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DISTRBUTION OF BIOSURFACTANT PRODUCING BACTERIA IN SOME AERAS OF EGYPT

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ABSTRACT

Biosurfactants are a unique class of biological compounds that have been shown to have a variety of potential applications. However, little knowledge is known about the distribution of biosurfactant - producing bacteria in the environment. The goal of this study was to evaluate how surfactant-producing bacteria are found in contaminated and uncontaminated sites. A twelve soil samples represent sandy or clay soils and a contaminated or uncontaminated areas were collected from different sites of Egypt and plated on nutrient agar medium. The 494 colonies were obtained and screened for biosurfactant production in mineral salts medium. A nine bacterial isolates were selected as a positive action on biosurfactant production. The nine isolates were purified and grouped which yielded 2 unique groups. In which consist of 2 strains and 4 species. In addition the surface tension results demonstrated that isolates produced different surfactant surface tension ranged between 25.1 to 50.2mN/m.

The potential production of biosurfactant is assessed based on the development of a fermentative process with a strain of *Pseudomonas aeruginosa* (PHM7) which was produced highly amount of biosurfactant with a high reduction in surface tension. These production using different carbon sources (hexadecane, paraffinic oil, glycerol and olive oil) and nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$ and CH_4NO_2) were tested. The more significant results were detected when glycerol was used as a carbon source, in amount of 2.0 g/L and $NaNO_3$ as a nitrogen source.

Keywords: Biosurfactant, Soil, Bacteria, *Bacillus*, *Pseudomonas*

INTRODUCTION

Surfactants constitute an important class of industrial chemicals widely used almost every sector of modern industry. About 54% of the total surfactant output is utilized in household and laundry detergents (Greek, 1991).

Most of the commercially surfactants are chemical and mainly petroleum-derived. However, rapid advances among consumers combined with expected new legislation, has provided further impetus for serious consideration of biological surfactants as possible alternatives to existing products (Sarney and Vulfson, 1995).

Biosurfactants have therefore, gained considerable interest in recent years due to their low toxicity, biodegradable nature and diversity. Their range of potential industrial applications includes enhance oil recovery, crude oil drilling, surfactant-aided bioremediation of water-insoluble pollutants and food processing (Lin, 1996; Desai and Banat, 1997 and Sullivan, 1998).

The development of this line of research is of paramount important, mainly in view of the present concern with protection of the environment. Therefore, the most significant advantage of microbial surfactant over chemical surfactant is its ecological acceptance because it is biodegradable and nontoxic to natural environments (Garcia, 1992).

A large variety of microorganisms produce potent surface-active agents, biosurfactant which vary in their chemical properties and molecular size. While, the low molecular weight of surfactants are often glycolipids, the high molecular weight of surfactants are generally either polyanionic heteropolysaccharides containing covalently-linked hydrophobic side chains or complexes containing both polysaccharides and proteins. The yield of the biosurfactant greatly depends on the nutritional environment of the growing organism. The enormous diversity of biosurfactants makes them an interesting group or materials for application in many areas such as agriculture, public health, food, health care, waste utilization and environmental pollution control such as in degradation of hydrocarbons present in soil (Karanth *et al.* , 2000).

The same authors detected that biosurfactants (BS) are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted intracellular and contain hydrophobic moieties that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface, respectively. A biosurfactant may have one of the following structures; mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein or lipopeptide, phospholipids or the microbial cell surface itself.

Considerable attention has been given in the past to the production of surface-active molecules of biological origin because of their potential utilization in food-processing, pharmacology, and oil industry. Although the type and amount of the microbial surfactants produced depend primarily on the produce organism, factors like carbon and nitrogen, trace elements, temperature and aeration also affect their production by the organism (Ramana and Karanth, 1989).

Biosurfactants are unique amphipathic molecules with properties that have been explored for a variety of industrial and biomediation applications (Banat *et al.* , 2000; Bodour and Maier, 2002; Kosaric, 2001 and Ron and Rosenberg, 2001).

