Isolation, Phylogenetic Analysis and Screening of Biosurfactants Producing By Locally Isolated Bacteria

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ABSTRACT

Biosurfactants or surface-active compounds are produced by microoaganisms. These molecules reduce surface tension both aqueous solutions and hydrocarbon mixtures. 45 bacterial strains previously isolated from petrochemical wastes around Oil Refinery Company in Tanta, Al Ggharbiyah, Egypt. Selection and screening of biosurfactant producer(s), via four different biioassays; (i) surface tension measurements, (ii) blood hemolysis test, (iii) drop-collapsing test, and (iv) emulsification test. Two isolates coded 3C.A and C1 respectively were chosen to be the best candidates for biosurfactant production. The highest production of biosurfactants produced by 3C.A isolate grown on medium supplemented with 3g/L ammonium sulfate as a nitrogen source and (4%) glucose as carbon source and incubated at 37°C. The biosurfactants were found capable of producing a relatively stable emulsion hydrocarbon at pH 7 and thermostable for 1 hour at 75 °C, based on the value of surface tension. The phylogenetic analysis based on 16S rDNA and physiological characzterization indicated that the isolates of strain 3C.A and C1 as well *Bacillus subtilis* Eg1 and *pseudonamnas sp* respectivly.

Keywords: ***Biosurfactant, Emulsification, surface tension, phylogentic analysis.***

INTRODUCTION

BIOSURFACTANTS are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface, respectively (Karanth and Veenanadig, 1999). They are structurally diverse group of surface-active molecules synthesized by microorganisms (Lu and Yaseen , 2007).

Rhamnolipids from *Pseudomonas aeruginosa*, surfactin from *Bacillus subtilis*, emulsan from *Acinetobacter calcoaceticus* and sophorolipids from *Candida bombicola* are some examples of microbial-derived surfactants. Originally, biosurfactants attracted attention as hydrocarbon dissolution agents in the late of 1960s, and their applications have been greatly in the past five decades as an improved alternative to chemical surfactants (carboxylates, sulphonates and sulphate acid esters), especially in food, pharmaceutical and oil industry(Desai and Banat, 1997; Banat and Cameotra ,2000). The reason for their popularity as high value microbial products is primarily because of their specific action, low toxicity, higher biodegradability, effectiveness at extremes of temperature, pH, salinity and widespread applicability, and their unique structure which provide new properties that classical surfactants may lack (Kosaric et al., 1992 ;Desai and Banat, 1997).

Biosurfactants possess the characteristic property of reducing the surface and interfacial tension using the same mechanisms as chemical surfactants. Unlike chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial fermentation processes using cheaper agro-based substrates and waste materials. During the past few years, biosurfactant production by various microorganisms has been studied extensively. Also various aspects of biosurfactants, such as their bioremediation, natural roles, production on cheap alternative substrates (Makkar and Cameotra,2002 ;Gautam and Tyagi, 2005) and commercial potential (Desai and Banat 1997; Banat et al., 2000), have been reviewed.

To the best of our knowledge, to describe the research and development strategies of making the biosurfactant production process cheaper and commercially attractive, no attempt has been made. Most of the work on biosurfactant applications has been focusing on bioremediation of pollutants (Mulligan et al., 2005) and microbial enhanced oil recovery **(**Banat et al., 1995). However, these microbial compounds exhibit a variety of useful properties and applications in various fields.

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MATERIALS AND METHODS

2.1 Isolation and enrichment of biosurfactant-producing microorganisms

Soil samples were collected from oil contaminated site in an oil refinery industry in middle Delta, Egypt (Tanta city, Alghrbia Government).One gram of different soils samples transferred to 500 ml Erlenmeyer flasks each containing 100 ml of synthetic basal medium SBM. The medium consist of: 1, K2HPO4; 0.5, KH2PO4; 2.NaNO3; .5, MgSO4,7.H20; amendedwith0. 5% (w/v) hexadecane and 1ml trace elements composed of CaCl2. 2H2O, 0.368; CuSO4. 5H2O, 0.624; FeSO4. 7H2O, 0.64; MnSO4 H2O, 0.594; ZnSO4 7H2O, 0.422; CoCl2.6H2O, 0.788; NaMoO4, 0.696, then PH adjusted to7.00. amended with 0.55(v/v) crude oil as a sole carbon source.

