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Genetic Improvement of Rhamnolipid Production from *Pseudomonas aeruginosa*

التحسين الوراثي لإنتاج الرامنولبيد من *Pseudomonas aeruginosa* بكتيريا

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ABSTRACT: Six bacterial isolates (isolated previously) were identified and/or ensured their identification. Results showed that these isolates belong to P. aeruginosa, and all isolates were capable of producing rhamnolipid, and the best one was P. aeruginosa RB67. In order to get rhamnolipid hyper producer mutants, mutagenesis of P. aeruginosa RB67 using UV light and MNNG were performed. Fifty colonies from each treatment (UV and MNNG) were selected and screened for their ability to produce rhamnolipid semi-quantitatively by replica plated on blood agar and CTAB-methylene blue agar. Based on the last method, twelve colonies from each treatment (UV and MNNG) were selected and used for measuring rhamnose concentration. The results showed that these mutants varied in their ability to produce rhamnolipid, and some of them showed an increase in rhamnolipid production. The highest rhamnose concentration (94 µg/mL) was achieved by the mutant (MOM12). Furthermore, FTIR spectroscopy results indicated that there were no apparent qualitative differences in rhamnolipid produced from mutants.
Keywords: *Pseudomonas aeruginosa , Mutagenesis , Rhamnolipid.*

المستخلص: تم تشخيص وتأكيد تشخيص ستة عزلات بكتيرية (معزولة سابقاً) وأظهرت النتائج أن جميع هذه العزلات تعود للنوع *Pseudomonas aeruginosa*، ولها القدرة على إنتاج الرامنولبيد وان افضل عزلة هي *Pseudomonas aeruginosa* RB67. ولاجل الحصول على طافرات ذات إنتاجية عالية من الرامنولبيد، طفرت بكتيريا *Pseudomonas aeruginosa* RB67 باستخدام الاشعة فوق البنفسجية ومادة MNNG، وانتخبت خمسين مستعمرة من كل معاملة (الاشعة فوق البنفسجية ومادة MNNG) وغربلت لقابليتها على الإنتاج شبه الكمي للرامنولبيد وذلك بتكرارها على وسط اكار الدم ووسط CTAB-methylene blue agar. واعتمادا على الطريقة الاخيرة انتخبت 12 مستعمرة من كل معاملة (الاشعة فوق البنفسجية ومادة MNNG) وتم قياس تركيز الرامنوز المنتج منها. اظهرت النتائج أن هذه الطافرات كانت متغايرة في قابليتها على انتاج الرامنولبيد، واظهر بعضها زيادة في إنتاج الرامنولبيد وان اعلى تركيز للرامنوز (94 مايكروغرام/مل) تم الحصول عليه بفعل الطافرة (MOM12). كما اظهرت نتائج FTIR عدم وجود تغيرات نوعية في الرامنولبيد المنتج من قبل الطافرات.
كلمات مدخلية: *Pseudomonas aeruginosa، التطفير، الرامنولبيد.*

INTRODUCTION

Surface-active agents are substances that have the ability to stabilize dispersions of one liquid in another, e.g. oil-in-water emulsions. Bio-surfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. The molecules reduce surface and interfacial tension in both aqueous solution and hydrocarbon mixtures, which make them potential candidates for enhancing oil recovery and emulsification process. Furthermore, they are used in food industries, hydrometallurgy, medicine, biotechnology and environmental protection in the separation of hydrocarbons and gases, and in the concentration and separation of amino acids, metal ions and other mixtures and suspensions (Rosenberg and Ron, 2000).

Rhamnolipid, a rhamnose containing glycolipidis, is one of the most important bio-

surfactant produced by *Pseudomonas aeruginosa*. It is considered to be one of the virulence-associated exo-products of *P. aeruginosa*. Furthermore, rhamnolipid possess antimicrobial activity against many Gram-positive and Gram-negative bacteria (Borjana *et al.*, 2002). In addition, rhamnolipid has been shown to be capable of binding to heavy metals, e.g., it can bind to cadmium, lead and zinc and removing them from soil (Sandrin *et al.*, 2000).

In this study genetic manipulation using physical (UV light) and chemical (MNNG) mutagenes were performed in order to improve rhamnolipid production from *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial Isolates

P. aeruginosa RB19, RB27, RB29 and RB31 were isolated and identified in a previous study (Nasir *et al.*, 2002). *Pseudomonas* RB55 and RB67 were obtained from Nasir, R.B. (Department of Biotechnology, College of Science, University of Baghdad). The isolates were maintained at 4°C on Brain Heart Infusion (Difco, USA) slants. BHI broth was used for the activation of the isolates.

