INDUCTION OF RHAMNOLIPID PRODUCTION BY *PSEUDOMONAS AERUGINOSA* A3 USING CHEMICAL AND PHYSICAL MUTAGENIC FACTORS

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ABSTRACT

This study was depend to select *Pseudomonas aeruginosa* A3 as a good producer of rhamnolipid (RL) biosurfactant after screening on agar plate where it was able to biosynthesize 4.3 g/L with emulsification index 52% and reducing the surface tension of water to 33.2 mN/m. Therefore, this study was aimed to increase the production of biosurfactant from selected isolate by exposure to several physical and chemical mutagenic factors like gamma radiation, nitrosoguanidine, ethyl methane sulfonate and lithium chloride. The results were shown that 0.2 g/L of nitrosoguanidine was the best mutant for increasing the production to about 2 folds (9.4 g/L) after 15 mins exposure to this material, as well as the emulsification index and surface tension of water were reached to 62% and 26.1 mN/m, respectively, comparing with non-mutant isolate. Also, the critical micelle concentration (CMC) and critical micelle dilution (CMD) of produced rhamnolipid were reached to 120 mg/L and 100 fold, respectively. The optimum conditions of RL production from mutant isolate were determined as 34ºC a best temperature, 6.5 optimum pH and incubation period of 108 h where the production was reached to 10.6 g/L and emulsification index 64% with surface tension of water 26 mN/m. characterization study of purified RL by using thin layer chromatography (TLC) analysis indicated that it was composed of a mixture of mono and di-rhamnolipid.

Keywords: biosurfactant, emulsification, gamma rays, nitrosoguanidine, chromatography.

مجلة العلوم الزراعية العراقية -2019 :50(4):1174-1185 فخري وآخرون

حث انتاج الرامنولبد بواسطة *PSEUDOMONAS AERUGINOSA* A3 باستخدام عوامل فيزيائية وكيميائية مطفرة

أيوب مرتضى الشيخ فخري ناظم حسن حيدر عبد الكريم جاسم هاشم
باحث أستاذ أستاذ

المستخلص

اعتمدت الدراسة الحالية إلى انتخاب العزلة *Pseudomonas aeruginosa* A3 كأفضل عزلة منتجة لمستحلب الرامنولبد بعد غربلتها على الوسط الصلب، حيث انتجت ما يعادل 4.3 غم/لتر من المستحلب بمعامل استحلاب 52% وبمعدل اختزال الشد السطحي للوسط إلى 33.2 ملي نيوتن/متر. لذا هدفت الدراسة إلى استخدام عدة مطفرات (فيزياوية وكيمياوية) لغرض تحفيز زيادة انتاج الرامنولبد من العزلة المنتجة وهي أشعة كاما، نايتروزوكوانيدين، اثيل ميثان سولفونيت وكلوريد الليثيوم. بينت النتائج ان مادة النايتروزوكوانيدين بتركيز 0.2 غم/لتر هي الأفضل حيث زاد الانتاج إلى الضعف تقريبا (9.4 غم/لتر) بعد تعريضها لمدة 15 دقيقة للمادة المطفرة، مقارنة بالعزلة الغير مطفرة. كذلك زاد معدل الاستحلاب إلى 62% وانخفض الشد السطحي للوسط إلى 26.1 ملي نيوتن/متر، كما وصل تخفيف المذيلات الحرج إلى 100 ضعف حيث كان تركيزه مساويا إلى 120 ملغم/لتر. كذلك بينت النتائج ان درجة الحرارة المثلى لإنتاج الرامنولبد من العزلة المطفرة هي 34ºم، مع افضل دالة حموضة 6.5 وبفترة حضن 108 ساعة، حيث وصل الانتاج إلى 10.6 غم/لتر بمعدل استحلاب 64% وشد سطحي للوسط 26 ملي نيوتن/متر. بينت الدراسة أن الرامنولبد هو مزيج من احادي وثنائي الرامنولبد بعد تنقية وتوصيف المنتوج باستخدام كروماتوغرافيا الطبقة الرقيقة.

الكلمات المفتاحية: مستحلب حيوي، استحلاب، أشعة كاما، نايتروزوكوانيدين، كروماتوغرافيا

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INTRODUCTION

To date, many microbial-produced biological macromolecules that exhibit highly surface activity and emulsifying potential, these compounds classified as biosurfactants, which are secondary metabolites, and as such, their biosynthesis corresponds with the beginning of the idiophase of microbial growth. They are superior over their chemical counterparts in biodegradability, biocompatibility, functional and stability at extreme temperature, pH and salinity, potentially high activities, low toxicity, and productivity from renewable substrates (39, 15). *Pseudomonas aeruginosa* is one of the opportunistic pathogens responsible of chronic lung infection in patients with cystic fibrosis. This bacterium is able to produce some extra-cellular virulence factors, including the biosurfactant rhamnolipid (RL) (38, 13). These are low-molecular-mass amphipathic molecules that possess both hydrophobic and hydrophilic moieties which allow them partitioning the interfaces between fluids with different degrees of polarity, such interfaces are oil-water or water-air phases (16). Such biosurfactants tend to decrease the surface tension at interfaces of hydrocarbon-water that results in pseudo-solubilization of these hydrocarbons through micelle or vesicle formation. This will increase the bioavailability and further degradation of such hydrophobic compounds (41, 33). The importance of these biosurfactants lies in motility, cell-cell interaction (pathogenicity, biofilm formation, amensalism, maintenance and maturation, and quorum sensing), substrate accession (direct interfacial contact with nutrients), as well as escaping toxic elements and compounds (12, 27). Rhamnolipids are well-characterized and composed basically of one or two hydrophilic rhamnose moieties bind glycosidically with lipid fatty acid tail, which are β-hydroxycarboxylic acid subunits (29, 1). The properties of this surfactant depend on its chemical structure and distribution of congeners that vary according to the producing strain, medium composition (carbon and nitrogen sources), and culture conditions (36). The carbon source is considered an important limiting factor that could alter both quantity and quality of the biosurfactants (10, 7). There are four types of rhamnolipids, mono-rhamno-di-lipidic (RL1) and mono-rhamno-mono-lipidic (RL3) which consist of one L-rhamnose molecule linked with two or one β-hydroxydecanoic acid chain, respectively. The other two types are di-rhamno-di-lipidic (RL2) and di-rhamno-mono-lipidic (RL4), which consist of two L-rhamnose molecules linked with two or one β-hydroxydecanoic acid chains, respectively (Figure 1) (42, 22). Although having important biological activities, RLs production on commercial level have many obstacles such as low productivity and costly production and recovery which are the most deterrents for using them in large industries. Different strategies exploited to overcome such hurdles, including optimization of the fermentation procedure, using of cost-effective substrates for microbial growth, development of genetically modified organisms as well as scaling up processes (25, 28). Therefore any successful alteration, even little, may improve the efficiency as well as economy of the process (20).

