Effect of Some Physical Mutagens on *Pseudomonas aeruginosa* H3 in Alginate Production

تأثير المطفرات الفيزيائية على قابلية بكتريا *Pseudomonas aeruginosa* H3 في إنتاج الالجينيت

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Abstract

Effect of physical mutagens on the ability of *Pseudomonas aeruginosa* H3 in alginate production was performed by subjection of the cell suspension of *P. aeruginosa* H3 to different doses of UV radiation (2, 4, 6, 8 and 10 J/m^2) and LASER for (30, 60, 90 and 120 sec.), then cell suspension was spread on LB agar plates and incubated at 37 ˚C for 24hrs. After that, random selection of thirty colonies that arose from the cells subjection to the effect of different doses of UV radiation and laser were made (90% of the suspended cells were killed and were tested for their alginate production). Results showed that the mutagenesis by UV radiation caused an increase in the ability of *P. aeruginosa* H3 in alginate production. Over-producer mutant, H3R1, was obtained from this treatment. The productivity of the alginate from this mutant was 170mg/l in comparison with the productivity of wild type (70mg/l). On the other hand, results also showed that mutagenesis using LASER radiation caused an increase in the ability of *P. aeruginosa* H3 in alginate production. Over-producer mutant, H3R42, was obtained from this treatment characterized by its high ability in alginate production (130mg/l) in comparison with the productivity of wild type (70mg/l). Results of Fourier Transform-Infra Red (FT-IR) for alginate produced by the wild type of *P. aeruginosa* H3 and over-producer mutants H3R1 and H3R42 at a wide range of wave lengths absorbance (400-4000) cm^{-1} showed that there were no any structural differences in the chemical structure of alginate produced by them on the basis of infra-red light absorption by different functional groups of alginate

المستخلص

درس تأثير المطفرات الفيزيائية على قابلية بكتريا *Pseudomonas aeruginosa* H3 في إنتاج الالجينيت باستخدام الأشعة فوق البنفسجية (2 , 4 , 6 , 8 و 10) جول/م^2 وأشعة الليزر (30 , 60 و90) ثانية. وقد تمت عملية التطفير مزارع فتية لبكتريا *P. aeruginosa* H3 مع كلا المطفرين كلا على انفراد ثم اخذ 100 مايكولتر من كلا المزرعتين ونشر على وسط LB المتصلب وحضنت الأطباق بدرجة 37 م لمدة 24 ساعة . تم الانتقاء العشوائي لـ 30 مستعمرة من المستعمرات التي تعرضت للتأثير القاتل للمطفر الذي يودي إلى قتل 90% أو

أكثر من الخلايا البكتيرية وغربلة قابليتها على إنتاج الالجينيت . أشارت النتائج إلى إن التطفيرالفيزيائي بالاشعة الفوق بنفسجية واشعة الليزر أدى إلى زيادة قابلية بكتريا*P. aeruginosa* H3 في إنتاج الالجينبت من خلال الحصول على عدد من الطافرات البكتيرية المتميزة باتتاجيتها العالية للالجينيت . إذ بلغ تركيز الالجينيت المنتج من أعلى الطافرات إنتاجا (H3R1 و H3R42) (130،170) ملغم/لتر بعد تعرضها للأشعة الفوق بنفسجية واشعة الليزر على التوالي بالمقارنة مع تركيز 70 ملغم/لتر المنتج من النوع البري لبكتريا *P. aeruginosa* H3 . وقد أشارت نتائج تحليل طيف الامتصاص الأشعة تحت الحمراء(FT-IR) على مدى واسع من الأطوال الموجية تراوح بين (400 – 4000) /$^{-1}$سم إلى عدم وجود أي اختلافات تركيبية في التركيب الكيمياوي للالجينيت المنتج من النوع البري لبكتريا *P. aeruginosa* H3 والطافرات البكتيرية عالية الإنتاج الناشئة عنها .