Identification of Bacterial Isolates

A number of morphological, physiological, biochemical and API20E system (Api Bio Merieux, Lyon, France) tests were performed to identify the isolates (Holt *et al.*, 1994).

Screening of Bacterial Isolates for Rhamnolipid Production

A fifty mL of mineral salt (Bushnell-Hass) medium (Patel and Desai, 1997) were dispensed into 250 mL Erlenmeyer flasks, and 2% of sunflower oil was added as a sole carbon source. Following sterilization by autoclaving, flasks were inoculated with 1% of 18 hrs bacterial culture, and then incubated in shaker incubator (180 rpm, 72 hrs, 30°C). After incubation the surface tension of the culture supernatant was measured by tensiometer (Kruss) (Saifour *et al.*, 2004).

Exposure of *P. aeruginosa* RB67 to UV Light

Culture of *P. aeruginosa* RB67 grown in brain heart infusion broth at 18 hrs was pelleted from 5mL, washed twice and re-suspended in the same volume of phosphate buffer (pH 7.0) (9.52 g of Na_2HPO_4 and 6.00 g of NaH_2PO_4 per liter of D.W.). The UV source was UV- transiluminator-Cross Linker [FLX-20-M, Vibler Lour mat, France]. The dose rate of UV irradiation was 2.5 J/m²/s. The experiment was performed under red light. Five mL samples of the bacterial suspension in phosphate buffer were irradiated in sterile petri dish for the following doses: 0, 5, 10, 15, and 20 J/m². Then, 0.1 mL samples from appropriate dilution of each treatment were spread on brain heart infusion agar plates, and then incubated at 37°C for 24 hrs to determine the total viable count.

Treatment of *P. aeruginosa* RB67 with MNNG

Culture of *P. aeruginosa* RB67 grown in brain heart infusion broth at 18 hrs was pelleted from 10 mL, washed twice and re-suspended in the same volume of phosphate buffer (pH 7.0), and dispensed into 250 mL Erlenmeyer flasks. N-methyl-N-nitro-N-nitrosoguanidine (MNNG) was added to the bacterial suspension at different concentration (0, 10, 20, 30, 40, 50 μg/mL). The mixture was incubated at 37 °C with quite shaking for 20 min. Then 0.1 mL samples from appropriate dilutions of each treatment were spread on brain heart infusion agar plates, and incubated at 37 °C for 24 hrs to determine the total viable count.

DETECTION OF RHAMNOLIPID HYPER PRODUCER MUTANTS

Semi-quantitative detection

According to the UV and MNNG survival curve, the treatment that led to a survival percentage of approximately 10% was suspected to have the higher mutation frequency. From this (selected) treatment of UV and MNNG, a number of colonies were picked up and replica plated on blood agar plates and incubated at 30 °C for 72 hrs. Fifty colonies from each selected treatment were replica plated on cetyltrimethyl-ammonium bromide (CTAB)- methylene blue agar plates (SW medium) (Siegmound and Wagner, 1991). Rhamnolipid producing colonies on SW agar plates were identified according to the formation of dark blue halos around the colonies on a light blue-plate background.

Quantitative detection

P. aeruginosa RB67 (wild type) and selected mutants, were grown on minimal salt medium containing 2% sunflower oil and incubated in shaker incubator (180 rpm) at 30°C for 72hrs. After incubation, the culture was centrifuged, then the pH of the 10 mL of the supernatant was adjusted to 2, and left for 24 hrs at 4 °C. After that, it was extracted with chloroform/methanol (2:1 V/V) and the solvent was evaporated using rotary evaporator. After evaporation the precipitate was dissolved in 2 mL of 0.1M sodium bicarbonate (Patel and Desai, 1997). The resultant was used to measure rhamnose concentration according to Dupois *et al.*, (1956). Rhamnolipid amount was determined according to rhamnose concentration measurement (Saifour *et al.*, 2004).

Characterization of Rhamnolipid using FTIR Analysis

The partially purified rhamnolipid obtained from *P. aeruginosa* RB67 (wild type, UV mutant (UOM12), and MNNG mutant (MOM12)), were subjected to Fourier Transform Infrared (FTIR). The FTIR spectrum will give the functional chemical groups that are found in the compounds in order to compare between them and identify if there is a qualitative differences between them or not.

RESULTS AND DISCUSSION

Identification of Bacterial Isolates

In this study the morphological, physiological and biochemical tests were performed to ensure the identification of *P. aeruginosa* (RB19, RB27, RB29 and RB31), and to identify the species of other two bacterial isolates (RB55, and RB67). Biochemical identification was also made using API 20E system to confirm the results.