Recently, several workers have presented intriguing data suggesting that biosurfactants are important for microbial growth and survival in the environment. For example, surfactin production is necessary for fruiting body formation by Bacillus subtilis (Branda *et al.* , 2001). Also, rhamnolipid is necessary for normal biofilm formation by *Pseudomonas aeruginosa* (Davey *et al.* , 2003 and Rashid *et al.* , 2000).

The present work is concerned on an initial study to screen for biosurfactant-producing microorganisms from twelve soil samples collected from both contaminated and uncontaminated areas in Egypt. An extensive survey on biosurfactant production among 494 bacterial isolates isolated from the rhizosphere of soil samples tested. A total of nine subgroups of bacterial strains producing biosurfactant were identified and classified by using API microtube system.

MATERIALS AND METHODS

Soil samples

Twelve soil samples were collected from various areas in Egypt represent clay and sandy soils (contaminated or uncontaminated). The

physical characteristics of such soils were found in Table (1). Soil samples were classified as control (uncontaminated) and soils contaminated with heavy metals or organic acids.

Table (1): Physical characteristics of different soil samples collected from various areas in Egypt

Soil number	Location	Type of contaminants	Texture	Organic matter (%)
S1	North Sinai	Heavy metal	Sandy	0.51
S2	North Sinai	Heavy metal	Sandy	0.36
S3	Ismialia	Uncontaminated	Sandy	3.22
S4	Ismialia	Uncontaminated	Sandy	1.35
S5	Ismialia	Organic fertilizer	Sandy	2.14
S6	Ismialia	Sewage sludge	Sandy	1.64
S7	El-Marg, Cairo	Uncontaminated	Sandy	0 24
S8	El-Marg, Cairo	Sewage sludge	Sandy	0.58
S9	Helwan	Heavy metal	Clay	2.70
S10	Helwan	Heavy metal	Clay	3.12
S11	Abo-Rwash, Giza	Sewage sludge	Clay	3.82
S12	Giza	Uncontaminated	Clay	4.78

Primary screening

The method used for screening the biosurfactant producing isolates was applied as all soil samples were air dried before sieving through 2-mm-diameter mesh screen. Ten grams of each soil samples was placed into a 500 ml conical flask containing 90 ml of distilled water and incubated at 25 °C with shaking rate of 250 rpm for two weeks. After 1, 4, 7, 10 and 14 days of incubation serial dilutions of each soil sample were carried out and a 1 ml of last 3 dilutions were placed into a Petri dishes containing nutrient agar and incubated at 28 °C for 3-5 days. After incubation, plates were examined and morphologically different bacterial colonies were selected for surfactant production.

Selective colonies were inoculated in 5 ml mineral salt medium (MSM) supplemented with 1% glucose. The MSM medium was a mixture of solution A and solution B. Solution A contained (per liter) 2.5 g of $NaNO_3$, 0.4 g of Mg SO_4 $7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05 g of CaCL $2H_2O$ and 10 ml of concentrated phosphoric acid (85%). The solution was adjusted to pH 7.2 with KOH. While, solution B contained (per liter) 0.5 g of $FeSO_4$ $7H_2O$, 1.5 g of Zn SO_4 $7H_2O$, 1.5 g of $MnSO_4$ H_2O, 0.3 of K_3BO_3, 0.15 g of $CuSO_4$ $5H_2O$ and 0.1 g of Na_2MoO_4 $2H_2O$. One milliliter of solution B was added to one liter of solution A to form MSM. The liquid cultures were incubated at 25 °C with shaking rate of 150 rpm for 5 days. The cell suspensions were then tested for the production of surfactant using the drop collapse method of Bodour and Maier (1998).

Qualitative test of surfactant

Qualitative drop–collapse test was performed in polystyrene lid containing 48 micro well. A thin coat of 10 w -40 oil was applied to each well. 5–µl aliquot of supernatant was put into the center of each well. If the drop

remained beaded, it means a negative result. While, if the drop spread, the result was recorded as a positive result or presence of biosurfactant.

