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Utilization of chemical and physical mutagens for increasing antibiotic production from *Oscillatoria amoena* isolated from Shatt Al-Arab

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Abstract

Three isolates of *Oscillatoria amoena* were screened for antibiotic production against both Gram positive and Gram negative test bacteria. Induced mutations were used to increase antibiotic production, the isolate named O_1 was chosen on the basis of its high antibiotic activity and subjected to the chemical mutagen N, methyl-N nitro. N. nitrosoguanidine (NTG) and physical mutagen ultraviolet light (UV-Radiation) of wave length 245 nm after sensitization with caffeine. These treatments provided mutants named O_1N_{50} (0.98 mg/100 ml); O_1N_{75} (1.2 mg/100 ml); O_1N_{100} (1.8mg/100 ml) and $O_1N_{100}U_1$ (2.2 mg/100 ml). The yields of the antibiotics produced by the mutants were compared with that of the wild type (mother strain O_1) (0.5 mg/100 ml).

Introduction

The science of antibiotic is one of the most important branche of modern knowledge. Most antibiotics have been isolated from cultures of bacteria, particularly actinomycetes (Sanglier *et al.*, 1993) and fungi, also from certain species of algae and cyanobacteria (Dipa and Sen, 1997; Mehdi and Al-Mousawi, 1999). The production of antibiotic depends greatly upon the conditions of cultivation of organisms, selection of a suitable medium and selection of highly antibiotic producing mutants produced mutants by using different chemical and physical mutagens such as nitrosoguanidine (NTG) and UV-radiation (Cruegers and Crueger,1984).

Selection of high antibiotic producing mutants takes place mainly in commercial laboratories and most of result are not published. Mutants which are specifically adapted to the fermentation process are used in the production of antibiotic (Sanglier *et al.*, 1993). The objective of genetic strain improvement program depends on fermentation process and the success of these programs depend greatly on the substance to be examined (Lam *et al.*, 1996). All development of high antibiotic yielding strains have come from empirical mutagen studies (Patrick, 1995).

Antibiotic properties of algae and their occurrence in nature are now being studied to enrich this science and practical medicine by the addition of very interesting antibiotic that one used in various branches

of medicine and industry (Egorov, 1985). A new active antibiotic against both Gram negative and Gram positive bacteria was isolated from blue-green algae *Oscillatoria amoena* from Shatt Al-Arab (Mehdi and Al-Mousawi, 1999). In order to enhance the production of the antibiotic by *O. amoena* the objective of the present study was therefore focused on developing highly antibiotic producing strain of *O. amoena* and optimizing the method for production.

Materials and Methods

The test bacteria:

The following test bacteria were used during the course of this study: *Staphylococcus aureus* NCTC 6571; *Bacillus subtilis* PCI 219; *Bacillus pumillus* NCTC 8241; *Escherichia coli* NCTC 5933; *Pseudomonas aeroginosa* NCTC 6750. The test bacteria were maintained in a well stopper vials containing nutrient agar (Difico).

Producer organisms:

Three isolates of *Oscillatoria amoena* producing antibiotic (Mehdi and Al-Mousawi, 1999) were maintained in a dilute chu-10D medium (Al-Mousawi, 1984). The pH of the final medium was adjusted to 7.6 by addition of NaOH and HCl (0.1 N each), before autoclaving at 121°C for 20 minutes. All cultures were placed in illuminated cabinet with 100 ? Em^{-2} sec^{-1} fluorescent light on 10:14 hrs. light: dark cycle at 29±1°C. Growth was monitored by determining the optical density at 750 nm using spectrophotometer. Chlorophyll-a and phaeophytine pigment (? g/vol. Of sample) extracted in 90% acetone and calculated from the formula of Lorenzen (Vollenweider, 1974).

Screening for antibiotic production by Osiciilatoria amoena:

The three isolates of *Osicillatoria amoena* were cultured by mixing measured volumes of algae suspensions with equal volumes of molten 1.2% W/V agar cooled to a temperature of 40°C and poured rapidly into a petridishes and incubated at 29 ± 1°C in light for 7 days. Five mm discs were used as inocula into liquid medium (Chu-10D medium).