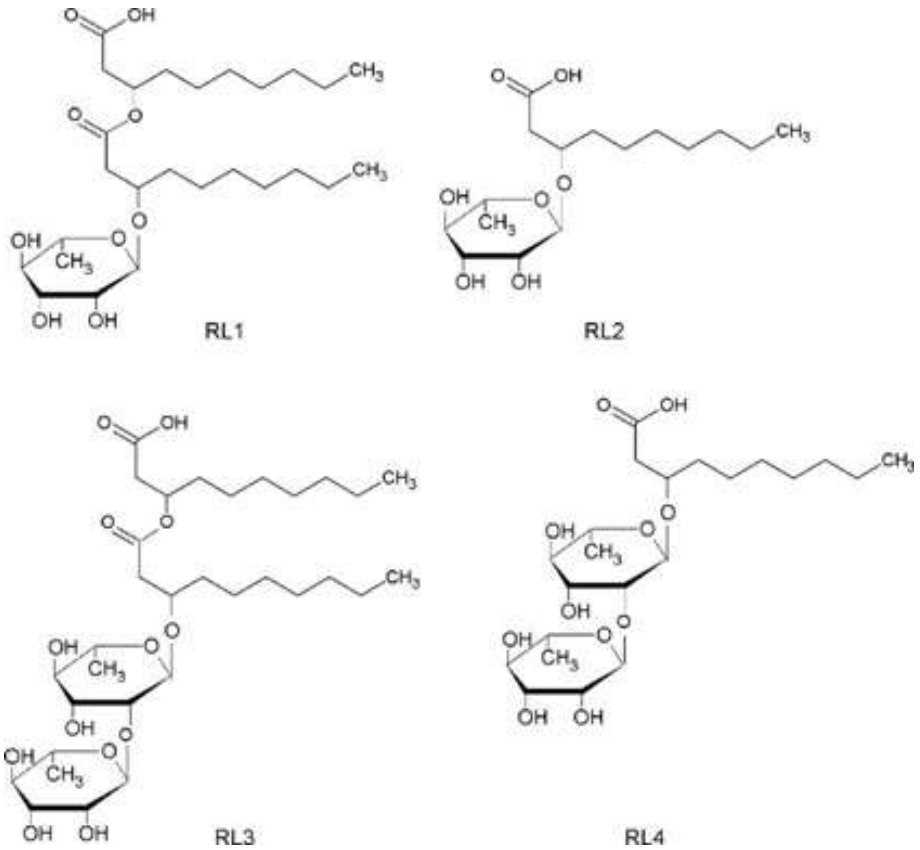

Figure 1. Structure of main rhamnolipid types (42).

There are many literatures that study the effect of physical and chemical mutagenic factors on RL production, like gamma radiation, N-methyl-N'-nitro-N-nitrosoguanidine, ethyl methane sulfonate and lithium chloride. Nitrosoguanidine is one of the most effective mutagenic compounds that able to induce mutation in the DNA repair pathway and considered to be more effective than UV light (40). The aim of this study is enhancement of RL production by chemical and physical mutagenic factors mentioned above using definitive concentrations with a brief exposure time.

MATERIALS AND METHODS

Selected microorganism

Serial ten-fold dilutions of 150 soil samples taken from hydrocarbons contaminated areas were cultured on cetramide agar plates supplemented with crude oil as a selective method for isolation of *Pseudomonas aeruginosa.* The isolates were diagnosed using Vitek 2 compact system and were preserved in brain heart infusion broth supplemented 20% glycerol at -20ºC (5). Tests were carried out according to manufacturer's procedures.

Screening method

The over-producing isolates of RL were detected using the semi-quantitative method that described by Siegmund and Wagner (35). Briefly, Proteose-peptone glucose ammonium slats (PPGAS) agar was supplemented with methylene blue-CTAB that forms ion-pairing with the anionic surfactant produced by *P. aeruginosa*. The composition of medium as follows (g/l): NH_4Cl, 1.0; KCl, 1.5; $MgSO_4$, 0.19; Tris-HCl, 14.5; proteose-peptone 10.0; glucose, 5.0; CTAB, 0.2; methylene blue, 0.0025; Agar, 15.0. The media was autoclaved for 15 mins at 121ºC and 2 ml of trace elements solution composed of (g/l): $FeSO4.7H_2O$, 2.0; $MnSO_4.H2O$, 1.5; $(NH_4)6Mo7O24.4H_2O$, 0.6; $ZnSO4.7H_2O$, 0.29; and $CuSO4.5H_2O$, 0.25; sterilized by cellulose acetate Millipore filter (0.22µm) was added to the medium when warmed down after autoclaving. Twenty five µl of 24 h old culture supernatant of selected isolate was added pre-cut wells in the agar plate and incubated for 48 h at 37ºC, the plates then placed in refrigerator 4ºC for 72 h to enhance the blue halo zone over the light blue background. The diameter of the halo is an indicator for the potency of the producing isolate.