Flasks were incubated at 30 C on a rotary shaker (160 rev min-1) for 4 days. After 4 days 1.0 ml of the culture was transferred to fresh media containing crude oil (2% wv-1) and re-incubated for another 4 days. Following five cycles of such enrichment, 1.0 ml of culture was diluted and plated on BSM agar plates containing crude oil as sole carbon source. The bacterial colonies obtained were further purified on Luria–Bertani slant 80^{o}C.

2.2 Analytical Methods

A) Hemolysis test

The first screening test for identification and isolation of BPB is hemolysis test. The bacteria strains for hemolytic activity by plating cell on to Blood Agar and incubated at 30°C for 72 h (Bicca et al., 1999).

B) Emulsification index (E24)

The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, vortexed at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm).The percentage of emulsification index calculated by using the following equation (Tabatabaee and Sajadian, 2005; Sarubbo et al., 2006).

$$E24 = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

C) Measurement of surface tension

Preculture of bacteria strains were prepared in NB in OD600=1.1ml of Inocula were added to 100ml MSS and 1% filtered oil as hydrocarbon source. The mixture with control samples were incubated at 30°C on shaker at 150 rpm for 3 days. The surface tension was meseared using a KRUSS F6 tensiometer (ABU-Ruwaida et al., 1991).

D) Investigation of pH effect on surface tension

The effect of pH on surface tension were carried out by changing pH (4.2-9.2) of BSM medium (pH=7.2). The cultures were incubated at 30°C on shaker at 200 rpm for 3 days in selected temperature (Yakimov et al., 1995)

E) Investigation of temperature effect on surface tension

Different temperature (25-50ºC) was investigated on surface tension. The cultures with control samples were placed on shaker at 200 rpm for 3 days in selected temperature (Yakimov et al., 1995)

2.3 Bacterium identification

The bacterial identification was carried out by determining the gene sequence coding for 16S rRNA and biochemical analysis. The PCR was carried out as conditions and temperature program described by (Kumar et al., 2006). The PCR product was separated by agarose gel electrophoresis and visualized by SYBR*1* Green 1 staining (Sigma, St. Louis, USA) and, finally purified by using a Wizard PCR Preps Purification System (MWG Company, Germany) according to the manufacturer's instructions. The purified DNA was sequenced as per procedure described below. To identify the isolated bacterium, the 16S rDNA consensus sequence, obtained by analyzing with DNAMAN version 5.2.9 (Lynnon BioSoft, Quebec, Canada, was then compared with 16S rRNA gene sequences from the public GenBank, EMBL, and DDBJ databases using the advanced gapped n-BLAST program, version 2.1. The program was run via Internet through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/). Sequences with more than 98% identity with a GenBank sequence were considered to be of the same species as the highest score-matching sequence on the public sequence databases. The biochemical reactions were also carried out according to Bergey's Manual of Determinative Bacteriology to identify the bacterium.

DNA sequencing

DNA sequencing reaction was performed with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the sequencing products were separated by capillary electrophoresis using a 310 Sequencer (Perkin–Elmer Corp., Applied Biosystems, USA).

2.4 Effect of some nutrients on biosurfactants production by 3C.A

3C.A isolate was reactivated in LB agar medium, cultivated at 30 C for 24h and then transferred to a 250

ml conical flask containing 50 ml liquid broth (LB). The flask was incubated at 35 C and 200rpm till OD600 (.73) to be used seed culture. Preliminary experiments were performed to optimize the culture medium for biosurfactnt production .the carbon source was conducted in SBM replacing crude oil by one of the following items: glucose, hexadecane, maltose, sucrose, galactose and lactose. Different types of oil were also tested as sole carbon source such as sunflower, paraffin oil and olive oil.

RESULTS AND DISCUSSION

3.1 Isolation and screening for Biosurfactants producing bacterial isolates

Fifty isolates representing different morphotypes were selected from 45 isolates and screening for biosurfactants production using three different techniques named blood hemolysis, drop collapase and surface tension. Emulsification index (%) was applied to evaluate the emulsification activity of the bacterial isolates.