The results showed that *Pseudomonas* RB19, RB27, RB29, RB31, RB55, and RB67 were all belonging to the species *P. aeruginosa*. The results of the morphological, physiological and biochemical performed tests were in agreement with Holt *et al.*, (1994).

Screening of Bio-surfactant Producing Isolates

The results, illustrated in Table 1, indicate that all isolates were able to produce surface-active compounds, and *P. aeruginosa* RB67 gave the best results by minimizing the surface tension to 30 mN/m, and hence *P. aeruginosa* RB67 was selected for further study. The growth of these isolates under such condition require the production of surface active compounds in order to emulsify the sun flower oil and increase its bioavailability for utilization by bacteria. Zouboulis *et al.*, (2003) indicated that insoluble hydrocarbons promote the bacteria to produce surface-active compounds that are capable of reducing the surface tension at the interface between liquids, solids, and gases, thereby allowing them to mix readily as emulsion in water.

Mutagenesis of *P. aeruginosa* RB67

P. aeruginosa RB67 was mutaginized in an attempt to increase its ability to produce rhamnolipid.

UV survival curve and Mutagenesis

The obtained results (Figure 1) indicated that *P. aeruginosa* RB67 was UV sensitive. The survival curve showed an increase of the lethal percentage with the increase of UV doses, and the highest lethal percentage or the less survival percentage was 4.7% when the bacterium exposed to 20 J/m^2 of UV irradiation. The survival percentage of *P. aeruginosa* RB67 was 10.4% (89.6% killing) when it was exposed to 15 J/m^2 of UV light. Therefore, this treatment was selected for mutants isolation. It is known that mutation in bacteria can be induced by agent such as UV irradiation whose mutagenesis is affected via miss-repair of damaged DNA by SOS repair system and have been termed indirect mutagens (Turner *et al.*, 2000).

Table 1. Surface tension of cell free supernatants of *P. aeruginosa* grown in minimal salt medium containing 2% sun flower oil with shaking (180 rpm), at 30 °C for 72 hrs.

Isolates	RB27	RB29	RB30	RB31	RB55	RB67	Control(-)	Control(+)	Methanol	Benzene
Surface Tension (mN/m)	32	40	33	32	41	30	58	68	23	28.9

Control (-): Medium without carbon source; **Control (+)**: Medium without bacteria.

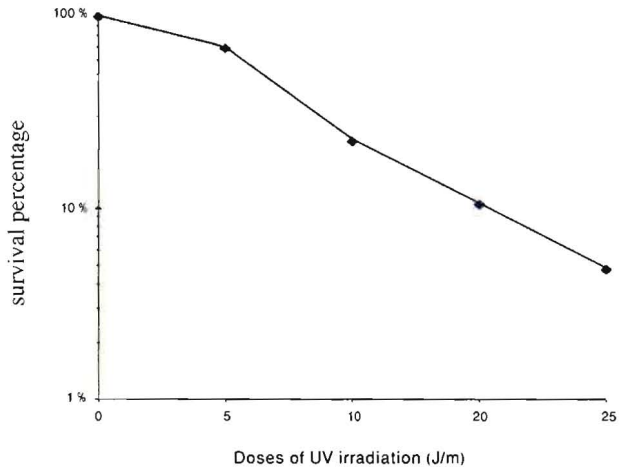

Fig. 1. Effect of UV irradiation on a suspension of *P. aeruginosa* RB67 in phosphate buffer (pH=7).

MNNG Survival Curve and Mutagenesis

Treatment of *P. aeruginosa* RB67 with different concentrations of MNNG indicated that this bacterium was sensitive to MNNG (Figure 2). The survival curve showed increase of the lethal percentage with the increase of MNNG concentration, and the highest lethal percentage (88.8%) or the less survival percentage was 11.2%, when the bacterium treated with 40 µg/mL of MNNG. Accordingly this treatment (40 µg/mL) was selected for mutants isolation.

It is known that MNNG is an effective mutagenic compound, it can generate mis-pairing lesion by adding alkyl group to various position on nucleic acids, and hence miss-replication of DNA, or miss-repair of damaged DNA (Turner *et al.*, 2000). In addition, it can induce mutation by an error prone DNA repair pathway (Abbas *et al.*, 2004).

