

## Hyper-Production of Biosurfactant by *Pseudomonas guguanensis* Strain Iraqi ZG.K.M through Random Mutagenesis

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### ABSTRACT

**Background:** With growing environmental concern, interest was increased in research and development of environmentally friendly substances. Microbial surfactants are nontoxic and biodegradable bioproducts that are widely used in medical, pharmaceutical, agricultural, industrial, food, cosmetics, and refinery applications. However, the main challenges in the production of biosurfactants are the high production cost and the limited yield. The improvement of biosurfactant production depend on many strategies, such as the use of low-cost raw materials, adjusting medium and growth conditions, and the selection of hype-producing strains. **Objective:**The study was conducted to obtain *Pseudomonas guguanensis* strain Iraqi ZG.K.M mutant with higher biosurfactant production, through random mutagenesis. **Materials and Methods:** Chemical mutagen (acridine orange) in different concentrations and the physical mutagen (UV irradiation) were used for the production of random mutagenesis in *Pseudomonas guguanensis* strain Iraqi ZG.K.M. The biosurfactant hype-producing strain was selected depending on CTAB test, E<sub>24</sub>%, and the measurement of surface tension. **Results:** A clear indication of the efficiency of UV light in producing biosurfactant mutant. In which, a higher E<sub>24</sub>% (78%) and the lowest surface tension (33 mN/m) were recorded with ZUVM2 mutant. **Conclusion :** UV light can successfully used to mutagenize *Pseudomonas guguanensis* strain Iraqi ZG.K.M to obtain hyper-producer mutants of biosurfactant that used in many applications.

**Keywords:** *Pseudomonas guguanensis* strain Iraqi ZG.K.M, Biosurfactant, hyper-producer.

## 1. Introduction

Microbial compounds which exhibit a clear surface activity are classified as biosurfactants. They belong to different groups including glycolipids, phospholipids lipopeptides, fatty acids, polysaccharide-protein complexes, and neutral lipids. These molecules can perform various natural roles in microbial growth and reproduction (1). Biosurfactants are organic, surface-active amphiphilic compounds that contain both hydrophobic and hydrophilic moieties, produced mainly by different microorganisms (bacteria, yeast, or fungi) on cell surfaces or can be secreted extracellularly (1, 2). They could accumulate between fluid phases and help in reduction the surface and interfacial tensions, making them ideal candidates for boost oil recovery, biodegradation, and bioremediation (3-8).

With a worldwide preference for renewable products, attention to microbial surfactants has been increased due to their diversity, selectivity, low toxicity, biodegradability, ecological acceptance, effectiveness at extreme pH and temperatures, the possibility to be produced through fermentation, and widespread applicability (4, 9, 10). Nowadays, biosurfactants are used in industries such as pharmaceuticals and cosmetics and have emerged as potential agents in food industries and agriculture, possessing several interesting properties of medical importance, bioremediation of petroleum pollutants, management and enhancing crude oil recovery, lubrication, solubilization, wetting, detergency and recently biosurfactants have been found to disrupt biofilm formation (2-9).

A wide range of bacteria has been reported as biosurfactant producers. The genera *Pseudomonas* and *Bacillus* are extremely studied as rhamnolipids and lipopeptides producers (10-13). *Pseudomonas guguanensis* is a member of the genus *Pseudomonas* that can be adapting to grow in crude oil, n-hexadecane, diesel, and kerosene-polluted environments, and exert emulsification activity for hydrocarbon bioremediation, due to their ability to produce biosurfactant (14).

Economic production is a limiting factor of biotechnology. The process of bio-industrial production of biosurfactant is frequently depends on the use of low-cost techniques, either by using cheap raw materials or by enhance biosurfactant productivity by altering microbial genetic makeup. Therefore, the current study aimed to enhance biosurfactant production through improving the strain *Pseudomonas guguanensis* strain Iraqi ZG.K.M by random mutagenesis, in which the mutant strain with high biosurfactant productivity was obtained (15, 16).

## 2. Material and Methods

### 2.1 Microbial strain

*Pseudomonas guguanensis* strain Iraqi ZG.K.M, isolated from petroleum-contaminated soil of the Al Dourah refinery and molecularly identified in a previous study was used to be mutagenized.