All fifty isolates tested demonstrated emulsification activity ranging from 71.9% to 93.33%, even those that did not reduce surface tension. The highest activities (93.33 and 92.3%) were achieved with isolates C1 and 3C.A, respectively (Table 1). It has previously been reported that most biosurfactant- producing bacteria were isolated from sites with a history of contamination by oil or it's by products **(**Bicca et al., 1999**).** Thus in our study, isolation was conducted with hexadecane as the sole carbon source. Bacterial isolates recovered from the isolation procedure were selected and subjected to several protocols to screen for biosurfactant/ bioemulsifier production. Quik, reliable methods for screening biosurfactant – producing microbes have been developed (Youssef et al., 2004). Blood hemolysis is widely used to screen for biosurfactant production and in some cases it is the sole method used (Banat et al., 1993). Although, none of the studies mentioned the possibility of biosurfactant production without a hemolytic activity, (Youssef et al., 2004) obtained anumber of false positive (16%) when using the blood agar lyses. In our study all isolates produced clear zone on agar plates indicating blood hemolysis. Only twelve of the fifty tested isolates gave positive results with the drop collaps method. This is aroid simple test (Bodour et al., 2003) in which a drop of a cell suspension is placed on an oil- coated surface, and drops containing biosurfactant collaps, wereas non surfactant containing drops remain stable.

3.2 Identification of potent bacterial isolates

Grouping of the fifty experimental bacterial isolates on genotypic traits was the main goal of this part. Identifying the most promising organisms was the other goal.

3.2.1. Biochemical confirmation tests

Historically, identification of bacteria relied on phenotypic characterization, which includes mainly colony and cell morphology, pigmentation, biochemical's tests and others. However, it is recognized that phenotypic characteristics are influenced by cultivation condition used to grow cells and these characteristics may change with the age or physiological state of the cells (Hirsch and Sigmund, 1995).

Table 1. Comparison of different methods for predicting Biosurfactants producing isolates

Isolate code	Haemolysis of blood	Drop collapse method	Emulsification index (%)	Surface* tension mN/m
1E	+	+	74.1	56.6
C.O	+	+	83.6	48.1
5E	+	+	85.8	50.3
C1	+	+	92.33	33.3
BE	+	+	90.32	34.4
3E	+	+	90.32	52.1
3C.A	+	+	94.9	31.2
4E	+	-	92.3	60.1
13B	+	+	64.9	48.5
14B	+	-	80.9	47.5
15B	+	+	84.6	42
17B	+	+	75.5	46
20B	+	+	77.9	51.5
21B	+	+	68.9	47.9
22B	+	-	69	51.3

* The surface tension value of uninoculated synthetic medium (control) was 70 mN/m.

* (+) Positive and (-) Negative

Selected isolates were examined morphologically after Gram staining. In order to confirm the relatedness between the experimental strains and the reference strain. As shown in (Table 2), all isolates strain showed positive reactions with respect to blood haemolysis, catalase, starch, casein and gelatin hydrolysis, nitrate reduction, glucose fermentation, Voges-Prauskauer tests and growth in presence of 10% NaCl. On the contrary, they recorded negative results with phenylalanine deamination, mannitol and xylose fermentation and gas production from glucose.

3.2.2 Molecular phylogeny of the selected bacterial isolates

The PCR products of the two selected bacterial isolates (3C.A and C1) were purified and sequenced on both strands using chain terminator method (MWG Company, Germany). The 16S rDNA sequences of the isolates were submitted to Gene Bank sequencing data and align the 16S rDNA sequences of Ribosomal Database project.

Table 3 Compiles GenBank accession number of 16S rDNA gene partial sequences of strains 3C.A and C1, the gighest sequence similarty as well as the closest neighbor(s). Sequences of the two isolates 3C.A and C1 were affiliated according to their 16S rDNA to members of genus Bacillus. 3C.A showd a high similarty (99%) to *Bacillus subtilis* Eg1. On the other hand, isolate C1 showed high similarty (99%) to *Psedomonas sp.*

The phylogenetic relationships of the experimental isolates and closely related species were analyzed using the multisequence alignment program and the results are presented in phylogenic dendograms (Figs 1 and 2).

Based on the previously obtained data, 3C.A selected for further investigations and was designated as *Bacillus subtilis* Eg1.