Production medium

Batch culture production of RL by selected *P. aeruginosa* isolate was performed in aqueous medium which is same as medium mentioned above (PPGAS medium) without agar and methylene blue, 50 ml of medium placed in 250 ml Erlenmeyer flask inoculated with overnight grown culture of the chosen isolate with turbidity of 0.65 measured by spectrophotometer at 600nm, which is corresponding to 10^8 CFU. The experiment carried out in thrice and incubated with shaking (150 rpm) at 30ºC for 96h (9).

Measurements of the produced RL properties

For evaluation of produced RL, emulsification index (E_{24}), surface tension, biosurfactant concentration, critical micelle dilution (CMD) and critical micelle concentration (CMC) were measured to assign the powerful of produced glycolipid, as these parameters are the most important to assess the activity of biosurfactant (30).

Emulsification assay

Emulsification index (E_{24}) was conducted according to Cooper and Goldenberg (8). In brief, equal volumes of supernatant and petroleum were mixed vigorously by vortex for 2 min and allowed to stand for 24 h. The E_{24} was evaluated by a ratio between the heights of emulsion to the total height of mixture.

Surface tension measurement

The surface tension of an aqueous solution was measured by the Wilhelmy platinum plate with a QBZY-2 Tensiometer (China). Fifteen ml of supernatant was poured into 50 ml glass beaker and put onto the tensiometer platform. The measurement was carried out at 25±1 ºC after dipping the plate in the solution until monitoring the value of supernatant surface tension following the procedure of measurement written in the manual of the instrument. Between each measurement, the Wilhelmy plate was rinsed with acetone and burned by alcohol burner. For more accurate value, the average of three records was used in the study (30).

Rhamnolipid extraction

The broth culture was extracted from biomass by centrifugation 10,000 rpm for 15 mins, the pH of crude culture was adjusted by 1 N HCl, left overnight for precipitation, then collected through centrifugation at 10,000 rpm for 20 mins. The supernatant was extracted with an equivalent volume of chloroform: methanol (2 : 1 v/v). The median scum layer was separated and the superior aqueous phase re-extracted with similar volumes of solvents above. The merged extracts were concentrated by evaporation at 40° C to obtain honey-colored dried extract that determined gravimetrically (37, 43).

Crtitcal micelle dilution (CMD) and critical micelle concentration (CMC)

CMC is a concentration at which the surface tension of a solution reached a point that any further addition of surface-active agents has little effect on reducing that value, and will aggregate as micelles. The CMC can be calculated by plotting the surface tension as a function of biosurfactant concentration as the curve slope abruptly changes at the point of CMC, which called the intersection point (34); while CMD is the maximum dilution that reduces the surface tension to the bottom level (6). For measuring critical micelle concentration (CMC) of rhamnolipid after extraction, concentrations range (5-300 mg/L) were prepared at room temperature, the surface tension of these solutions were recorded (23).

Exposure to physical factors

Gamma radiator device was used to mutate actively growing *P. aeruginosa* by applying two different doses exposure of 100 and 600 rad corresponding to 4 and 24 h, respectively. The treated cells were used to inoculate fresh PPGAS broth medium which constitute same chemicals mentioned above except of methylene blue. The production medium was incubated at 30ºC for 96 h with 150 rpm shaking. Surface tension, E_{24} and other tests were performed in comparison with control (26).

Exposure to chemical factors

Overnight culture of the selected *P. aeruginosa* isolate was inoculated into phosphate buffer (pH=7) and adjusted with 0.5 tube MacFarland to obtain 1.5×10^8 CFU. The previous buffer solution contains different concentrations of each of nitrorsoguanidine, ethyl methane sulfonate and lithium chloride (0.05, 0.1, 0.2, 0.5 and 1.0 g/L) incubated at

37ºC for 15 min in oscillatory incubator. The treatment was terminated by diluting the solution 20 times with prepared mineral salt medium and incubated at 30º C in shaker incubator for 96 h. The experiment performed in thrice and compared with a non-treated culture as a control (42, 2).

Effect of environmental factors

Different environmental factors were studied to evaluate the ability of isolate to produce maximum RL amount regarding the intensity of each factor. These include incubation temperatures ranged from 25-40, pH of production medium (4, 5, 6, 6.5, 7 and 8) and incubation time ranged from 10 to 168h.

Biosurfactant purification

The RLs components were separated from the crude mixture as follows. Heat activated silica gel 60 slurry in chloroform was decanted onto a column of a glass chromatography (3.5 × 30cm). Two grams of crude extracted RL were dissolved in chloroform (4 ml) and added onto the column. Then the column washed with chloroform at a flow rate of 60 ml/h to elute neutral lipids totally, followed by two steps of chloroform/methanol addition at volumes s of 250 and 200 ml with 50:3 and 50:5 ratios, respectively, for separation of mono-RL. Final elution was done by same solvents above at 50:50 ratio and pure methanol, each volume of 100 ml, to elute di-RL. The fractions composition was checked by thin layer chromatography (32).