Quantitative test of surfactant

The selective isolates with the positive drop- collapse test were inoculated in 25 ml of MSM supplemented with 1% glucose for 5 days. After, the incubation period the broth cultures were centrifuged at 10,000 rpm for 10 mins. (Beckman centrifuge). The supernatants of each isolate were transferred to 50 ml glass beaker. A surface tensionat (Fisher Scientific, Model 21) was used for assay surface tension of each isolate supernatant.

Growth condition and biosurfactant production

The selective *Pseudomonas aeruginosa* was activated in a triptic soyer agar medium (TSA, Merck), cultivated at 30 °C for 48 hours. After the incubation period, the culture transferred to a 250 ml flask containing 50 ml of TSA. The flasks were shaked at 200 rpm for 24 hours at 30 °C. The bacterial cells were harvested by centrifugation at 10,000 rpm for 15 minutes. The harvested cells were suspended in a culture medium supplemented with 1.0 % (v/v) glycerol (Venkata and Karanth, 1989).

Biosurfactant production by *P. aeruginosa* was assayed during one week incubation period. The condition applied were temperature of 30 °C, rotary shaker of 150 rpm and agitation. The carbon sources tested in the production process were N-hexadecane (Oxide), paraffinic oil, glycerol and olive oil (vegetable oil). Also, sodium nitrate ($NaNO_3$), ammonium sulphate ($(NH_4)_2SO_4$) and urea (CH_4N_2O) were also tested as nitrogen sources.

Determinations

Bacterial growth was monitored by measurement of absorbance at a wavelength of 520 nm. Samples of 20 ml broth culture were centrifuged at 10,000 rpm for 15 minutes. The harvested cells were suspended in 5.0 ml of distilled water and the biomass was expressed as dry weight (g/L).

Rhamnolipids determination expressed as rhamnose (g/L) was measured in the cell free culture medium by using the phenol sulfuric acid method (Dubois *et al.* , 1956). Also, glycerol was assessed by the enzymatic colorimetric method for triglyceride content (Hafesburg *et al.* , 1986).

RESULTS

The twelve soil samples collected from different areas in Egypt i.e., El-Marg, Giza, Ismalia, Helwan and North Sinai were screened for biosurfactant producers. Eight of soil samples were a sandy soil and the rest of them were clay soil. The total organic carbon content of such soil samples tested was ranged from 0.24 to 4.78%. The microbial density of such soils was ranged between $5.6X10^4$ to $8.8X\ 10^7$ CFU g^{-1} dry soil.

The primary screening for bacterial isolates counted on nutrient agar medium was yielded a total of 494 isolates. These isolates were subcultured in MSM-glucose broth for five days. After the incubation period, the

subcultural isolates were tested for purity, Gram stain, motility and biosurfactant production with drop-collapse test. The data presented in Table (2) show that a nine biosurfactant producing isolates (1.7%) for the total isolates tested under the screening conditions.

Table (2): Occurrence of biosurfactant – producing bacteria in different soil samples collected from Egyptian soils

Soil No.	Total colony counts (X 10^5 cfu g^{-1} dry soil)	No. of isolates collected	No. of isolates producing biosurfactant
S1	6.40	38	2
S2	1.20	32	-
S3	740.00	39	1
S4	54.00	42	-
S5	39.00	35	1
S6	18.00	31	-
S7	0.56	41	-
S8	7.20	22	-
S9	94.00	23	-
S10	340.00	44	2
S11	680.00	64	-
S12	880.00	83	3
Total		**494**	**9**

The results contained also showed that from the twelve soil samples tested, five (41.7% of them) were biosurfactant producers. Three of them were sandy soil samples (37.0%) and two of such samples collected were clay soil samples (50%). Moreover, four isolates were isolated from heavy metal contaminated soils, only one isolated from organic contaminated soils and four isolates were isolated from the uncontaminated soils.