3.3 Effect of carbon source on biosurfactant production

In this experiment crude oil was replaced by one of the following different carbon sources: glucose, hexadecane, maltose, sucrose, galactose and lactose. The concentration used was equivalent to 20g/L glucose. Sugars were separately sterilized and supplemented to flasks each containing 100 ml of SBM. Each flasks was inoculated with 1% (v/v) bacterial suspension from a pre culture (OD600 0.7). From thae data obtained in (Fig. 3) it is clear that growth of 3C.A strain was affected by the nature of the carbon source. Highest growth (OD600 2.631) was with glucose, whereas, lowest growth (OD600 1.66) was recorded with sucrose. The bacterium was able to produce bioemulsifier of variable activities when grown on different carbon sources. The highest emulsification activity (98.2%) was observed for supernatants of cultures containing glucose. These results revealed that there was no relationship between cell growth and biosurfactant production, similar to that reported by other investigators (Ries et al., 2004).

Table 2. some biochemical characteristics of the bacterial isolates

Test	Isolated code							
	1E	*C.O*	5E	C1	BE	3E	3C.A	4E
Blood heamolysis	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Hydrolysis of casein	+	+	+	+	+	+	+	+
Hydrolysis of gelatin	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+	+	+
Phenylalanine deamination	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+
Glucose fermentation	+	+	+	+	+	+	+	+
Mannitol fermentation	-	-	-	-	-	-	-	-
Xylose fermentation	-	-	-	-	-	-	-	-
Gas from glucose	-	-	-	-	-	-	-	-
Voges – prauskauer reaction	+	+	+	+	+	+	+	+
Growth in 10% NaCl broth	+	+	+	+	+	+	+	+

* (+) Positive and (-) Negative

Table 3. Accession numbers and similarity percentage to the nearest neighbors of the selected isolates

Isolates code	GenBank accession number of nucleotide Sequences	Similarity	Nearest neighbor(s)
3C.A	**GU247131**	**99 %**	***Bacillus subtilis* Eg1**
C1	**JF806950.1**	**99 %**	***Psedomonas sp.***

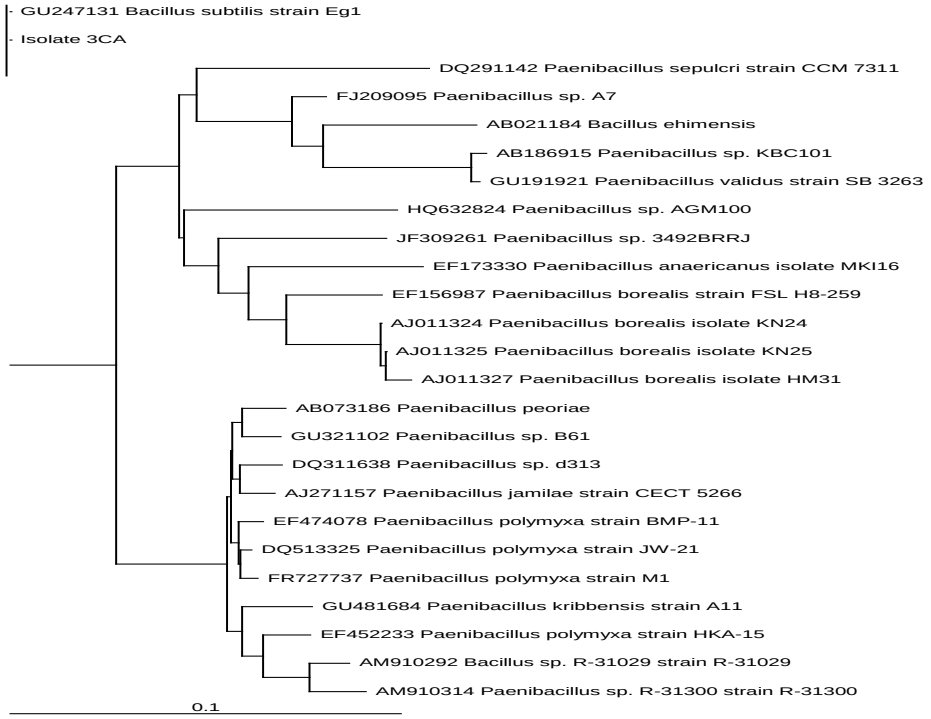

Fig. 1 Phylogenetic relationships using isolate 3C.A and the most closely related bacterial species. The dendrogram was generated from MEGA software

Fig. 2. Phylogenetic relationships among isolate C1 and the most closely related bacterial species. The dendrogram was generated from MEGA software