### 2.2 Treatment with Acridine Orange

Sub-Inhibitory Concentration (SIC) of Acridine Orange (AO), described in (17), was determined with minor modifications. An overnight bacterial culture was diluted with sterile LB medium until an optical density OD<sub>600</sub> of 0.1 was reached. An aliquot of 1 ml bacterial culture was inoculated into a 4 ml sterile LB medium containing different concentrations of AO (200, 400, 600, 800, 1000, and 1200 mg/l). All tubes were incubated at 30°C for a period of (15, 30, 45, 60, 100, and 120 min). Then, 0.1 ml from each tube at a certain time was spread on plates of nutrient agar and incubated for 24 hrs. at 30°C. The resulting colonies were collected and test their ability to produce biosurfactant.

### 2.3 Treatment with Ultraviolet (UV) irradiation

The direct plate irradiation technique, described in (16,18) was used with minor modifications. An overnight bacterial culture was diluted with sterile LB medium to 0.1 of an optical density OD<sub>600</sub>. A 50 µL of bacterial suspension was spread on plates of nutrient agar medium, and expose the plates to UV rays (from UV-transilluminator - Cross Linker of 254 nm) at 15 cm distance for (10, 20, 30, 60, 120, 300, 600, and 900 sec). The

experiment was conducted in the dark to avoid photo-reactivation. After irradiation, all plates were covered with aluminium foil and incubated for 24 hrs. at 30°C. The resulting colonies were collected and test their ability to produce biosurfactant.

#### 2.4 Detection of biosurfactant hyper producer mutants

Colonies obtained from each mutagenized culture (colonies grown on the high concentrations of the AO and colonies grown on plates exposed to UV) were cultivated in flasks containing mineral salt media (MSM) with 2% crude oil and incubated at 30 °C in a 150 rpm shaker incubator for 4 days. MSM composed of (g/l): KH<sub>2</sub>PO<sub>4</sub> (1.0), K<sub>2</sub>HPO<sub>4</sub> (1.0), NaCl (1.0), CaCl<sub>2</sub> (0.05), (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> (1.0), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.5), FeCl<sub>3</sub> (0.002) and Yeast extract (0.1) (19).

##### 2.4.1 Screening in blue agar medium (CTAB test)

From MSM mutagenized cultures, 50 µl of supernatant was transferred into well punctured blue agar medium plates, (minimal salt agar medium supplemented with CTAB (0.2 g/L) and methylene blue (0.005 g/L)), and incubated at 30°C for 48 hrs. (20). Biosurfactant hyper-producer mutants were identified following the formation of a definite clear bluish halo zone around each well, as compared with the parent strain.

##### 2.4.2 Measurement of emulsification index (E<sub>24</sub>%)

Emulsification index (E<sub>24</sub>%) was employed to describe the emulsifying activity of biosurfactants. In a 10 ml test tube, equal volumes (v/v) of cell-free supernatant and toluene were injected and mixed rapidly for 2 min in a vortex. Test tubes were left vertically at room temperature. The height of the emulsion layer was recorded after 24 h (21). The E<sub>24</sub>% was calculated as the percentage of emulsion layer height (mm) to the total height of mixture (mm), multiplied by 100.

$$E_{24} = (\text{Height of the emulsion layer} / \text{total height of mixture}) \times 100\%$$

##### 2.4.3 Measurement of surface tension

Surface tension is substantial parameter in evaluating the surface activity, determined by K6 tensiometer at room temperature, using the du Nouy platinum ring technique. The tensiometer must be calibrated by distilled water (72 mN/m) prior to sample measurement. From MSM mutagenized cultures, about 20 ml of cell-free supernatant was transferred into a clean glass beaker and placed on the sample table. The height of sample trough was changed so that the platinum ring, hanging from the balance hook, was submerged for 15 min under the sample surface to be equilibrated and then pulled up carefully. The microbalance record the force applied on the ring while pulling it out the liquid surface. When the platinum ring emerges from the liquid, the value is shown as a surface tension of that sample (2, 21).