Morphological characteristics

From the nine biosurfactants isolates, six of them (66.7%) were Gram-positive bacteria and three of them (33.3%) were Gram-negative bacteria. (Table 3). Upon examining the types of soils tested, the results revealed that, Gram-positive isolates were dominated in soil contaminated with heavy metals and arid region representing 66.7% of the total soil contaminated with heavy metal and the Gram-negative isolates were found in uncontaminated soils with the presence of 100% of the uncontaminated soil samples.

Table (3): Distribution of biosurfactant in different types of soils

Type of soil	No. of samples	No. of biosurfactant producing isolates	G^+ spore-former	G^- short rods
contaminated with heavy metals	4	4	4	-
contaminated with organic acid (organic fertilizer + sewage sludge)	4	3	1	-
Uncontaminated	4	2	1	3
Total	12	9	6	3

These data revealed the ability of microorganisms to survive in such soils or depending on the type of contaminant present in such ecosystems.

The last nine isolates were characterized; firstly by determination of surface tension of culture supernatants for each isolate grown under optimum conditions. The values recorded were ranged from 25.10 to 50.20 mN/m; secondly according to API microtube system (20B, 20E and 50 CHE); the isolates were classified to two genera *Bacillus* spp. (6 isolates) and *Pseudomonas* spp. (3 isolates) Table (4).

The six *Bacillus* isolates obtained were identified as *Bacillus subtilis* (83.3%) and *B. licheniforms* (16.7%). While, the three *Pseudomonas* isolates collected were positioned as *Pseudomonas* spp. (66.6%) and *P. aeruginosa* (33.4%).

One strain of genes *Pseudomonas* (*P. aeruginosa)* was chosen for the biosurfactant production because such strain was recorded a highly surface tension value.

Table (4): Classification of biosurfactant producing isolates collected from different areas of Egypt

Isolates number and code	Genera	Surface tension (mN/m)
BHM-1	*Bacillus subtilis*	50.20
BSW-2	*Bacillus subtilis*	40.10
BSW-3	*Bacillus subtilis*	32.60
BUC-4	*Bacillus subtilis*	30.40
BUC-5	*Bacillus licheniforms*	38.20
BUC-6	*Bacillus subtilis*	34.80
PHM-7	*Pseudomonas aeruginosa*	25.10
PSW-8	*Pseudomonas* spp.	27.40
PUC-9	*Pseudomonas* spp.	32.30

B= *Bacillus* spp., P = *Pseudomonas* spp.
HM = Heavy metal, SW = sewage sludge, UC= Uncontaminated,

Effect of various carbon sources on biosurfactant production

The production of rhamnolipid by a *P. aeruginosa* (PHM7) using different carbon sources such as hexadecane, paraffinic oil, glycerol and olive oil was studied for one week incubation period. The recorded data show that such strain was able to use hexadecane within a week of fermentation period with producing 118.0 mg/L of rhamnose with a 44.5% drop in surface tension (about 39.96 mN/m). while the use of paraffinic oil (a very complex and heterogeneous carbon source) produced a highly amount of rhamnolipids (270.0 mg/L) but with a very low in drop of surface tension (16.4%). Also, using olive oil as a carbon substrate, the bacterium produced 186.0 mg/L of rhamnolipids with a drop of 37% in surface tension. While, using of a medium supplemented with glycerol, the *Pseudomonas* produced 1070.0 mg/L of rhamnose with a high drop in surface tension of 57.3% (Fig. 1 and 2).