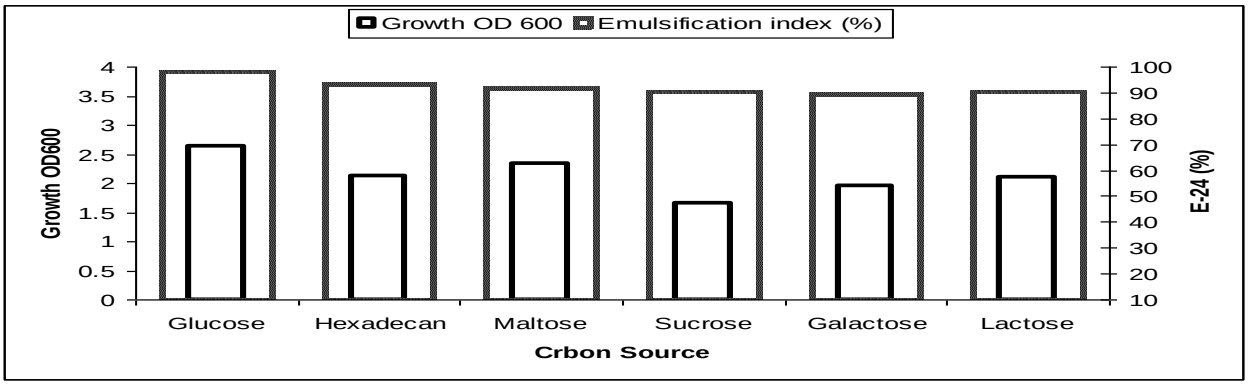

Fig. 3. Biosurfactants production by *Bacillus subtilis* Eg1in SBM supplemented with different carbon sources

*Cells were incubated for 24h at 30 C with 200 rpm agitation rate.

Being the most promising carbon source, the bacterium was studied for the production of biosurfactants under batch fermentation with different glucose concentration (1, 2, 3, 4, and 8 w/v) .Highest emulsification activity (98.1%) was recorded in supernatants of cultures containing 4% glucose (Fig. 4).

3.4 Effect of nitrogen source on biosurfactant production

In this experiment, sodium nitrate in the SBM medium was replaced by peptone, soybean, ammonium sulphat and ammonium nitrate at a concentration equivalent to .43 g N/L. Glucose was added at 5 %(w/v) level. Each flask received standard inoculums of 1% (v/v) from 24h old pre-culture. After 24h incubation at 30^{o}C shaken at 200 rpm, growth was measured and cultures were centrifuged. The data presented in (Fig. 5). Demonstrate that supernatant of cultures amended with ammonium sulphate as the sole nitrogen source gave the highest emulsification activity (96.3 %). In good agreement with our data are those observed by (Duvnjak and N. Kosaric ,1983) who reported that ammonium salts and urea were prefeed nitrogen sources for biosurfactant production by *Arthrobacter paraffineus*.

Different concentration (1, 2,3,4,5 and 11 g/L) of ammonium sulphate was used in presence of 5 g / L glucose. The incubation was done as mentioned before. The biosurfactants production was monitored by measuring emulsification index. The results illustrated graphically in Fig. 6 indicated that under the experimental conditions, the best concentration of ammonium sulphate was found to be 3g/L which allowed the bacterium to produce a biosurfactant of highest emulsifying activity (98.2 %).

The highest activity of the bioemulsifier produced by *Bacillus subtilis Eg1* was achieved when carbon : nitrogen ratio of 13:1 had achieved. Similarly, nitrogen limitation caused increased biosurfactant production in many microbial species including *P. aeruginosa* (Suzuki et al., 1974; Ramana and Karanth., 1989). and *Nocardia* satrain SFC-D(Kosaric et al., 1990).