Analyzing of RL by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) technique was used to characterize the biosurfactants after purification method to determine their types. The process was conducted by TLC plates (10 × 10 cm) coated with silica gel. Separation and analysis of the produced biosurfactant proceed through a solvent system comprised chloroform, methanol and acetic acid in a ratio of 65:15:2, respectively. After that, the paper sprayed with resolving solution composing of 0.15g of orcinol dissolved in 8 ml of 60% H_2SO_4 and the final volume completed to 50 ml with deionized distilled water, left for 10 mins in oven at 110ºC. The appearance of brown spots was considered as positive test for the glycolipid biosurfactant and compared with standard RL and according to the R_f value which is rate of retention flow calculated by the formula:

R_f= Distance of sample spot/ Distance of solvent (46).

RESULTS AND DISCUSSION

Selection of a good RL-producing isolate

An outstanding biosurfactant-producing isolate means that this secondary metabolite is able to manifest high emulsifying activity and reduction in surface tension of water to minimal value (19). A total 168 microbial pure cultures were isolated from hydrocarbon contaminated soil samples. The best screening method on solid medium so far used for the detection of extracellular glycolipids including RL is the methylene blue-CTAB agar. The ability of the anionic biosurfactant to form insoluble ion-pairing with the cationic surfactant CTAB and appeared as gloomy blue halo in contrast to the surrounding light blue color of the agar plate is a good indicator for RL-producing *P. aeruginosa*. Only 32 isolates were able to form dark-blue halo zones on light background of CTAB-methylene blue agar (Figure 2). Five of these were able to reduce the surface tension below 35 mN/m with E24 greater than 50% after using the supernatant that contain the extracellular biosurfactant. The *P. aeruginosa* A3 was the best isolate to fulfill this task producing 4.3 g/L RL and was selected for further experiments.

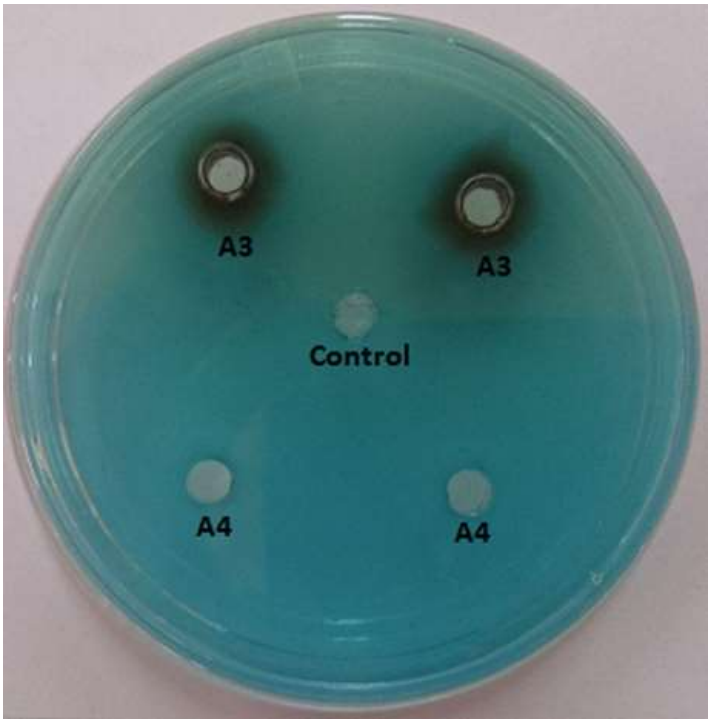

Figure 2. Dark blue halos of anionic biosurfactant of CTAB-methylene blue agar incubated for 48h at 37ºC

Mutagenesis of *P. aeruginosa*

It was found that different treatments of each gamma radiation, ethyl methane sulfonate and lithium chloride lead to equal or less quantities of RL compared to control (data not shown), while nitrosuguanidine have a positive impact in certain concentration. *Pseudomonas* is sensitive to nitrosoguanidine; there was a decrease in bacterial No. with increasing exposure to this mutagenic agent. The fact that NTG is a potential mutagenic agent is known through the ability of this compound to create mispairing lesion by inserting methyl group to different sites on DNA therefore miss its replication or damage repairing mechanism (14). The maximum amount of rhamnolipid produced was 9.4 g/L when the cells treated with 0.2 g/L of NTG. This was more than two-fold of untreated cells. The results were shown that the lowest value of surface tension (26.1 mN/m) was gained with the supernatant of that experiment with emulsification activity of petroleum 62%, which is similar value observed by George and Jayachandran (15) when emulsifying kerosene. Other treatments revealed no such increase in the amount of RL produced (Table 1). The mutation probably happened in genes responsible for regulation of RL production (rhlR and rhlI) which control the biosurfactant production in *P. aeruginosa* (2). A study by Iqbal *et al*., (17) concluded that *P. aeruginosa* subjected to 200-800 rad of gamma rays becane hyperproductive by 2-3 times compared with the parent isolate.

Table 1. Properties of RL produced during the experiments

Treatment	Surface tension mN/m	EI24 (%)	Rhamnolipid (g/L)
Fresh medium	**58.0**	**0.0%**	**0.0**
untreated *P. aeruginosa*	**29.0**	**52%**	**4.3**
NTG 1 g/L	**41**	**32%**	**2.9**
NTG 0.5 g/L	**34.0**	**41%**	**3.8**
NTG 0.2 g/L	**26.1**	**62%**	**9.4**
NTG 0.1 g/L	**28.8**	**57%**	**6.7**
NTG 0.05 g/L	**32.3**	**45%**	**4.6**

A similar approach conducted by Tahzibi *et al*., (42) and found that *P. aeruginosa* PTCC1637 treated with 0.1 g/L NTG was able to increase biosurfactant production almost 10 fold compared to wild strain at 5th day of incubation. The heavy foam formed during the fermentation process is not a negative factor as it can be used for biosurfactant recovery (45). *P. aeruginosa* revealed enhanced production of RL when treated with nitrosoguanidine. This approach could be followed for overproduction of biosurfactant and could be

applied for large-scale production of glycolipid as an alternative for synthetic non-green chemical surfactants. It was found that biosurfactant production was increased gradually even after the microbial growth had attained the stationary phase of growth. This confirms that the biosurfactant is a microbial secondary metabolite.