### 3. Results and Discussion

#### 3.1 Results

##### 3.1.1 Treatment with Acridine Orange

At various periods (15, 30, 45, 60, 100, and 120) min, the effect of different AO concentrations on *P. guguaneensis* strain Iraqi ZG.K.M was studied. The bacterial strain demonstrated resistance to AO at concentrations of 200 mg/l and 400 mg/l even after increasing the incubation period as the colonies grew well without changing their shape and characteristics. No growth was recorded in plates treated with 1000 mg/l or 1200 mg/l of AO. While, 60 min exposure of bacteria to AO at concentrations of 600 mg/l or 800 mg/l resulted in 10% and 1% survival, respectively. Additionally, bacterial growth was inhibited with an increasing incubation period in the presence of the mutagenic agent. Figure (3.1) showed the survival curve of *P. guguaneensis* strain Iraqi ZG.K.M after exposure to 600 mg/l and 800 mg/l of AO in different incubation periods.

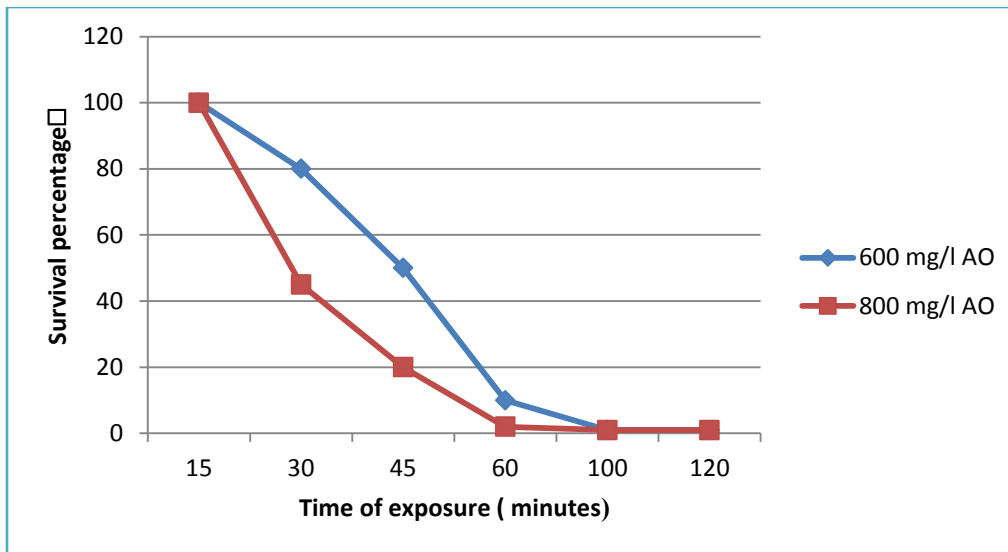


Figure (3.1): Survival curve of *P. guguensis* strain Iraqi ZG.K.M After exposure to AO (600 mg/l, 800 mg/l) at different time interval.

### 3.1.2 Treatment with Ultraviolet (UV) irradiation

The survival count was determined at intervals (10, 20, 30, 60, 120, 300, 600, and 900 sec). Figure (3.2), showed that 97% of cells were killed within 300 seconds of UV exposure and it was mostly less affected by short-term UV exposure (0-60 seconds), while no bacterial growth was recorded when exposed to UV rays for 600-900 seconds.

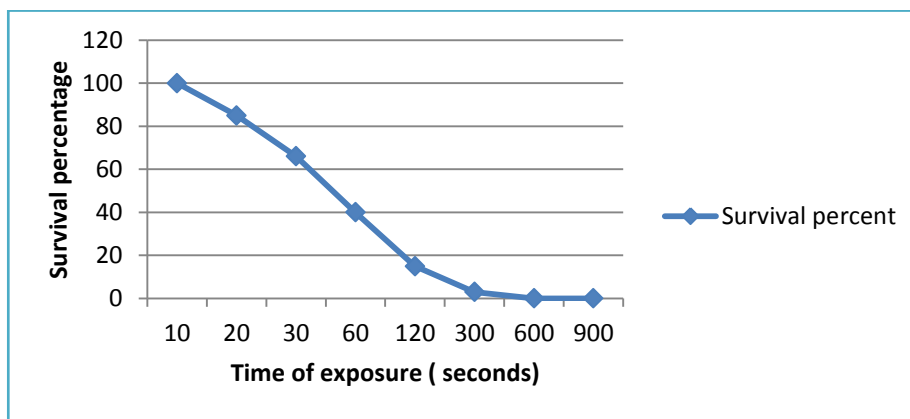
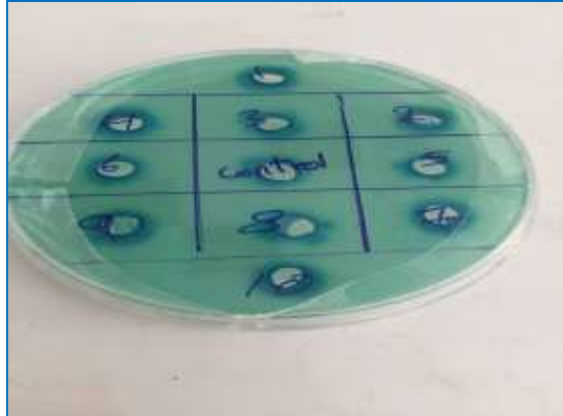