Introduction:

Alginate is a linear unbranched polymer of D- Mannuronic acid and its C5 epimer L-guluronic acid, which are linked by β-1, 4-glycosidic bonds. In bacteria, alginate is modified by the addition of O-acetyl groups on some D-mannuronic acid residues [1, 2]. Alginates are one of the exopolysaccharides which act as extracellular material that allows the formation of differentiated biofilms, with restrict diffusion of clinical antibiotics and protect embedded cell against human antibacterial defense mechanisms [3]. These polysaccharides are commercially important because of the gelling and colloidal properties. It has a variety of uses, as an ingredient of photographic emulsions, dental impression material and as additive in various food stuff [4]. It was also used as thickening agent in the textile, paper industries, wound healing and immobilization by microencapsulation [5].

Alginate is one of the few polymers synthesized by some eukaryotic and prokaryotic organisms. The eukaryotic source of alginate is mainly the marine algae. However, amongst the prokaryotes, two bacterial genera *Azotobacter* and *Pseudomonas* are known to contain species capable of alginate production [4]. The genetic and biosynthesis pathway of alginate in *Pseudomonas aeruginosa* has been extensively studied due to its role in the disease of cystic fibrosis which is a cause of morbidity and mortality [6].

Because of the importance of alginate as a perfect microbial polysaccharide for different uses and applications, this study was aimed to enhance the ability of highly alginate producer isolate *P. aeruginosa* H3 in alginate production by physical mutagenesis using UV and LASER radiation.

Materials and Methods

Bacterial isolate:

Pseudomonas aeruginosa H3 characterized by its high ability in alginate production was obtained from [7]. This isolate was maintained on nutrient agar plates. Ability of this isolate in alginate production before and after mutagenesis was tested using production medium described by [7] which consists of (g/l): KH2PO4, 0.05; MgSO4.7H2O, 0.2; CaCl2.2H2O, 0.1; FeSO4.9H2O, 0.05; Na2MO4.2H2O, 0.007; commercial baker's yeast, 1; and date extract, 4%v/v.

Physical Mutagenesis:

Fresh culture of *P. aeruginosa* H3 was used to inoculate LB medium and incubated at 37 °C till mid log phase, then cells were precipitated at 6000 rpm for 15 min., washed and resuspended in 5 ml phosphate buffer, to prepare for mutagenesis by subjecting to the physical mutagen UV and LASER respectively. For the former, mutagenesis was achieved according to [8], by subjecting *P. aeruginosa* H3 cell suspension with Phosphate buffer to (0, 2, 4, 6, 8 and 10) J/m^2 of UV radiation in a dark place, while mutagenesis with LASER was done according to [9], by subjecting the cell suspension of *P. aeruginosa* H3 to LASER for different periods (0, 30, 60, 90 and 120 sec). For UV radiation, 0.1ml aliquots of cell suspension was taken after each treatment and diluted properly, then spread on LB agar plates. But for the LASER, 0.1ml aliquots was taken from the cell suspension after each time of irradiation and spread on LB agar plates. In both cases, the plates were incubated at 37°C for 24 hrs to account the viable and screening the survivals for its ability in alginate production.

Extraction of Alginate

Alginate was extracted from culture medium using alcohol precipitation method [10], to determine alginate dry weight.

Characterization of Alginate Using FT-IR

Chemical structure of alginate produced by the wild type and over producer mutants of *P. aeruginosa* H3 was examined using Fourier Transform-Infrared Spectrometry (FTIR) that identify functional groups in the alginate structure, and determine the wave length absorbance of each group.