The bulges of stock culture were added to 100 ml of the liquid medium in 500 ml Erlenmeyer flasks and incubated for 5 to 7 days at 29±1°C in a shaking incubater (GFL) at 140 rpm photon flux density of 100 ? $Em^{-2}sec^{-1}$ fluorescent light on 10:14 hrs. light: dark cycle. Shaking was necessary to maintain growth in homogeneous suspension (Mehdi and Al-Mousawi, 1999).

Supernatant of algae growth was obtained by settling under gravity using centrifuge at 2000 g for 20 minutes. The supernatant was tested for the production of antibiotic by using agar plate diffusion method
(Vladimir, 1983) by using standard test bacteria mentioned above.

Development of isolates with enhanced antibiotic production:

Mutation and selection program:

Induced mutation:

a- Isolation of mutants resistant to different concentration of NTG:

NTG was added in concentration from (10-250) ? g/ml to Chu-10D agar

medium on which isolates were then cultivated (Rhodes *et al.*, 1981).

Two groups of plates were prepared in triplicates, the first one contained Chu-10 D agar medium for cultivating mother producer strain while the second contained the same medium with increasing concentrations of NTG for the isolation of mutants resistant to these chemical compound concentrations. The algal plates without NTG were inoculated with 0.1 ml of decimally diluted algal suspension growth while the plates with NTG were inoculated with 0.2 ml of an undiluted alga suspensions. All plates were incubated at 29 ± 1°C in light for 7-10 days.

Colonies of organisms from plates with NTG were again inoculated on the Chu-10D agar medium containing the same concentration of NTG to ensure the presence of resistance to NTG.

b- Treatment with UV-radiation

Algal growth of O_1N_{100} and mother strain O_1 were harvested from 10-15 days old cultures grown at 29±1°C in light on Chu-10D agar plates.

Algal suspension was divided in to five portions each portion of 3 ml in glass Petridishes of 9 cm diameter with addition of mg/ml solution of caffeine (Sannders and Holt, 1989). The first portion was regarded as a control and the remaining portions were irradiated by bactericidal UV lamp giving 95% of its output at 254 nm for different time intervals (0.5; 1; 1.5; 2) minutes.

During irradiation, the suspension of algal growth was agitated by magnetic stirrer, the mutation frequency was calculated by inoculation the irradiation culture Chu-10D agar medium and incubated at 29±1°C for 10-15 days in light. The survival was calculated and compared with control on the same medium, each mutant was tested for antibiotic activity described above.

Testing of mutants:

1. **Antibiotic activity against test bacteria:**

This was achieved by determination the diameter of inhibition zone (IZ mm) and comparison with mother strain (wild type) (Mehdi and Al-Mousawi, 1999).

2. **The activity of antibiotic biosynthesis of mutants:**

The activity of mutants to produce antibiotic was determined as described by (Mehdi and Al-Mousawi, 1999) and compared with mother strain under the same condition of cultivation.

Factors effecting the production of antibiotic:

A detailed study has been conducted on factors effecting the yielding of antibiotic in fermentation $_{medium}$ (Chu-10D medium) including incubation period, shaking, temperature and initial pH.

Results

Three isolates of *Oscillatoria amoena* producing antibiotic were given symbols O_1, O_2, O_3. No significant differences were recorded among the three isolate. All isolates produced antibiotic active against both Gram-positive and Gram-negative bacteria (table-1).

Table (1) Antibiotic activity of three isolates of *Oscillatoria amoena* against test bacteria.

Test bacteria	Diameter of inhibition zone (mm)		
	O_1	O_2	O_3
Staphylococcus aureus NCTC 6571	13.5	11.5	11
Escherichia coli NCTC 5933	8	7	6.5
Bacillus pumillus NCTC 8241	12	11	11
B. subtilis PCI 219	13.5	11	11.5
Pseudomonas aeroginosa NCTC 6750	6	5.5	5

The activity of produce antibiotic is varied among isolates (table 2).

Table (2) Antibiotic and chlorophyll-a produced by three isolate of *Oscillatoria amoena* (O_1, O_2, O_3) in fermentation medium.