Figure (3.2): Survival curve of *P. guguensis* strain Iraqi ZG.K.M. After exposure to UV irradiation at a different time interval.

### 3.1.3 Detection of biosurfactant hyper producer mutants

#### 3.1.3.1 Screening in blue agar medium (CTAB test)

In blue agar medium, the formation of definite clear bluish halo zones around wells was considered as biosurfactant overproducer mutants of *P. guguensis* strain Iraqi ZG.K.M. Figure (3.3) showed the ability of mutants to form definite clear bluish halo zones compared with the parent.



**Figure (3.3):** Screening of mutants *P. guguaneensis* strain Iraqi ZG.K.M in blue agar medium. Mutants with biosurfactant overproduction can form a definite clear bluish halo zone compared with the parent.

Three colonies from each treatment (AO and UV), (termed ZAOM1 to ZAOM3 and ZUVM1 to ZUVM3, respectively), which gave the largest dark blue halo zone were selected to estimate the emulsification index and surface tension.

### 3.1.3.2 Measurement of emulsification index ( $E_{24}\%$ )

Results, illustrated in figure (3.4), showed a variable emulsification ability of the AO and UV mutants, compared with the wild type strain that recorded  $E_{24}\%$  of 42%. For AO mutants, ZAOM1 showed a high  $E_{24}\%$  similarity to the wild type (41%), while ZAOM2 showed decreasing in emulsification index (23%) as compared with the wild type. However, the ZAOM3 mutant gave an emulsification index higher than the wild type (52%).

For UV mutants, ZUVM1, ZUVM2, and ZUVM3 recorded a high emulsification index compared with the wild type strain. Where, the  $E_{24}\%$  of ZUVM1 and ZUVM3 were 67% and 68%, respectively. ZUVM2 recorded the highest  $E_{24}\%$  of 78%.

### 3.1.3.3 Measurement of surface tension

The measurement of surface activity is the most straightforward screening method that gives a strong indicator for the production of biosurfactant. The wild type strain that grows in optimum conditions recorded a reduction in surface tension of the culture medium to 38 mN/m. In AO mutants, ZAOM1 showed high similarity to the wild type (37 mN/m). ZAOM2 showed increasing in surface tension (45 mN/m) as compared with the wild type, while ZAOM3 mutant can lower the surface activity to 35 mN/m. In contrast, the surface tension of ZUVM1 and ZUVM3 mutants was highly similar to that of the wild type, recorded at 38 mN/m and 37 mN/m, respectively. While ZUVM2 can reduce the surface tension to 33 mN/m. Figure (3.4) showed the  $E_{24}\%$  and the surface activity of *P. guguaneensis* strain Iraqi ZG.K.M mutants after exposure to AO or UV irradiation, compared with the wild type.