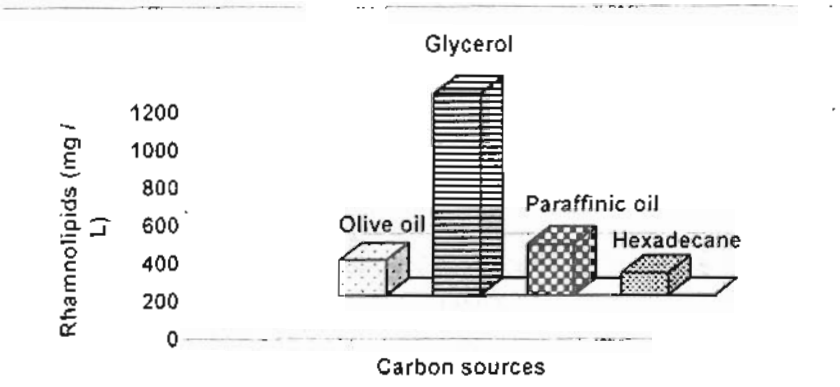

Fig. (1): Effect of different carbon sources on biosurfactant production by *Ps. aeruginosa*

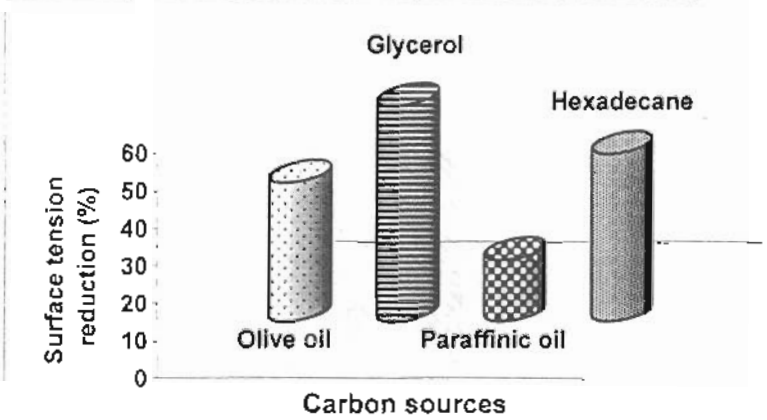

Fig. (2) Reduction in surface tension (%) due to the production of biosurfactant by *Ps. aeruginosa*

Effect of various nitrogen sources on biosurfactant production

Sodium nitrate, ammonium sulphate and urea were tested as nitrogen sources for cultivation of *P. aeruginosa* in liquid media. The obtained results indicated that, use of sodium nitrate was more effective than ammonium sulphate and urea. Sodium nitrate implies a better productivity (0.94 g/g) followed by ammonium and urea (0.65 and 0.38 g/g), respectively specially when glycerol (2.0% v/v) was used as a carbon source (Fig 3 and 4). While, *Ps. aeruginosa* (PHM7) was able to use different nitrogen sources such as nitrate or ammonium, however, the results indicted that to obtain a highly concentrations of biosurfactant, it is necessary to apply the preferred and optimum concentration of nitrogen.

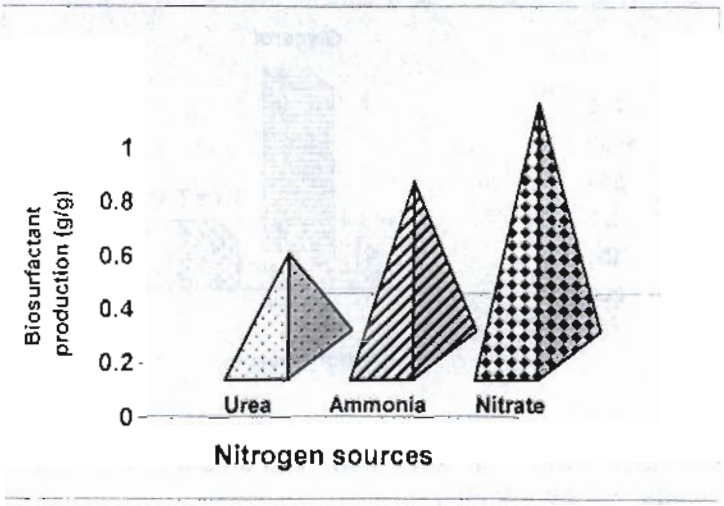

Fig (3): Biosurfactant production by *Ps. aerugenosa* as affected by different nitrogen sources