Strains	Weight of antibiotic (Mg/100ml)	Chlorophyll-a (Mg/100ml)
O_1	0.5	10.5
O_2	0.25	8.5
O_3	0.28	8.1

Maximum antibiotic production occurred after 7 days of incubation at 29 ± 1°c with shaking (140 rpm).

These results were found to be significantly different when tested by analysis of variance ($f = 13.7$, $P > 0.05$).

O_1 was chosen among recovered isolates on the bases of its high antibiotic production and subjected to chemical induced mutation (NTG) and physical mutation (exposure to UN-radiation), for increasing its ability for antibiotic production.

Development of isolates:

a- Induced mutation (resistance to NTG):

Antibiotic production of isolate resistant to NTG in comparison with mother strain (wild type) was shown in Table (3). The mutant, which was resistant to 100mg/ml, gave the maximum activity against test bacteria both Gram positive and Gram-negative bacteria the difference in antibiotic yield among resistant isolates was found to be highly significant when tested by analysis of variance (R.L.S.D. = 43.588 at 0.01, R. L. S. D. = 33.44 at 0.05). This treatment provided three mutants namely O_1N_{50} (0.98 mg/100 ml); O_1N_{75} (1.2 mg/100 ml) and O_1N_{100} (1.8 mg/100 ml).

Table (3) Antibiotic activity produced by O_1 (*Oscillatoria amoena*) resistant to different concentration of NTG against test bacteria.

Concentration of NTG (mg/ml)	Weight of antibiotic (mg/100ml)	Diameter of inhibition zone (mm)				
		Test bacteria				
		Staphylococcus aureus NCTC 6571	*Escherichia coli* NCTC 9533	*Bacillus pumillus* NCTC 8241	*B. subtilis* PCI 219	*Pseudomonas aeroginosa* NCTC 657
0	0.5	13.5	8	12	13.5	6
10	0.55	13.5	8	12	13	6.5
25	0.58	11	8	12	13	6.5
50	0.98	14.5	9.5	13	13.5	7
75	1.2	16	10	13.5	15	7
100	1.8	17.5	11.5	15	15.5	9
150	0	0	0	0	0	0
200	0	0	0	0	0	0
250	0	0	0	0	0	0

b- Treatment with UV-radiation:

It is apparent from table (4) that production of antibiotic by O_1N_{100} and the wild type O_1 after being exposed to UV-radiation for (0.5; 1; 1.5; 2) minutes have increased significantly in comparison with (0) time.

Table (4) Effect of UV-radiation on growth and antibiotic production by different strains of *Oscillatoria amoena*.

Isolates	pH		Exposure time to UV radiation (min)	Weight of produced antibiotic (mg/100ml)	Chlorophyll-a (? g/100ml)	Phaeophytine (? g/100ml)	Diameter of inhibition zone (mm)				
							Test bacteria				
	Initial	After 7 days					*Staphylococcus aureus* NCTC 6571	*Escherichia coli* NCTC 9533	*Bacillus pumillus* NCTC 8241	*B. subtilis* PCI 219	*Pseudomonas aeroginosa* NCTC 657
O_1	7.6	6.5	0	0.5	10.5	1.2	13.5	8	12	13.5	6
	7.6	6	0.5	0.8	9.5	2.3	14	9	12.5	9	7
	7.6	6	1	0.2	9.5	3.1	15	10	10	10	10
	7.6	7.5	1.5	0.001	1.8	4.8	1.5	1	1.5	0	0
	7.6	7.5	2	0.0	0.24	3.8	0	0	0	0	0
O_1N_{100}	7.6	6.5	0	1.8	10	1.2	17.5	11.5	15	15.5	9.5
	7.6	6	0.5	1.8	9.5	2.3	17	11	15	15	9.5
	7.6	6	1	2.2	10.5	1.5	18	12.5	15.5	16	10
	7.6	7.6	1.5	0.002	0.4	3.85	1.5	1	1	1.5	0
	7.6	7.6	2	0	0.05	3.1	0	0	0	0	0

The activity of mother O_1 and O_1N_{100} dropped to 99% when irradiated for 1.5 minuets in comparison with (O) time, but when the irradiation time was prolonged to 2 minuets the activity was completely disappeared. Statistical analysis proved that all strains (mutant and mother) exhibited highly significant differences in their antibiotic yield (R.L.S.D. = 41.8). this treatment provided mutant with highly antibiotic activity named O_1N_{100} U_1. (Table-4).