Extraction of RL

The honey-colored extracted organic phase after dehydration was weighted and the amount of RL was determined gravimetrically. The remaining aqueous phase after extraction was measured by Tensiometer to insure that al the biosurfactant was collected by solvents and no remnants were left without extraction. The surface tension was 61 mN/m which emphasizes that no RL remained in medium without extraction. The quantification analysis by gravimetrical method was consistent with measurements of surface tension. Also, it was concluded from Figure 3 that the CMC of crude RL produced by mutated *P. aeruginosa* was 120 mg/L compared with the 170 mg/L for the non-mutant isolate crude extract, while the maximum CMD obtained after 100-fold dilution compared to only 10-fold dilution of control (Figure 4). The results have shown the effectiveness of rhamnolipid produced by mutant *P. aeruginosa* isolate compared to non-mutant one. A study by Saikia *et al*., (30) resulted into 90mg/L for CMC of RL produced by *P. aeruginosa* RS29 grown on glycerol, while the CMD^{-1} and CMD^{-2} of the cell-free supernatant were 28.3 and 40.0 mN/m, respectively. A surface tension of 34 mN/m was corresponding to CMC of 18.75 mg/L (24), while the CMC for mono-RL and di-RL were 15 and 30 mg/L, respectively, which indicates that mono-RL has more surface activity (31). The surface tension of culture broth is a good indicator for production of microbial-surfactant, but it's not a direct measurement for biosurfactant quantity. For pure RLs or their derivatives, the CMC depends largely on the chemical structure of the different species, but generally it falls in the range 50-200 mg/l (3).

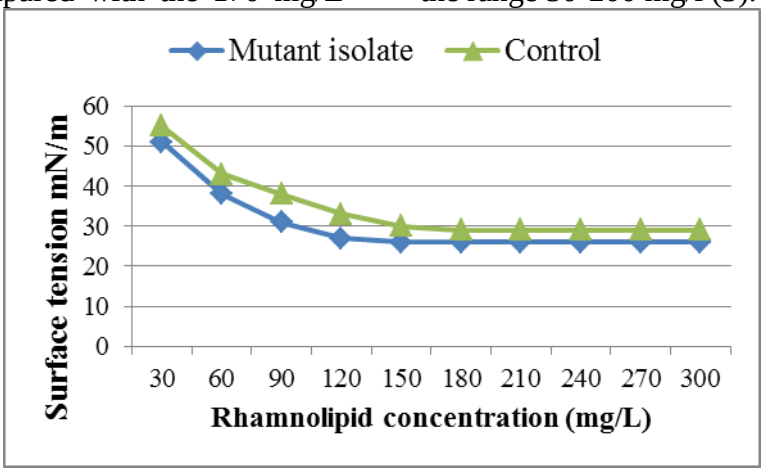

Figure 3. Critical micelle concentration of mutant *P. aeruginosa* and control after 96 h of incubation

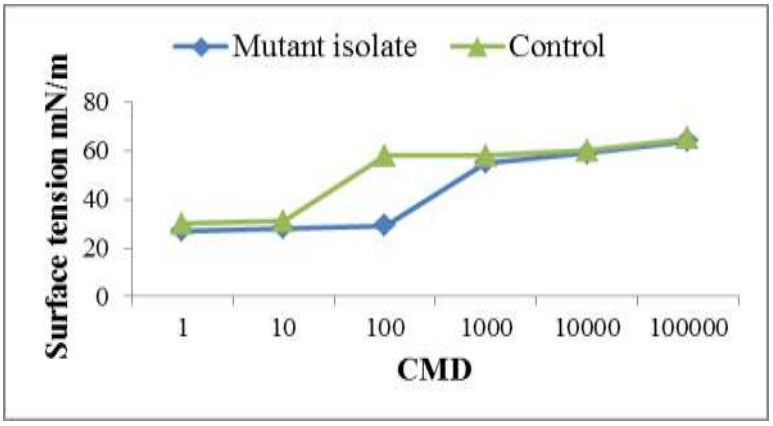

Figure 4. Critical micelle dilution of mutant *P. aeruginosa* and control after 96 h of incubation

Effect of temperature

As indicated in Figure 5, Rhamnolipid production was increased as the temperature raise from 25ºC to 30ºC, remained constant at 34ºC, a slight decrease in production observed at 37ºC, then a sharp decrease appeared as the temperature shifted to 40ºC, so the optimum temperature range for biosurfactant production by *P. aeruginosa* A3 was in the range 30-34ºC when the surface tension was 26 mN/m and the E_{24} was 63%. These observations were in agreement with a study conducted by Wei *it al*., (45), while the optimum temperature for production of RL by *P. aeruginosa* was 35ºC (18). *P. aeruginosa* TMN produced di-RL at 37ºC as optimum temperature, above this there was decrease in production (24). This difference in optimum temperatures for production depends largely on the isolate used in the production process