Results and Discussion

In order to improve the ability of P. *aeruginosa* H3 in alginate production, this isolate was subjected to physical mutagenesis by UV and LASER radiation respectively. Results indicated in figure (1) showed the killing effect of UV on bacterial cells, total viable count of *P. aeruginosa* H3 was decreased from 1.31×10^9 CFU/ml in the zero time to 0.35×10^9 CFU/ml after the first exposure to $2J/m^2$ of UV radiation. Survival percent was 26.7% after the first exposure followed by severe reduction to 5.34%, 4% and 2% for the next different UV radiation doses. Colonies obtained after subjection to the killing effect of mutagen (90% killing and more) was screened to examine its ability in alginate production because the most possible mutagenic effect of UV ray occurs at killing effect between 90-100 % [11]. These were achieved by random selection of 30 colonies and were used to inoculate LB broth medium, incubated at 37 °C for 24 hrs. with shaking (150rpm), then 0.1ml of the culture medium was used to inoculate alginate production medium. Results mentioned in table (1) showed that there are numbers of over producer mutants characterized with their high ability in alginate production. Alginate produced by the highly over producer mutant was 170 mg/l in comparison with the productivity of wild type (70 mg/l), while the productivity of other over producer mutants was ranged between 80-160 mg/l. The increase of alginate

production by these mutants may be due to the effect of the mutagen in the inactivation of regulatory genes (*muc* A, *muc* B, *muc* C and *muc* D) leading to an increasing in alginate biosynthesis by direct action of *alg* D promoter or indirectly by up- regulation transcription of another regulatory gene *alg* R as it was mentioned by Browning *et al* and Martin *et al* [12, 13].

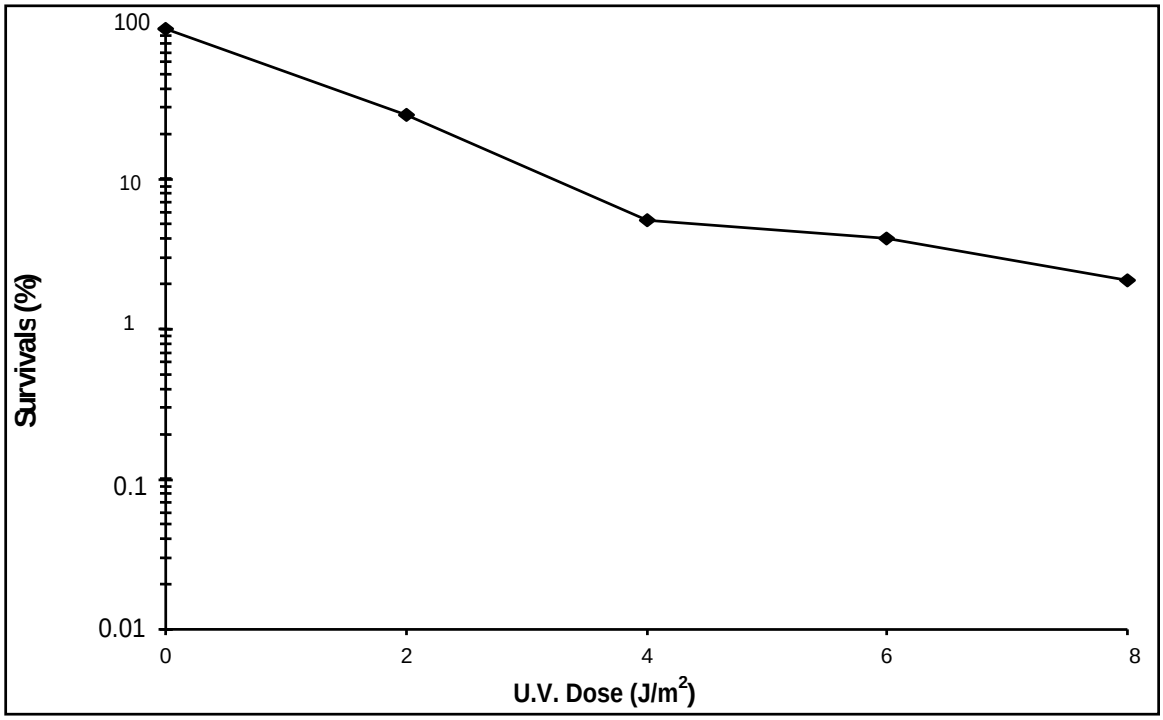

Figure (1): Effect of different doses of UV radiation on the survival of *P. aeruginosa* H3.