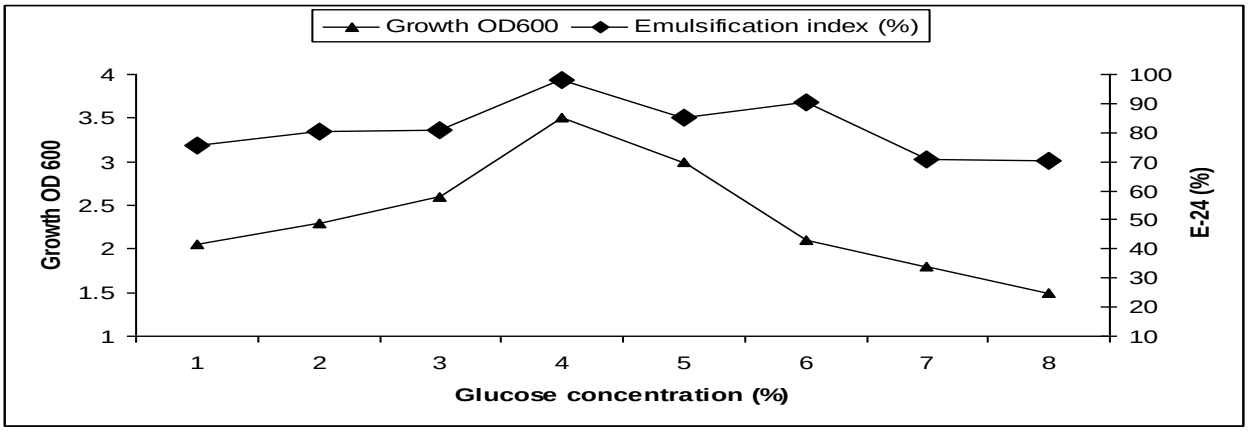

Fig. 4. Effect of glucose concentrations on growth of *Bacillus subtilis* Eg1 and Emulsification index of cell-free extract

*Cells were incubated for 24h at 30 C with 200 rpm agitation rate.

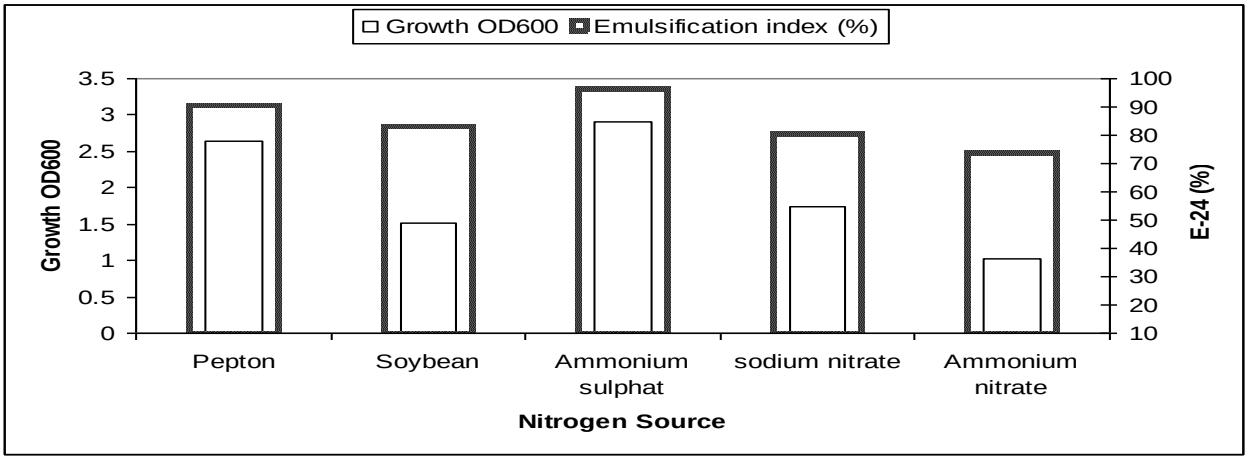

Fig. 5. Effect of inorganic sources as well as some natural substances on the growth of *Bacillus subtilis* Eg1and Emulsification activity of supernatants

* Cells were incubated for 24h at 30°C with 200 rpm agitation rate.