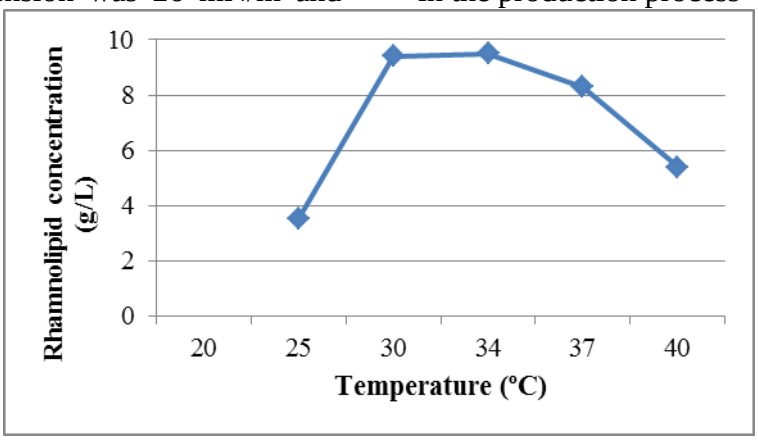

Figure 5. Effect of temperature on RL production by *P. aeruginosa* A3 in batch culture after 96 h incubation

Effect of Ph

pH is an important factor as metabolism is sensitive to different values that affect all chemical reactions in living cells. Figure 6 revealed that the effect of different values of pH on the quantity of RL obtained. There was an increasing in RL as the pH elevated from 4 to reach maximum at 6.5 (9.8 g/L), then sharply decreases with increasing pH values. The E_{24} of supernatant was 63% and the surface tension was 26.1 mN/m. Wei *it al*., (45) found that the best pH range for RL production was 6-6.8, which is similar to observations in this study, while Kaskatepe *et al*., (18) found the best was 6.8. A study by Moussa *et al*., (24) concluded that the optimum pH for production of di-RL *P. aeruginosa* TMN was 7, which is also close to the value obtained in this study. As the pH optimum range for the growth of this bacterium is 6-7, it was also the optimum range for RL production

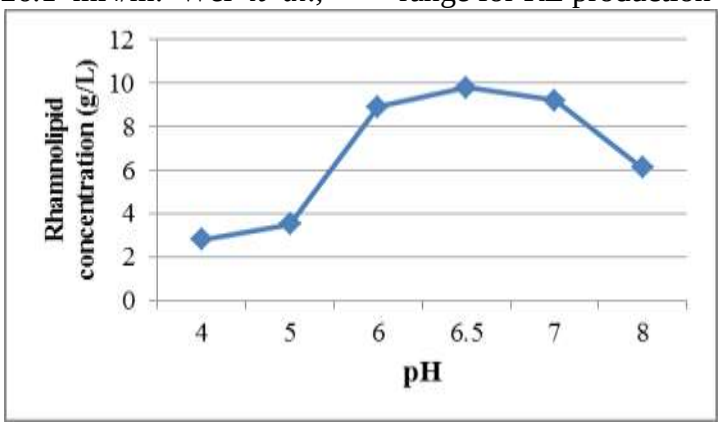

Figure 6. Effect of pH on RL production by *P. aeruginosa* A3 in batch culture incubated at 34ºC for 96 h

Production period

The RL production was increased with time until it reaches the maximum level after 108 h of incubation where 10.6 g/L was obtained, any further incubation period have no additional amount of biosurfactant and was decreased with time (Figure 7). This gives indication that RL may be degraded or

consumed by bacteria. The emulsification index was 64% and the surface tension 26 mN/m. These findings confirm that RL was majorly produced during slowing down of cell growth, while production is limited during the exponential growth phase, which is in agreement with previous conclusion by Maier and Sorbeon-Chavez (21) that this product is a secondary metabolite of *P. aeruginosa* during the stationary phase of growth. A study by Kaskatepe *et al*., (18) shown that 7 days was the optimum incubation period, while Twigg *et al*., (44) obtained maximum amount after 72 h

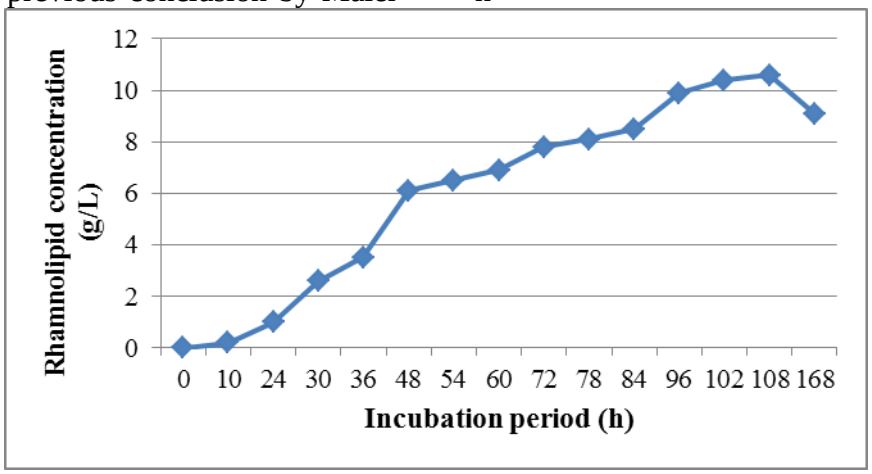

Figure 7. Effect of incubation period on RL production by *P. aeruginosa* A3 in batch culture

TLC

After purification of crude RL by column chromatography, the separated fractions were analyzed using TLC. The spots appeared on the TLC plates were corresponding to rhamnsoe domains (R_f value 0.41 and 0.17) which represent mono- and di-RLs respectively (Figure 8). This indicates that this isolate (*P. aeruginosa* A3) produce a mixture of mono- and di-RL. The preliminary characterization of this type of biosurfactant was a glycolipid (15). Three distinct peaks (0.17, 0.34 and 0.53) were observed in the study conducted by Bharali *et al*., (4) to produce RL from *P. aeruginosa* grown on biodiesel, while only two spots (R_f of 0.9 and 0.6) observed by Das *et al*., (11) for mono and di-RL, respectively. These differences in R_f values is correlated to the congeners of the biosurfactant mixture depending on the production isolate and growth conditions.