Table (1): Ability of alginate production by *P. aeruginosa* H3 mutants after irradiation with UV Radiation.

Bacterial mutant	Alginate concentration (mg/l)
P. aeruginosa H3 wild type	70
P. aeruginosa H3R1	170
P. aeruginosa H3R2	160
P. aeruginosa H3R3	70
P. aeruginosa H3R4	100
P. aeruginosa H3R5	30
P. aeruginosa H3R6	90
P. aeruginosa H3R7	60
P. aeruginosa H3R8	100
P. aeruginosa H3R9	100
P. aeruginosa H3R10	80
P. aeruginosa H3R11	90
P. aeruginosa H3R12	140
P. aeruginosa H3R13	120
P. aeruginosa H3R14	40
P. aeruginosa H3R15	60
*P. aeruginosa*H3R16	140
P. aeruginosa H3R17	80
P. aeruginosa H3R18	60
P. aeruginosa H3R19	90
P. aeruginosa H3R20	100
P. aeruginosa H3R21	70
P. aeruginosa H3R22	70
P. aeruginosa H3R23	80
P. aeruginosa H3R24	70
P. aeruginosa H3R25	70
P. aeruginosa H3R26	80
P. aeruginosa H3R27	60
P. aeruginosa H3R28	60
P. aeruginosa H3R29	70
P. aeruginosa H3R30	70

On the other hand mutagenesis using UV radiation caused a decrease in the ability of the other mutants of *P. aeruginosa* H3 (H3R5, H3R7, H3R14, H3R15, H3R18, H3R27 and H3R28) in alginate production. Alginate produced by these mutants ranged between 30 mg/l to 60 mg/l in comparison with the productivity of wild type (70 mg/l). Reduction in alginate production by these mutants may be due to the genetic mutations induced in the structural genes responsible for alginate biosynthesis pathway (4), or due to the mutations that may occur in the regulatory genes (*alg* U, *muc* A, *muc* B and *muc* C) which compromise the main switch controlling the conversion to the mucoidal growth (alginate production) [12].

Results mentioned in table (1) also showed that the ability of the other mutants (H3R3, H3R21, H3R22, H3R24, H3R25, H3R29 and H3R30) do not affected during mutagenesis by UV, and this may be due to the genetic mutations in different genes of the chromosomal DNA of *P. aeruginosa* other than those responsible for production and regulation of alginate biosynthesis [12,13]. UV irradiation affects via miss repair of the damaged DNA by SOS repair system and termed indirect mutagen [14, 15].

Another type of Physical mutagens (LASER) was used to generate over producer mutants of alginate from *P. aeruginosa* H3. Results indicated in figure (2) showed that this mutagen had a lethal effect on the bacterial cells of *P. aeruginosa* H3; this can be noticed from the reduction of the total viable count of bacterial cells from $6.3x10^{10}$ CFU/ml in the zero time to $2.2x10^{10}$ CFU/ml after the first time of exposure (30 sec) to LASER. Survival percent was decreased after exposure to this dose to 34.9%, while the next incubation periods (60, 90, 120 sec.) caused severe reduction in the survival percentage (26.9%, 9.68%, 2% respectively). After subjection to the mutagen for the previous periods, 100µl aliquots of the cell suspension subjected to the killing effect of mutagen (90% killing and more) was taken and spread on LB agar plates and incubated at 37 °C for 24 hrs., then the resultant colonies were screened to detect its ability in alginate production by random selection of 30 colonies and were used to inoculate LB broth medium, incubated at 37 °C for 24 hrs. with shaking (150 rpm),then 0.1ml of the culture medium was taken and used to inoculate alginate production medium. Results illustrated in table (2) showed that there is highly over producer mutant (H3R42 mutant) characterized with its high ability in alginate production.