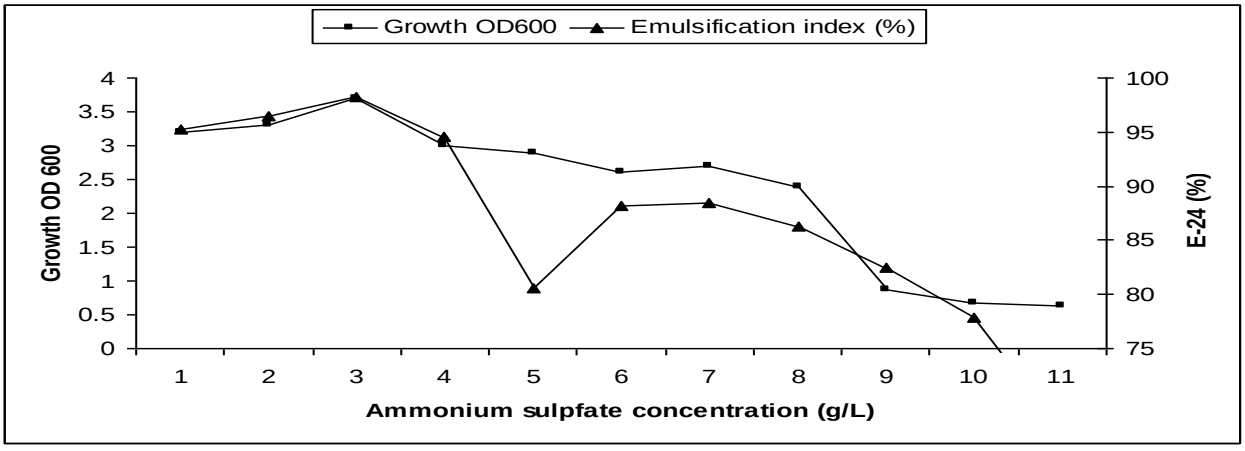

Fig. 6. Growth and emulsification index of *Bacillus subtilis* Eg1 grown on liquid SBM amended with 5 % glucose as carbon source and different concentrations of ammonium sulphate

* Cells were incubated for 24h at 30 C with 200 rpm agitation rate.

Morover, (Guuerra–Santos et al.,1984) showd maximum rhamnolipid production after nitrogrn limitation at a C: N ratio of 16:1 to 18:1 and no surfactant production below a C: N ratio of 11:1 was observed.

4.4.3. Characterization of *Bacillus subtilis* Eg1 strain biosurfactant

The biomolecule produced by Bacillus subtilus. Eg1 was recovered using ethanol and purified as described in the material and methods section. The purified product was characterized with respect to its ability to reduce surface tension and emulsification activity.

- **Effect of pH on surface tension**

The surface tension of whole broth of selected strains maintained nearly constant at all tasted pH (4.2-9.2) at 30ºC, indicating that pH variation has no appreciable effect on surface tension. But maximum of surface tension reduction was at pH 6.2-7.2 (Fig. 7). They found the surface tension reducing activity of *Bacillus subtillis* Eg1was stable to pH over the range of pH of 5.0-9.5. Also (Kumar et al., 2006) observed biosurfactant production of *Rhodococcus* was ranged from 6.5 to 7.2 that determined by surface tension.

- **Effect of temperature on surface tension**

All of the strains reduced surface tension in tasted temperatures but the best temperature for selected strains was proven to be between 30-40 (Fig.8). In this study, strains found to be reduced surface tension in tasted temperatures but the best temperature for selected strains was ranged 30- 40°C. Also (Banat et al., 1993) found the optimum biosurfactant production of *Rhodococcus.* sp at 37ºC.