Figure 8. Analyzing of rhamnolipid by TLC

CONCLUSIONS

In this study, the ability of local isolate of *Pseudomonas aeruginosa* to produce RL increased by more that 2-fold after a brief exposure to certain concentration of nitrosoguanidine compared to non-treated one; both emulsification index and surface tension were improved by this treatment. Optimization of culture conditions revealed further increased production at 34ºC, 6.5 pH and 108 h incubation period with better properties of biosurfactant

REFERENCES

1. Abdel-Mawgoud A. M., F. Lepine and E. Deziel. 2010. Rhamnolipids: diversity of structures, microbial origins and roles. Applied Microbiology and Biotechnology. 86: 1323-1336

2. Al-Kazaz, A.A., S.J. Al-Awadi, M.H. Al-Gelawi and R.S. Al-Mohamed. 2008. Genetic mutation of *Pseudomonas aeruginosa* OM13 to improve its production of antifungal compound. Iraqi Journal of Biotechnology. 7(1): 117-132

3. Benincasa, M., A. Abalos, I., Oliveira and A. Manresa. 2004. Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. Antonie van Leeuwenhoek. 85: 1-8

4. Bharali, P., S.P. Singh, N. Dutta, S. Gogoi, L.C. Bora, P. Debnath and B.K. Konwar. 2014. Biodiesel derived waste glycerol as an economic substrate for biosurfactant production using indigenous *Pseudomonas aeruginosa*. RSC Advances. 4: 38689-38706

5. Bonilla, M., C. Olivaro, M. Corona, A. Vazquez and M. Soubes. 2005. Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. Journal of Applied Microbiology. 98: 456-463

6. Cheng, T., J. Liang, J. He, X. Hu, Z. Ge and J. Liu. 2017. A novel rhamnolipid-producing *Pseudomonas aeruginosa* ZS1 isolate derived from petroleum sludge suitable for bioremediation. AMB Express. 7(120): 1-14

7. Chong, H. and Q. Li. 2017. Microbial production of rhamnolipids: opportunities, challenges and strategies. Microbial Cell Factories. 16: 137.

8. Cooper, D.G. and B.G. Goldenberg. 1987. Surface-active agents from two *Bacillus* species. Applied Environmental Microbiology. 53(2): 224-229.

9. Costa, S.G.V.A.O., M. Nitschke, F. Lepine, E. Deziel and J. Contiero. 2010. Structure, properties and applications of rhamnolipids produced by *Pseudomonas aeruginosa* L2-1 from cassava wastewater. Process Biochemistry. 45: 1511-1516.

10. Das, P., S. Mukherjee and R. Sen. 2009. Biosurfactant of marine origin exhibiting heavy metal remediation properties. Bioresources Technology. 100: 1015-1019.

11. Das, P., X. Yang and L.Z. Ma. 2014. Analysis of biosurfactants from industrially viable *Pseudomonas* strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity. Frontiers in Microbiology. 5: 696

12. Dusane, D.H., S.S. Zinjarde, V.P. Venugopalan, R.J. McLean, M.M. Weber and P.K. Rahman. 2010. Quorum sensing: implications on rhamnolipid biosurfactant production. Biotechnology and Genetic Engineering Reviews. 27: 159-184

13. Faure, E., K. Kwong and D. Nguyen. 2018. *Pseudomonas aeruginosa* in chronic lung infection: How to adapt within the host? Frontiers in Immunology. 9: 2416

14. Friefelder, D. 1987. Molecular Biology. 2nd ed. Yones and Bttett. Boston. USA. Pp: 1-934

15. George, S. and K. Jayachandran. 2012. Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D. Journal of Applied Microbiology. 114: 373-383

16. Gudina, E.J., J.F.B. Pereira, L.R. Rodrigues, J.A.P. Coutinho and J.A. Teixeira. 2012. Isolation and study of microorganisms from oil samples for application in microbial enhanced oil recovery. International Biodeterioration and Biodegradation. 68: 56-64.

17. Iqbal, S., Z.M. Khalid and K.A. Malik. 1995. Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of *Pseudomonas aeruginosa*. Letters in Applied Microbiology. 21: 176-179

18. Kaskatepe, B., S. Yildiz, M. Gumustas and S.A. Ozkan. 2015. Biosurfactant production by *Pseudomonas aeruginosa* in kefir and fish meal. Brazilian Journal of Microbiology. 46(3): 855-859

19. Lan, G.H., Q. Fan, Y.Q. Liu, C. Chen, G.X. Li, and Y. Liu. 2015. Rhamnolipid production from waste cooking oil using *Pseudomonas* SWP4. Biochemical Engineering Journal. 101: 44-54.

20. Lotfabad, T.B., N. Ebadipour, R. Roostaazad, M. Partovi and M. Bahmaei. 2017. Tow schemes for production of biosurfactant from *Pseudomonas aeruginosa* MR01: Applying residues from soybean oil industry and silica sol-gel immobilized cells. Colloids and Surfaces B: Biointerfaces. 152: 159-168.

21. Maier, R.M. and G. Sorbeon-Chavez. 2005. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential application. Applied Microbiology and Biotechnology. 54: 625-633.

22. Mehdi, S., J.S. Dondapati and P.K.S.M. Rahman. 2011. Influence of nitrogen and phosphorus on rhamnolipid production by *Pseudomonas aeruginosa* DS10-129 using glycerol as carbon source. Biotechnology. 10(2): 183-189.