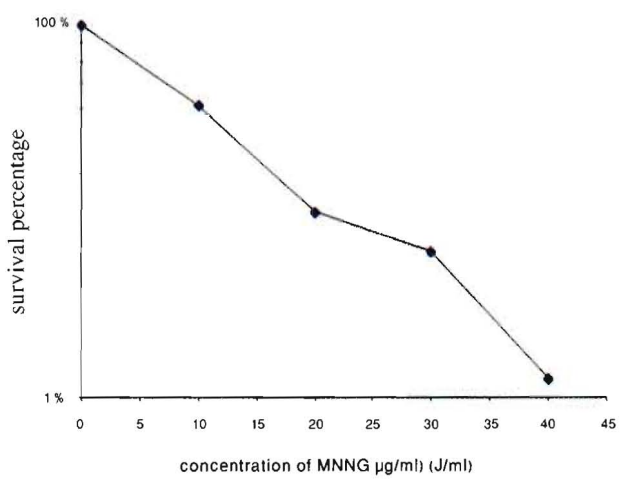

Fig. 2. Effect of MNNG on a suspension of P. aeruginosa RB67 in phosphate buffer (pH=7).

Selection of hyper producer

Mutated cultures from the selected treatments were screened for haemolytic activity exerted by rhamnolipid by replica plating on blood agar. Results indicated that all colonies showed approximately the same haemolytic zone. However, it can not be excluded that hemolytic zone formation on blood agar plate might be due to divalent ions and other hemolysins produced by the microbe under investigation (Mulligan *et al.*, 1989). For this reason, an alternative method for the detection of rhamnolipid was employed, in which fifty colonies from each UV and MNNG treatment were replica plated on CTAB-methylene blue agar plates. The diameter of the dark blue region has been shown to be semi-quantitatively proportional to the concentration of rhamnolipid bio-surfactant (Siegmund and Wagner, 1991). Twelve colonies from each treatment (UV and MNNG) were selected (designated UOM1 to UOM12 and MOM1 to MOM12, respectively), which gave the largest dark blue halo and used for measuring rhamnose concentration.

The obtained results (Table 2) showed that the rhamnose concentration produced by *P. aeruginosa* RB67 (wild type) was 70 µg/mL, while the concentration of rhamnose varied among the twelve mutants of each treatment. However, mutants UOM11, UOM12 and MOM12 showed a significant increase in concentration of rhamnose (85 µg/mL, 89 µg/mL and 94 µg/mL, respectively).

UV light and MNNG were used successfully as mutagens to get different mutants from different bacteria. Al-Delaimi and Al-Gelawi (2006) and Baho (2006) used UV light to mutate *Xanthomonas campestris* and *P. aeruginosa,* respectively and they successfully get hyper producer mutants for protease and lectins, respectively. It was used also by Abbas *et al.*, (2004) to mutate *P. aeruginosa*, to isolate rhamnolipid hyper producer mutants.

The results indicated that both UV light and MNNG caused random mutation in the rhamnolipid gene(s). The mutation may have occurred in the regulatory gene site that regulate the production of rhamnolipid positively, or in the structural gene that code for rhamnolipid production, and hence, this might have led to decrease in the production of rhamnolipid from these mutants. In addition, a mutation in the structural gene (RhlG) which is responsible for the formation of fatty acid moiety of rhamnolipid could inhibit rhamnolipid production (Jesus *et al.*, 1998).

Table 2. Rhamnose concentration produced from *P. aeruginosa* RB67, (A) after exposure to UV light (15 J/m^2), (B) after treatment with MNNG (40 μg/mL).

A		B	
P. aeruginosa	**Rhamnose Concentration (μg/mL)**	**P. aeruginosa**	**Rhamnose Concentration (μg/mL)**
Wild TypeRB67	70	Wild Type RB67	70
Mutant UOM1	52	Mutant MOM1	38
Mutant UOM2	54	Mutant MOM2	45
Mutant UOM3	61	Mutant MOM3	48
Mutant UOM4	61	Mutant MOM4	57
Mutant UOM5	64	Mutant MOM5	61
Mutant UOM6	68	Mutant MOM6	65
Mutant UOM7	69	Mutant MOM7	65
Mutant UOM8	78	Mutant MOM8	73
Mutant UOM9	81	Mutant MOM9	74
MutantUOM10	82	MutantMOM10	78
MutantUOM11	85	MutantMOM11	82
MutantUOM12	89	MutantMOM12	94

The increase in the rhamnolipid production from other mutants may be attributed to two reasons. The first is that the mutation may occur in promoter region that render it to be more similar with that of the consensus sequence, and as a result the promoter strength increase, resulting in an increase in the efficiency of transcription. It was referred that promoter strength is directly proportional to the degree of similarity with the consensus sequence (Turner *et al.*, 2000).

Secondly, a mutation might occur in the structural gene (Rh1C) that codes for dirhamnolipid synthesis. This mutation may prevent the formation of dirhamnolipid, so abundance of rhamnose will accumulate in the cell leading to the synthesis of large amount of monorhamnolipid (Rahim *et al.*, 2001).