Factors affecting the production of antibiotic by Oscillatoria amoena:

a- Incubation period:

Determination of optimum period of antibiotic production is shown in table (5). It is concluded that incubation for seven days gave the optimum production, increasing time resulted in decreasing antibiotic production.

b- Shaking

Analysis of variance exhibited highly significant difference in antibiotic production (R.L.S.D = 29.55) between unshaken and shaken culture of strains. The production in shake culture was 10 fold more than in unshaked one. The optimum production appeared at 140 rpm (Table 5).

c- Temperature

Production of antibiotic was estimated under three selected temperature (Table 5). It was found that production remained almost constant when temperature raised to 29-30°C, then it dropped when temperature raised to 35°C and vanished at 45°C.

The anova test revealed a highly significant difference between antibiotic production at 35°C and 29 to 30°C for each strains (R.L.S.D. = 9.2, $P < 0.01$).

d- Initial pH:

Table (5) shows the effect of initial pH on the production of antibiotic. It is apparent that the best initial pH of the medium was 7.6. This result was confirmed biostatistically (R.L.S.D. = 15.9).

Table (5): Effect of shaking, temperature and initial pH on the production of antibiotic by different strains of *Oscillatoria amoena*.

Strain	Factors							
	Incubalim period (day)	Weight (mg/100ml)	Temp. (°C)	Weight (mg/100 ml)	Shaking (rpm)	Weight (mg/100 ml)	Initial pH	Weight (mg/100 ml)
O_1	3 5 7 10	0.04 0.09 0.5 0.035	29 35 45	0.5 0.03 0	0 100 140 180	0.01 0.05 0.50 0.03	6 7.6 8	0.2 0.5 0
O_1N_{100}	3 5 7 10	0.09 1.2 1.8 0.055	29 35 45	1.8 0.09 0	0 100 140 180	0.03 0.08 1.8 0.04	6 7.6 8	0.03 1.8 0.002
$O_1N_{100}U_1$	3 5 7 10	0.06 1.3 2.1 0.067	29 35 45	2.1 0.09 0	0 100 140 180	0.03 0.09 2.2 0.03	6 7.6 8	0.035 2.2 0.032

Discussion

In the present study three isolates of *Oscillatoria amoena* produced antibiotics have been checked and given the symbol (O_1, O_2 and O_3). The O_1 isolate was chosen on the basis of it's high antibiotic production ability and was studied extensively for increasing production by improving the genotype of the isolates and inoculum condition (Al-Rubeai *et al.*, 1998).

The species *O. amoena* was chosen in the present study to complete the research began at 1999 aiming to find a strain with high antibiotic producing capability. Since this species was found to produce a new antibiotic active against both Gram positive and Gram negative test bacteria (Mehdi and Al-Mousawi, 1999).

Strain development (Induced mutation):

The isolates were subjected to prior treatment with chemical mutagen (NTG) and then to the physical mutagen UV radiation with the addition of caffeine.

a. Increased resistance to NTG:

This mean obtaining mutant with genotypes different from mother strain (O_1). It is suggested that mutant resistant to NTG might influence the genetic material and causes errors during replication of nucleotide sequence producing a new genotype that has the ability to produce high activity of antibiotic due to the influence of enzymatic activity of biosynthesis of antibiotic and as a result the biosynthesis of antibiotic has increased by such type of mutation, table (3) (Mehdi, 1997).

b. Treatment with UV radiation:

In the present study, inducing mutation in *O. amoena* isolates by exposing to UV-radiation was carried out. The results showed (Table-4) that at 1 minute exposure time to UV-radiation the production of antibiotic increased and brought about high yield mutant such as $O_1N_{100}U_1$ (2.2 mg/100 ml). This have represents the best exposure increasing the highest producing ability (Goldat, 1961).