23. Morikawa, M., Y. Hirata and T. Imanaka. 2000. A study on the structure & function relationship of lipopeptide biosurfactants. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1488: 211-218

24. Moussa, T.A.A., M.S. Mohamed and N. Samak. 2014. Production and characterization of Di-rhamnolipid produced by *Pseudomonas aeruginsoa* TMN. Brazilian Journal of Chemical Engineering. 31(4): 867-880

25. Mukherjee, S., P. Das and R. Sen. 2006. Towards commercial production of microbial surfactants. Trends in Biotechnology. 24(11): 509-515.
26. Raza, Z.A., M.S., Khan and Z.M. Khalid. 2007. Evaluation of distant carbon sources in biosurfactant production by a gamma ray-induced Pseudomonas putida mutant. Process Biochemistry. 42(4): 686-692
27. Rikalovic, M.G., G. Gojgic-Cvijovic, M.M. Vrvic, and I.M. Karadzic. 2012. Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san-ai. Journal of Serbian Chemical Society. 77: 27-42
28. Rikalovic, M.G., M.M. Vrvic, and I.M. Karadzic. 2015. Rhamnolipid biosurfactant from *Pseudomonas aeruginosa*: From discovery to application in contemporary technology. Journal of Serbian Chemical Society. 80(3): 279-304
29. Robert, M., M.E. Mercade, M.P. Bosch, J.L. Parra, M.J. Espuny, M.A. Manresa and J. Guinea. 1989. Effect of carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnology Letters. 11: 871-874.
30. Saikia, R.R., S. Deka, M. Deka and H. Sarma. 2012. Optimization of environmental factors for improved production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* RS29 on glycerol. Journal of Basic Microbiology. 52: 446-457
31. Samadi, N., N. Abadian, R. Ahmadkhaniha, F. Amini, D. Dalili, N. Rastkari, E. Safaripour and F.A. Mohseni. 2012. Structural characterization and surface activities of biogenic rhamnolipid surfactant from *Pseudomonas aeruginosa* isolate MN1 and synergistic effects against methicillin-resistant *Staphylococcus aureus*. Folia Microbiologica. 57(6): 501-508
32. Sanchez, M., J.A. Teruel, M.J. Espuny, A. Marques, F.J. Aranda, A. Manresa and A. Ortiz. 2006. Modulation of the physcial properties of dielaidolphos-phatidylethanolamine membranes by a dirhamnolipid biosurfactant produced by *Pseudomonas aeruginosa*. Chemistry and Physics of Lipids. 142: 118-127.
33. Santos, D.K.F., R.D. Rufino, J.M. Luna, V.A. Santos and L.A. Sarubbo. 2016. Biosurfactants: Multifunctional biomloecules of the 21st century. International Journal of Molecular Sciences. 17(401): 1-31.
34. Sheppard, J.D. and C.N. Mulligan. 1987. The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. Appllied Microbiology and Biotechnology. 27: 110-116
35. Siegmund, I. and F. Wagner. 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnology Techniques. 5: 265-268.
36. Silva, S.N., C.B. Farias, R.D. Rufino, J.M., Luna and L.A. Sarubbo. 2010. Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. Colloids and Surfaces. B: Biointerfaces. 79: 174-183.
37. Sim, L., O.P. Ward and Z.Y. Li. 1997. Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. Journal of Industrial Microbiology & Biotechnology. 19: 232-238
38. Smith, R.S. and B.H. Iglewski. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Current Opinion in Microbiology. 6: 56-60
39. Sorbeon-Chavez, G., F. Lepine and E. Deziel. 2005. Production of rhamnolipids by *Pseudomonas aeruginosa*. Applied Microbiology and Biotechnology. 68: 718-725
40.. Stonesifer, J. and R.H. Blatz. 1985. Mutagenic DNA repair in Streptomyces. Proceeding of the Nathional Academy of Sciences of the United States of America. 82: 1180-1183.
41.. Suthar, H., A. Hinguaro, A., Desai and A. Nerurkar. 2008. Evaluation of bioemulsifier mediated microbial enhanced oil recovery using sand pack column. Journal of Microbiology Methods. 75: 225-230.
42.. Tahzibi, A., F. Kamal and M.M. Assadi. 2004. Improved production of rhamnolipids by *Pseudomonas aeruginosa* mutant. Iranian Biomedical Journal. 8(1): 25-31.
43. Thaniyavaran, J., A. Chongchin, N. Wanitsuksombut, S. Thaniyavaran, P. Pinphanichakaran, N. Leepipatpiboon, M. Morikawa and S. Kanaya. 2006. Biosurfactant production by *Pseudomonas aeruginosa* A41 using palm oil as carbon source. The Journal of General and Applied Microbiology. 52: 215-222.

44. Twigg, M.S., L. Tripathi, A. Zompra, K. Salek, V.U. Irorere, T. Gutierrez, G.A. Spyroulias, R. Marchant and I.M. Banat. 2018. Identification and characterization of short chain rhamnolipid production in a previously uninvestigated, non-pathogenic marine pseudomonad. 102: 8537-8549

45. Wei, Y.H., C.L. Chou and J.S. Chang. 2005. Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. Biocemical Engineering Journal. 27: 146-154.

46.. Wittgens, A., T. Tiso, T.T. Arndt, P. Wenk, J. Hemmerich, C. Muller, R. Whichmann, B. Kupper, M. Zwick, S. Wilhelm, R. Hausamnn, C. Syldatk, F. Rosenau and L.M. Blank. 2011. Growth independent rhamnolipid production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. Microbial Cell Factories. 10(80): 1-17.