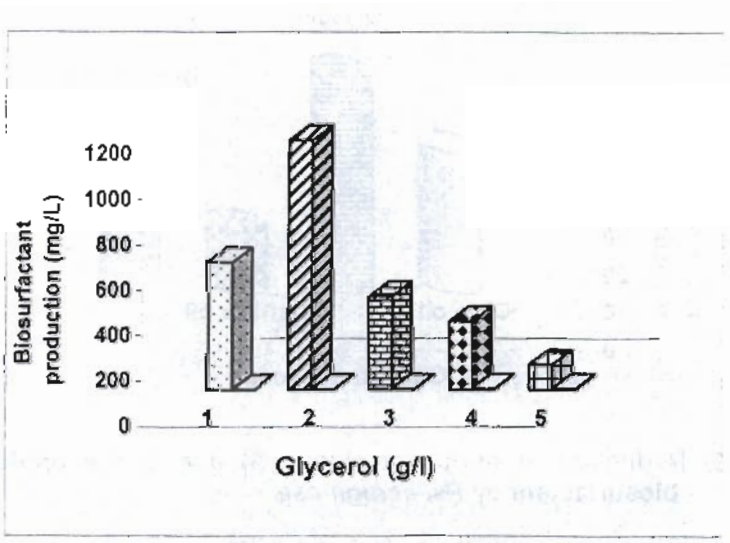

Fig (4): Effect of different concentrations of glycerol on biosurfactant production by *Ps. aeruginosa*

DISCUSSION

As has been showed from the data obtained, the biosurfactant-producing organisms were found in some ecosystems specially the environmental polluted with heavy metals or industrial wastes. These biosurfactant materials may be had an important tool for survival of such microorganisms (Bodour and Maier, 2002 and Konz *et al.* , 1999).

The obtained results indicted that a low biosurfactant producing population as well as bacterial isolates isolated from the soil samples tested were detected. The low number of bacterial densities and bacterial isolates may be due to the not enrichment screening conditions used for the soils tested. Where, many investigators detected that a further difficulty with screening for biosurfactant producers is that biosurfactant production depends both on the type of carbon source present and on the types and amounts of other nutrients in the screening medium (Adamczak and Bendnarski, 2000; Davis *et al.* , 1999, Mulligan *et al.* , 1989 and Robert *et al.* , 1989).

Moreover, there are many factors influence on the diversity and activity of soil bacterial populations. Hence, when looking at *Pseudomonas* population in tomato and flax rhizosphere in different soil types, Latour *et al.* (1996) observed that diversity was almost influenced by soil type and less by crop type. Also, Bachmann and Kinzel (2001) suggested that soil type was the most important factor determining the overall diversity of bacterial populations in the rhizosphere.

The obtained results detected that, two biosurfactant-producing genera, *Bacillus* and *Pseudomonas* were isolated from the soil samples tested. The low numbers of biosurfactant isolates may be revealed that, other biosurfactant-producing genera were found in such ecosystems but not enriched well by the screening conditions used in this study.

In addition, the surface tension reduction activities of the biosurfactant-producers were different depending upon the strain tested and the structure of biosurfactant assay. So the data recorded showed that supernatant of *Bacillus* strains reduced the surface tension values from 72 (water) to many values ranged between 30.4 to 50.2 mN/m. While, the *Pseudomonas* supernatant gave a surface tension values more different in their activities and ranged between 25.1 to 32.1 mN/m. The differences in these surface tension activities values is a significant. Where, a reduction in surface tension from 72 to 50.2 mN/m is considered a low active, while a reduction in its activity to 25.1 mN/m meaning a highly active. The last results were confirmed with the results obtained by Bodour *et al.* (2003).

Moreover, biosurfactant production appears to have evolved in an independent yet parallel fashion, for examples, rahmnolipid (*P. aeruginosa*) biosurfactant genes are completely different from surfactin (*B. subtilis*) biosurfactant genes (Bodour *et al.* , 2003)

The data recorded about effect of various carbon sources on production of biosurfactant showed that hexadecane produced low amount of rhamnose but with high surface tension. This fact could probable due to the formation of an emulsion during fermentation period, which interfered in the quantification of the surface tension. This fact was agreement with the results detected by Boulton and Ratledge (1987). Where, they showed the use of vegetable oil and glycerol as carbon sources to produce rhamnolipids seems to be an interesting and low cost alternative.