If the exposure is carried out for more than 1.5 minutes the production of the antibiotic will decrease which is contributed to greater death rates and increased mutation frequency. Especially this may interfere with biosynthesis pathway of the antibiotic and the enzymatic activity which are responsible for antibiotic production. A wave length of 254 nm was applied in this study which caused damaging of DNA due to dimmers formation between adjacent pyrimidines of complementary strands which results in cross-links causing induced mutation and production of a new genotypes (Crueger and Crueger, 1984). The addition of caffeine has increased the production due to the inhibition of excision repair after exposing to UV-radiation (Mehdi, 1997).

Factors effecting the production:

The conditions under which microorganisms are cultivated are no tess important than strain improvement for increased yield of antibiotic. The cultivation conditions include aeration degree, temperature and pH. The yield of antibiotics can be increased three time by using a suitable condition for cultivation of each selected isolate (Kamei *et al.*, 1991).

Many antibiotic producing microorganisms grow better in media with

neutral pH (about 7) (Perry and Staley, 1997). The pH of fermentation media will change during growth (table-5) of a particular microorganism and this depends basically on the source of nitrogen used, consequently the media for the cultivation of antibiotic producers should be so composed that the pH of the media during growth should remain within the allowable limits so that synthesis antibiotic would not be affected adversely. So far as, the temperature is concerned, antibiotic producers will optimally produce antibiotic only if a certain optimum temperature of cultivation is ensured, the optimum temperature in the present study was 29-30°C
(Table 5) at which they can develop normally and synthesis the antibiotic.

Deviation of temperature from the optimum range will retard the growth of algae and decrease the yield of antibiotic due to the substantial effect of the temperature on the activity of the enzymes (Perry and Staley, 1997).

Shaking of the culture is also very crucial for biosynthesis of antibiotic. In the present study, the optimum degree of shaking which gave the optimum yield was at 140 rpm (Table 5).

The culture is most sensitive to interruption in aeration, when the degree of shaking is below 140 rpm the growth of the antibiotic producer slows down significantly and the yield decreases. Saturation of culture with oxygen depends not only on the quantity of air bubbled per unit volume and time but also on the growth degree of the producer and the stirring of the medium (Perry and Staley, 1997). In addition to the speed of the stirrer, the composition of the medium and on the temperature of the cultivation.

Shaking of the culture ensures removal of the metabolites and the products of lysis from producer cells and promotes a better oxygen distribution in the medium (Vladimir, 1983).

During cultivation of isolates in flasks on shaker the aeration degree depends on the number of reciprocation of the shaker per minute and on the volume of the culture. The smaller volume of the medium in the flask, the better it's aeration. Aeration degree of the liquid medium increasing depth of the layer (Kamei *et al.*, 1991).

In conclusions, it was shown from the results that the optimum temperature for biosynthesis 29±1°C with 140 rpm at 7.6 initial pH gave the maximum yield of antibiotic.

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الملخص

استخدمت ثلاث عزلات من الطحلب *Oscillatoria amoena* لاختبار فعالية المواد المضادة التي تنتجها ضد الجراثيم القياسية السالبة والموجبة لصبغة كرام. ولزيادة إنتاج المضاد الحيوي اختبرت العزلة المسماة O_1 ذات الإنتاجية العالية من المضاد، وأخضعت إلى المواد الكيمياوية والعوامل الفيزياوية المحفزة للإنتاج العالي مثل N, methyl-N-nitro-N-nitrosoguanidine (NTG). والتعريض للأشعة فوق البنفسجية (UV-radiation) وبطول موجي 245nm مصحوباً بإضافة مادة الـ caffeine على التوالي.

أدت هذه المعاملات إلى الحصول على عزلات مطفرة ذات انتاجية عالية مقارنة مع العزلة الأم (O_1) (0.5 ملغم/100سم^3) وأطلق على هذه العزلات الأسماء التالية O_1N_{50} (0.98 ملغم/100 سم^3) و O_1N_{75} (1.2 ملغم/100 سم^3) و O_1N_{100} (1.8 ملغم/100 سم^3) و $O_1N_{100}U_1$ (2.2 ملغم/100 سم^3).