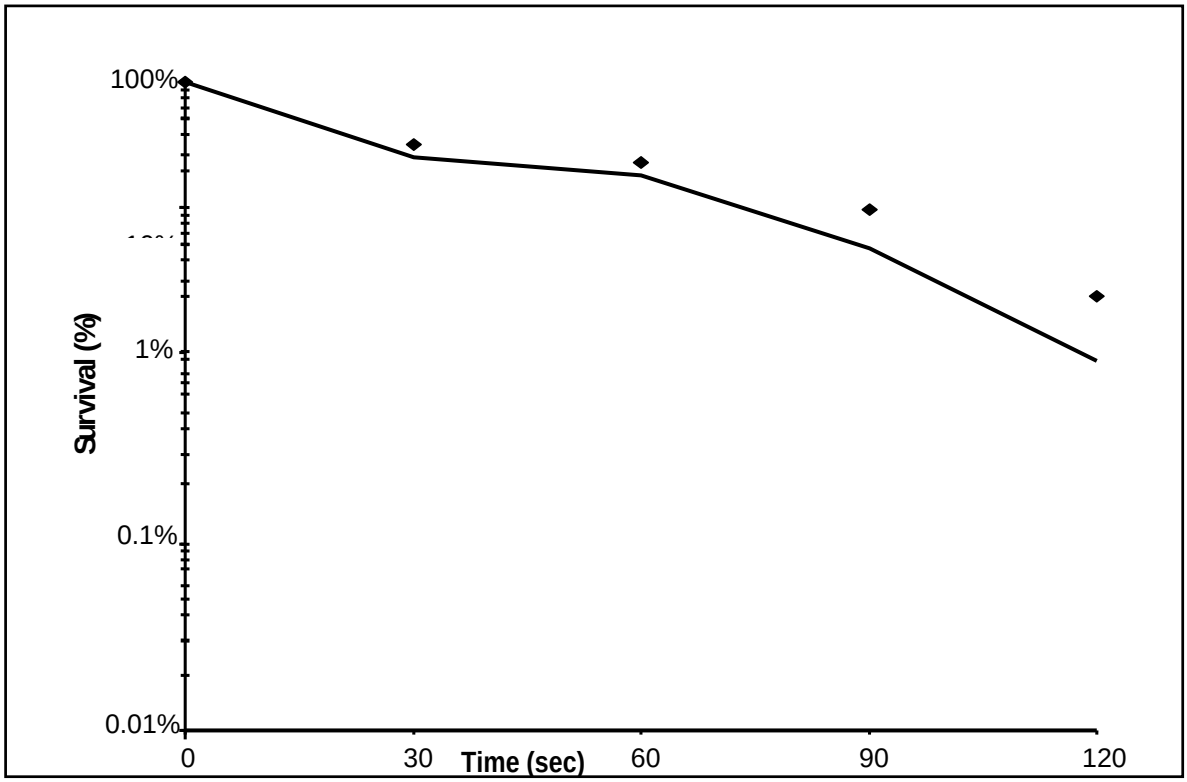

Figure (2): Effect of LASER radiation on the survivals of *P. aeruginosa* H3 after exposure for different time periods.

Productivity of crude alginate produced by this mutant was 130 mg/L in comparison with the productivity of the wild type (70 mg/l) which represents the enhancing effect of LASER to the ability of *P. aeruginosa* H3 in alginate production. On the other hand there is other less over producer mutants (H3R31, H3R32, H3R34, H3R36, H3R38, H3R39, H3R40, H3R41, H3R43, H3R46, H3R47, H3R49, H3R50, H3R53 H3R56 and H3R58) were obtained after mutagenesis with LASER. The productivity of crude alginate from these mutants ranged between 80 to 120mg/l. The high ability of alginate production by all these over producer mutants may be due to the genetic mutations that may be inactivate one or more of the regulatory genes, that regulates negatively alginate production by *P. aeruginosa* H3 [16].

