5:5). Aliquots of 20 µl of supernatant of the sample extracts were injected separately into the HPLC system with the flow rate of 2.5 ml/min with a back pressure of 250 psi. The compounds were read at 235 nm. The samples were run for 20 min and the retention time (RT) was noted; the RT was compared with the standard and the antibacterial compounds were identified.

RESULTS AND DISCUSSION

Ten actinobacteria were isolated from the sediment samples and checked for their antagonistic activity against potential human pathogens, i.e. *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. With the exception of strain A-3 (which showed an inhibition zone greater than 24 mm against *E.coli*), all of the isolates produced inhibition zones of less than 24 mm when tested against all the tested human pathogens (Table1).

Isolates A-1, A-3, A-6, A-7 and A-9 which showed antibacterial activity against human bacterial pathogens were exposed to UV to determine study the effect of mutation on their antibacterial activity. Mutated isolates were checked for their antibacterial activity against four human bacterial pathogens.

When compared to the non-mutated A-1strain, A-1 showed an increase in the inhibition

Table 1. Antibacterial activity of A-3 and A-3 Mutant strains

S. No.	Test microorganisms	Zone of Inhibition (mm)	
		A-3	A-3 Mutant
	Gram Positive organisms		
1.	*Bacillus subtilis*	16	18
2.	*Staphylococcus aureus*	14	20
	Gram Negative organisms		
1.	*Escherichia coli*	18	26
2.	*Pseudomonas aeruginosa*	18	24

zone against *B. subtilis* (+2mm) and *E.coli* (+2mm), but a decrease of inhibition against *S.aureus* (-1mm) and *P.aeruginosa* (-1mm). Mutated strain A-3 exhibited an increase in the diameter of inhibition zone against *B. subtilis* (+3mm), *S.aureus* (+2mm), *E.coli* (+5mm) and *P.aeruginosa* (+2mm). A-6 showed increased diameter of inhibition zone against *E.coli* (+1mm) and *P.aeruginosa* (+1mm) but a decrease in zone size against *B.subtilis* (-1mm) and *S.aureus* (-1mm). A-7 showed an increase in the inhibition zone against *B. subtilis* (+2mm) and *E.coli* (+3mm) but a decrease in inhibition against *S.aureus* (-2mm) and *P.aeruginosa* (-1mm). A-9 exhibited increased inhibition activity against *E.coli* (+2mm) and *S.aureus* (+3mm) and but a decrease in inhibition against *B. subtilis* (-2mm) and *P.aeruginosa* (-1mm).

Isolates exposed to UV radiation showed a variation in the zone of inhibition produced against all the four tested bacterial pathogens when compared to the non-UV radiation exposed strains. Antibacterial activity of the strain A-3 was increased against all the tested pathogens. The UV-mutated actinobacteria showed increased antibiotics production when compared to non-mutated actinobacteria; similar increased antibiotic production by the fungus *Penicillium chrysogenum* following UV exposure has been reported[12].

In the present study, mutation of strain A-3 enhanced its antibacterial activity against all the tested human bacterial pathogens. Isolates A1, A6, A7 and A9 however, showed decreased antibacterial activity against some of the tested human bacterial pathogens. This can be ascribed to the fact that the mutation of the active gene of this strain, responsible for the production of antibiotics might have been partially inactivated; the removal of *scbA* gene by UV-mutation has for example been shown to enhance the antibiotics production in *Streptomyces lividans*[13].

The mutant which showed maximum antibacterial activity (i.e.A-3) was selected for further studies. The morphological and cultural characteristics of the A-3 mutant were studied using the International Streptomyces Project (ISP) media. The different ISP media used for the morphological study were ISP-1, ISP-3, ISP-4, ISP-5 and ISP-7 and growth characteristics and the presence of aerial mycelium and soluble pigments were observed. Based on all the above characteristics, mutant strain (A-3) was identified as *Streptomyces griseus* (Fig.1).

It is desirable to optimize each and every component of a fermentation media by varying the concentration of media constituents so as to achieve the maximum antibiotic production. The purpose of media optimization is to support the efficient growth of microorganisms. Different combinations of medium constituents and

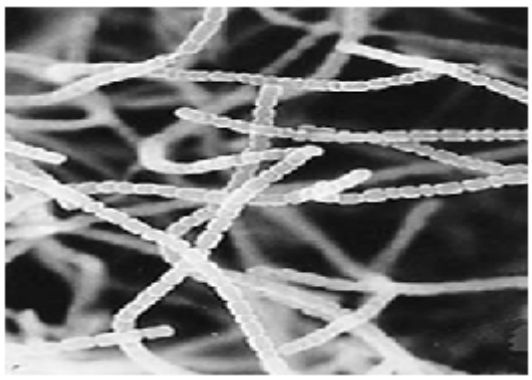

Fig. 1. Spore chains of mutantstrain- A3 Rectiflexibles (RF)

sequences of optimized fermentation conditions need to be investigated in order to determine the optimum growth conditions, which produce biomass with best suited physiological state constituted for antibiotic production.