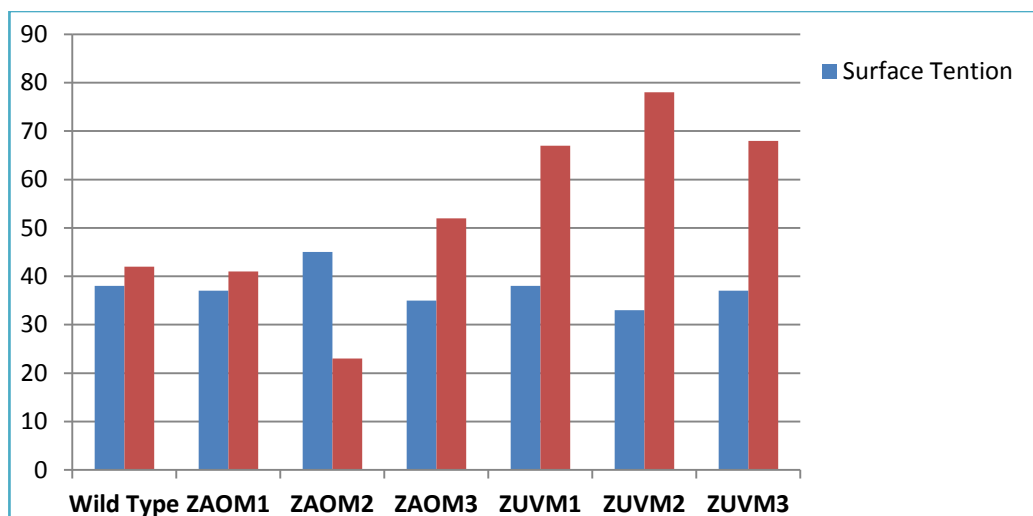


Figure (3.4): The emulsification activity and the surface tension of *P. guguensis* strain Iraqi ZG.K.M mutants after exposure to AO or UV irradiation. The highest E<sub>24</sub>% (78%) and lowest surface tension (33 mN/m) were recorded with the ZUVM2 mutant.

### 3.2 Discussion

Strain improvement is one of the most essential strategies used worldwide to make the cost of biosurfactant production competitive (16). Attempts have been made to raise biosurfactant production, depending on nutrition and environmental factors (19). However, the process of production cannot be commercially viable until the ultimate product yield is naturally high. Improving bacterial strains provides significant opportunity to reduce the cost of production while increasing the desired product (15, 16). Mutation is one of the techniques that can alter the microbial genetic makeup, thereby stimulating their activity and affecting the yield of all biotechnological products (18). Therefore, *P. guguensis* strain Iraqi ZG.K.M was randomly mutagenized, using chemical mutagen Acridine Orange (AO) and physical mutagen (UV light), in an attempt to increase biosurfactant productivity.

#### 3.2.1 Treatment with Acridine Orange

Acridine orange AO is an organic mutagenic agent that intercalates between DNA base pairs, promoting nucleotide bases insertions and deletions during replication. The resultant mutation shifts the translated reading frame of the coded information in the mRNA transcript, resulting in alteration in amino acids sequence at the location of insertion and/or deletion (16, 22).

According to the results, high AO concentration can inhibit bacterial growth due to stress and the toxicity of the chemical agent (18). However, the best concentrations are those that permit the bacteria to grow slowly, which were determined in this study to be 600 mg/l and 800 mg/l. additionally, increasing the incubation period of bacteria with AO may decline the percentage of survival, while the mutant frequency increases. Therefore, 60 min exposure of bacteria to AO at a concentration of 600 mg/l or 800 mg/l was the best incubation period for isolation of mutant, because increasing in incubation period may prevent bacterial growth. Therefore, 60 min incubation period of concentrations 600 mg/l or 800 mg/l was selected for mutant's isolation.

Tahzibi and his colleagues obtained *P. aeruginosa* mutants, derived from random mutagenesis by AO, which improved rhamnolipid production 10 times higher than the parent strain (23). Also, Das and his colleagues used AO and acriflavine to obtain three mutants in *P. aeruginosa* that improved the efficiency of bioremediation and removal of heavy metal-containing waste pollutants (24). In another study, bacterial strains were treated with AO to obtain metal-resistant mutants, particularly of copper and zinc in samples collected from copper factory effluents (25).

### 3.2.2 Treatment with Ultraviolet (UV) irradiation

UV radiation is a toxic mutagenic agent that has biological effects on bacteria mostly via the interaction of oxygen species on the cell membrane, phospholipid bilayer rearrangement, and pore formation (26). Therefore, UV penetration across the cell membrane makes the cross-linked DNA to be closed and prevents DNA replication and transcription (27).

Results indicated that *P. guguaneensis* strain Iraqi ZG.K.M was UV sensitive; it lost its viability exponentially. The long period of exposure to UV leads to a decline in the percentage of bacterial survival, due to the destruction of bacterial strain, while the mutant frequency increased. UV irradiation was the most widely used physical mutagen, that was used successfully with *P. aeruginosa* (16, 18).

### 3.2.3 Detection of biosurfactant hyper-producer mutants

Blue agar medium assay was used to detect biosurfactant hyper-producer mutants as it is a qualitative and semi-quantitative assay. The diameter of the dark blue region is semi-quantitatively proportional to the concentration of biosurfactant (16).