Table (2): Ability of *P. aeruginosa* H3 mutants in alginate production after irradiation with LASER.

Bacterial mutant	Alginate concentration (mg/l)
P. aeruginosa H3 wild type	70
P. aeruginosa H3R31	100
P. aeruginosa H3R32	90
P. aeruginosa H3R33	70
P. aeruginosa H3R34	80
P. aeruginosa H3R35	50
P. aeruginosa H3R36	90
P. aeruginosa H3R37	60
P. aeruginosa H3R38	100
P. aeruginosa H3R39	100
P. aeruginosa H3R40	80
P. aeruginosa H3R41	90
P. aeruginosa H3R42	130
P. aeruginosa H3R43	120
P. aeruginosa H3R44	70
P. aeruginosa H3R45	60
P. aeruginosa H3R46	110
P. aeruginosa H3R47	80
P. aeruginosa H3R48	60
P. aeruginosa H3R49	90
P. aeruginosa H3R50	100
P. aeruginosa H3R51	70
P. aeruginosa H3R52	70
P. aeruginosa H3R53	80
P. aeruginosa H3R54	70
P. aeruginosa H3R55	70
P. aeruginosa H3R56	80
P. aeruginosa H3R57	60
P. aeruginosa H3R58	90
P. aeruginosa H3R59	70
P. aeruginosa H3R60	70

Results indicated in table (2) also showed that there are other mutants symbol H3R35, H3R37, H3R45, H3R48 and H3R57 respectively with low ability in alginate production. Crude alginate produced by these mutants ranged between 50 to 60 mg/l, in comparison with the productivity of wild type (70 mg/l).This results may be due to the genetic mutations occurred in the regulatory genes, that regulates positively alginate production,

the other mutants are with no difference in their ability in alginate production in comparison with the wild type (70 mg/l).This result may be may be due to the incidence of the mutations in different genes other than alginate biosynthesis genes on the chromosomal DNA of *P. aeruginosa* H3 [17] . LASER induced cell inactivation is due to the induction of DNA damage [18].

From these results it could be concluded that Physical mutagens by UV radiation successfully enhanced alginate production from *P.aeruginosa* H3 since its productivity 2.43 fold higher than the alginate produced by the wild type. Also, laser irradiation successfully developed the alginate productivity about 1.86 fold higher than the alginate produced by the wild type. By comparing the highest alginate concentration 170mg/l for mutant (H3R1) that obtained after UV radiation and 130mg/L for mutant (H3R42) after Laser exposure, UV radiation was better than Laser in enhancing alginate productivity.

Alginate produced by wild type and mutants of *P. aeruginosa* H3 after mutagenesis with Physical mutagens was analyzed using FTIR chromatography on a wide range of wave lengths (400-4000) cm^{-1}, to detect any structural differences in the chemical structure of alginate. Results mentioned in Table (3) showed that there is no any structural differences in the chemical structure of alginate produced by the wild type of *P. aeruginosa* H3 and the highly over producer mutants (H3R1 and H3R42) that arose after mutagenesis with UV and LASER irradiation respectively. The absorbencies of the active groups of alginate structure occur nearly the same wave lengths (for hydroxyl group, carbonyl group and ether group). From the mentioned results, O-H broad band of alginate from the wild type (H3) and mutants (H3R1and H3R42) were approximately the same. Also for the C=O and C-O-C bands show no differences.

Table (3): Absorbance of the active groups of alginate produced by the wild type of *P. aeruginosa* H3 and over-producing mutants.

Bacterial mutant	Mutagen	Functional Group	Wave Length (cm^{-1})
P. aeruginosa H3 (wild type)	—	O-H	3417.63
		C=O	1743.53
		C-O-C	1249.79
P. aeruginosa H3R1 mutant	UV radiation	O-H	3425.34
		C=O	1743.53
		C-O-C	1249.79
P. aeruginosa H3R42 mutant	LASER	O-H	3379.05
		C=O	1743.53
		C-O-C	1249.79

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