For the optimization of fermentation process, the basal medium used contained 0.3% yeast extract, 0.35% $CaCO_3$ and 0.5% NaCl for the antibiotic production (as was suggested by a literature survey); this medium was then optimized for antibiotic production by varying a variety of parameters, including the type of carbon and nitrogen source, incubation period, incubation temperature, pH, and DO_2. Antibacterial activity was maximum after 72 h at a temperature of 29°C, a pH of 7, 60% DO_2, and lactose and peptone (1%) as the preferred carbon and nitrogen sources respectively.

The nature of the carbon sources used as well as other medium constituents are known to influence the microbial production of antibiotics and nutrition plays an important role in determining the onset and intensity of secondary metabolism; for example, limiting the supply of an essential nutrient is often an effective means of restricting growth and thereby inducing specific metabolic and regulatory effects[14,15, 16].

The production of antibiotic was studied under optimized parameters and media composition. When the growth of the microorganisms reached to idiophase, the fermented broth was collected, filtered and centrifuged for separation of cell debris, and supernatant was collected. From the supernatant which is separated from the fermentation broth was subjected to the solvent extraction using various

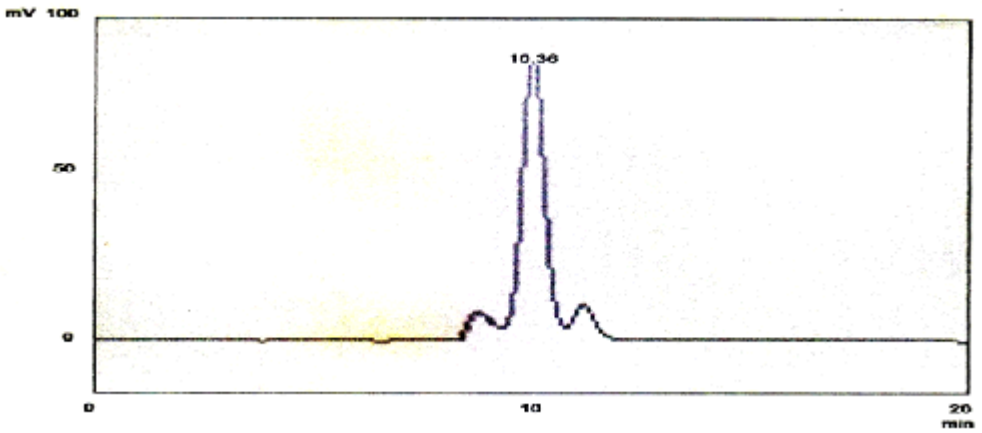

Fig. 2. Chromatogram of antibacterial compound of A3 mutant strain

organic solvents like petroleum ether, chloroform, ethyl acetate etc. The cell biomass collected during fermentation broth filtration, is subjected to cell disintegration using sterile sand to check the intracellular antibiotics.

In solvent extraction study, the same proportion of organic solvent and fermentation broth were extracted. Each solvent was studied, initially, for its antibacterial properties using cup-plate method. It was found that ethyl acetate is an ideal solvent for the isolation of antibiotics from fermentation broth, since it did not modify the antibiotic and this solvent was the most efficient (of the solvents tested) in terms of antibiotic extraction solvents. A variety of solvents have been used to extract antibiotics from actinomycetes with varying degrees of success, although ethyl acetate and methanol are often found to be the most effective[17,18,19].

In the present study the HPLC profile of the antibacterial compounds of *Streptomyces griseus* showed absorption peaks at retention time (min) 10.36 (Fig. 2). The retention 10.36 (min) of antibacterial compound from *Streptomyces griseus* on HPLC was similar to streptomycin, an antibiotic previously widely reported[20]. The retention time 3.263 (min) of antimicrobial compound *from Streptomyces* sp. RM17 on HPLC was similar to

oxohexaene (3.23 min), a relatively new antifungal agent has been previously reported[21]. Further investigation is now needed to determine the structure of the active compound isolated here and to scale up the production for in vivo testing.

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