Characterization of Rhamnolipid Using FTIR

The IR spectrum of the rhamnolipid produced from *P. aeruginosa* RB67 (wild type, and mutants UOM12, and MOM12) were shown in figures 3a, 3b, and 3 (Figure 3). The results showed that the OH band of the rhamnolipid produced from these three bacterial isolates were approximately the same. The same values can be seen with C-H group and C=O group between rhamnolipid of these three isolates.

The results also indicate small differences in the band domain between these three isolates, and may be attributed to the presence of impurities in the partially purified rhamnolipid compounds, which may lead to a slight divergence in the band domain.

By comparing the rhamnolipid produced from *P. aeruginosa* RB67 (wild type), UOM12, and MOM12, it can be concluded that there is a quantitative increase in rhamnolipid production, but with no apparent qualitative changes observed.

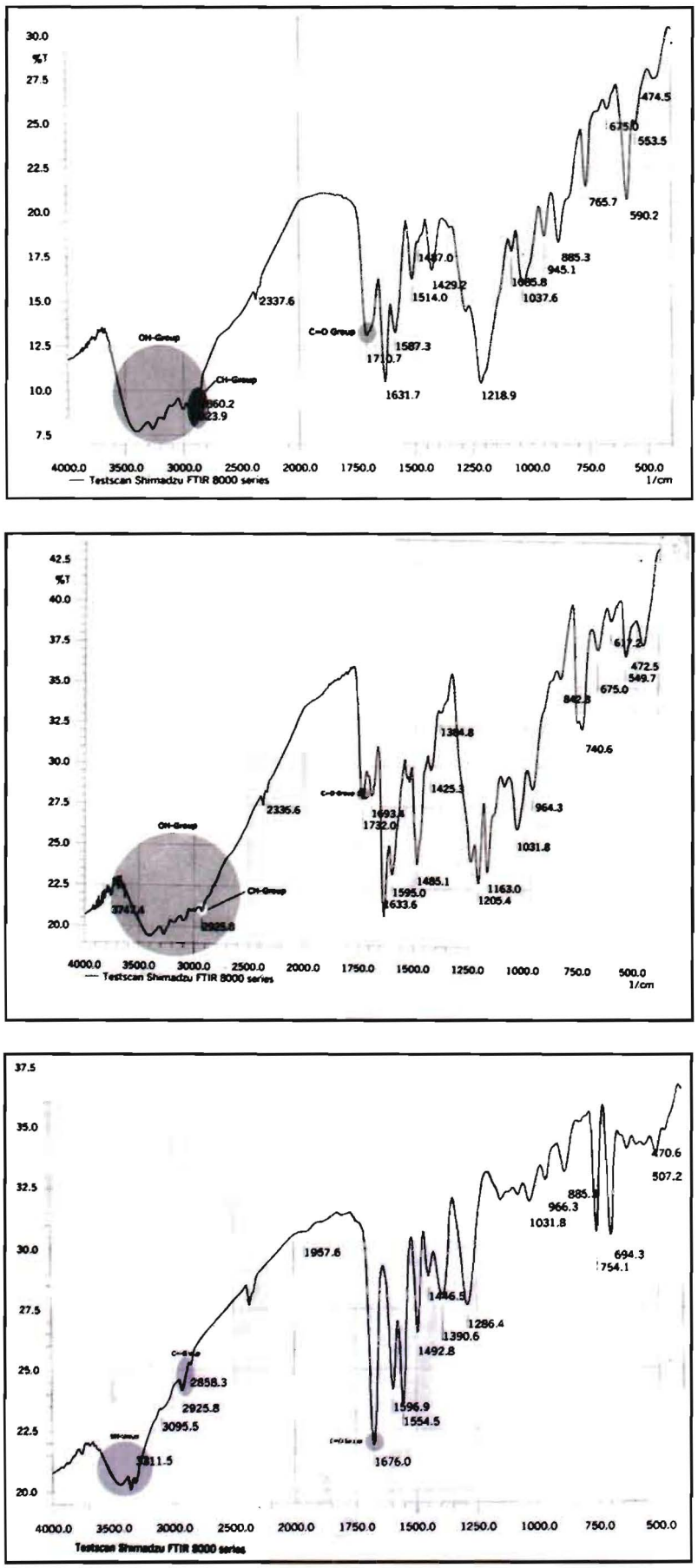

Fig. 3. FTIR Spectroscopy of Partially Purified Rhamnolipid Produced from *P. aeruginosa* RB67 (wild type) (A),UOM12 mutant (B), and MOM12 mutant (C).

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