Results indicated that both AO and UV light can cause random mutation. UV light was more efficient in producing biosurfactant mutant than AO, whereas, the highest  $E_{24\%}$  (78%) and lowest surface tension (33 mN/m) were recorded with ZUVM2 mutant. This mutation may occur in the structural gene(s) encoded for biosurfactant, or in the regulatory gene(s) sites that regulate its production positively or negatively. Mutations can impact numerous metabolic pathways involved in the control of byproduct synthesis, which explains the variation in biosurfactant synthesis across mutants (28). In addition, UV rays can cause a loss in the cell membrane segment, thereby increasing cell permeability to metabolites (26).

UV light and AO were successfully used to mutagenize different bacteria. Al- Makadci used UV light to mutagenize *P. aeruginosa*. He successfully isolates hyper-producer mutants of rhamnolipid (29). While AO was used to mutagenize *P. putida*, *P. aeruginosa*, and *A. radioresistens*, mutants that tolerate different concentrations of oil sludge were obtained (17).

## 4. Conclusions

Through random mutagenesis, UV light can successfully use to mutagenize *Pseudomonas guguaneensis* strain Iraqi ZG.K.M to obtain hyper-producer mutants of biosurfactant compared with the wild type. The ZUVM2 mutant can be used for the commercial production of biosurfactant which can be used in many applications.

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## تحسين إنتاج المستحلب الحيوي من بكتريا *Pseudomonas guguanensis* Strain Iraqi ZG.K.M باستخدام الطفرات العشوائية

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### الخلاصة

**خلفية عن الموضوع:** مع تزايد الاهتمام بالبيئة ، ازداد الاهتمام بالبحث والتطوير في مجال المواد الصديقة للبيئة. ان المستحلبات الحيوية الميكروبية الخافضة للشد السطحي هي منتجات حيوية غير سامة وقابلة للتحلل والتي تستخدم على نطاق واسع في التطبيقات الطبية والصيدلانية والزراعية والصناعية والغذائية ومستحضرات التجميل والتكرير. مع ذلك ، فإن التحديات الرئيسية في إنتاج المستحلبات الحيوية الخافضة للشد السطحي هي ارتفاع تكلفة الإنتاج ومحدودية المردود. يعتمد تحسين إنتاج المستحلب الحيوي على العديد من الاستراتيجيات ، مثل استخدام المواد الخام منخفضة التكلفة، وتعديل الظروف و وسط نمو البكتريا ، واختيار السلالات ذات الإنتاجية العالية. **الهدف من الدراسة:** أجريت الدراسة للحصول على سلالة طافرة من بكتريا *Pseudomonas guguanensis* Strain Iraqi ZG.K.M ذات قدرة إنتاجية عالية للمستحلب الحيوي الخافض للشد السطحي من خلال استخدام الطفرات العشوائية. **المواد والطرق العمل:** تم استخدام المطفر الكيميائي (الأكردين البرتقالي) بتركيز مختلفة و المطفر الفيزيائي (الأشعة فوق البنفسجية) لإنتاج الطفرات العشوائية في *Pseudomonas guguanensis* Strain Iraqi ZG.K.M. تم اختيار السلالة ذات الإنتاج العالي للمستحلب الحيوي اعتماداً على اختبار CTAB،  $E_{24}$  % ، وقياس الشد السطحي. **النتائج:** أوضحت النتائج كفاءة الأشعة فوق البنفسجية في إنتاج سلالة طافرة ذات إنتاج عالي للمستحلب الحيوي . حيث تم تسجيل أعلى مؤشر استحلاب  $E_{24}$  % (78) وأقل شد سطحي (33 ملي نيوتن/متر) في السلالة الطافرة ZUVM2. **الخلاصة:** يمكن استخدام الأشعة فوق البنفسجية لإحداث طفرات في *Pseudomonas guguanensis* Strain Iraqi ZG.K.M للحصول على سلالات طافرة ذات إنتاج مفرط للمستحلب الحيوي الخافض للشد السطحي والمستخدم في العديد من التطبيقات.

**الكلمات المفتاحية:** *Pseudomonas guguanensis* strain Iraqi ZG.K.M ، المستحلب الحيوي الخافض للشد السطحي ، فرط الإنتاج.