Explanation of the present results was that the nitrate first undergoes to dissimilatory nitrate reduction to ammonium and then assimilation by glutaminc-glutamate metabolism. This means that assimilation of nitrate as

nitrogen source is sol slow (Santa Anna *et al.* , 2002). Also, nitrate is more suitable nitrogen source than ammonia and urea. These results were in agreement with data reported by Syldatk *et al.* (1985); Ochsner *et al.* (1995) and Arino *et al.* (1996).

CONCLUSIONS

From the present study, it could be concluded that biosurfactant produced by various strains are very useful and can be used in different applications. So that, it is very important that use of any screening process must be discriminatory enough to permit to most or all isolates producing biosurfactant to grow better and produce a high amount of biosurfactant. Also, more deep study must be taken in consideration on biosurfactant in order to know the structure, activity and the optimum conditions for production as well as identify genera involved in the synthesis of these biomolecules.

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توزيع البكتريا المخفضة للتوتر السطحى الحيوية فى مختلف المناطق فى مصر

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قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة – جيزة – مصر

تعتبر مخفضات التوتر السطحى كأحد المركبات النادرة و الهامة والتى يمكن استخدامها فى العديد من الأغراض الحيوية. اى يمكن استخدامها فى كثير من التطبيقات الهامة. الأ أن كمية المعلومات المتوفرة عن مثل هذه المخفضات للتوتر السطحى وكذلك أماكن تواجدها فى الأوساط البيئية المختلفة مازالت حتى الآن قليلة. ولهذا يتركز هذا البحث على دراسة مدى تواجد مثل هذه الأنواع البكترية فى الأوساط البيئية المختلفة سواء الملوثة منها أو الغير ملوثة.

تم دراسة أثنتا عشر عينة تربة مختلفة (رملية – طينية) سواء كانت ملوثة أو غير ملوثة من عدة أماكن مختلفة فى مصر بزراعتها فى بيئة أجار مغذى وفحص المجاميع البكترية النامية. تم فحص ودراسة ٤٩٤ مجموعة بكترية من حيث مقدرتها على إفراز مخفضات التوتر السطحى فى بيئة أملاح معدنية. تم اختيار ٩ عزلات بكترية أظهرت نتيجة موجبة فى مدى مقدرتها على إفراز مخفضات التوتر السطحى. حيث تم تنقيتها وتقسيمها إلى مجموعتين رئيسيتين مشتملتين على سلالتان وأربعة أجناس مختلفون. أظهرت النتائج المتحصل عليها أن العزلات التسعة تنتج كميات مختلفة من مخفضات التوتر السطحى بقيم تتراوح ما بين ٢٥,١ إلى ٥٠,٢ .

وحيث أن الإنتاج الأمثل لمثل هذه المخفضات التوتر السطحى تعتمد أساسا على مدى تطور مراحل الإنتاج المختلفة. فقد تم اختيار أفضل السلالات المعزولة وهى من جنس الباسيدوموناس أريجونوزا من حيث المقدرة العالية على الإنتاج بكمية كبيرة وكذلك على خفض التوتر السطحى بمعدل عالى. حيث تم دراسة تأثير استخدام مصادر كربون مختلفة مثل الهكسانان – زيت البرافين – الجلسرول – زيت الزيتون وكذلك مصادر النتروجين المختلفة مثل نترات الصوديوم – كبريتات الأمونيوم – اليوريا. ولقد أظهرت النتائج أن استخدام الجلسرول (٢ جم/لتر) تعطى أعلى إنتاجية من مخفضات التوتر السطحى خاصة عند استخدام مادة نترات الصوديوم كمصدر للنتروجين.