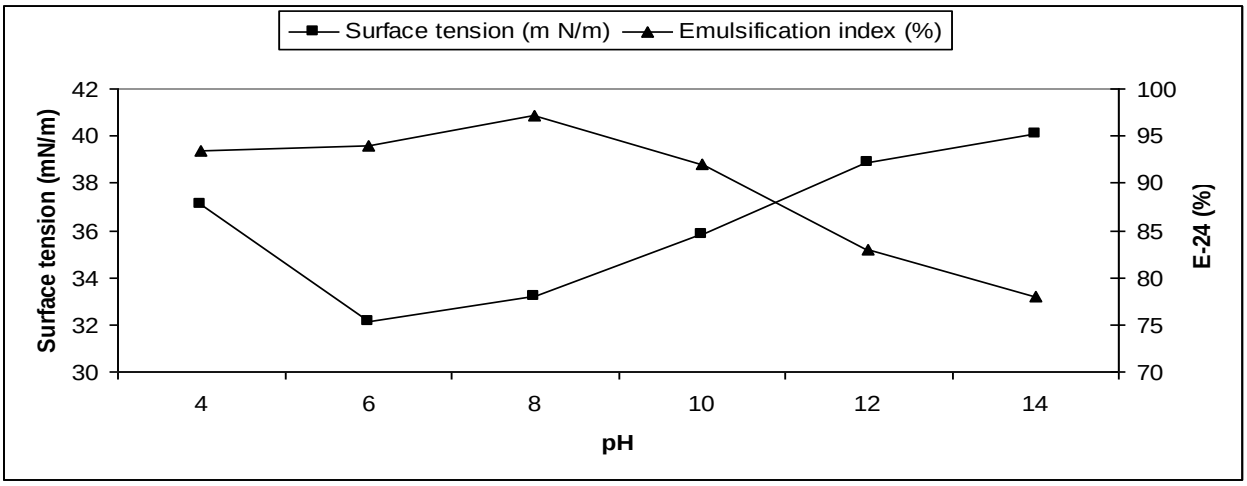

Fig.7. Effect of different pH on biosurfactant production

*Incubation was done at pH 7 for 24h with 200rpm agitation rate.

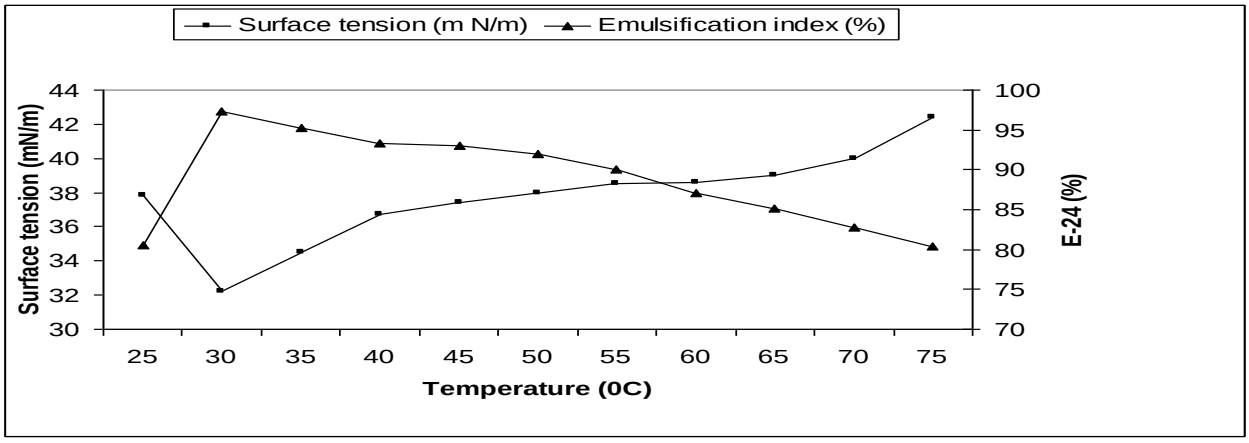

Fig. 8. Effect of different temperatures on biosurfactant production

* Incubation was done at pH 7 for 24h with 200rpm agitation rate

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الملخص العربي

عزل سلالات بكتيرية محلية قادرة على انتاج مخفضات التوتر السطحى وعمل شجرة التطور الوراثى لهما

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تتكون المركبات المعروفة بمخفضات التوتر السطحى من شطرين أحدهما محب والأخر كاره للماء ولذا فهى تتجزأ تفضيلياً على السطح الواقع بين سائلين مختلفين فى القطبية مثل السطح المشترك بين الماء والزيت مؤدية الى خفض التوتر السطحى وتكوين مستحلب يمكن ان يذوب فى الماء أو العكس. وتمنح هذه المركبات خواص ممتازة فى التطهير، والتنظيف والإستحلاب، وتكوين رغوة كما أن لها صفة التشتيت مما يجعل صناعة مخفضات التوتر السطحى أحد الصناعات الكيميائية متعددة الإستعمالات. وتنتج الغالبية من هذه المركبات فى الوقت الحالى كيميائيا من البترول ومع ذلك فلقد تزايد فى الآونة الأخيرة الإهتمام بإنتاج هذه المركبات من الكائنات الدقيقة ويرجع ذلك الى تنوعها الكبير وطبيعتها الآمنة بيئيا وامكانية انتاجها بواسطة التخمير. وتطبيقاتها الفعالة فى مجال البيئة واستخلاص الزيت الخام والرعاية الصحية والمبيدات الحيوية الزراعية وكذلك الصناعات الغذائية المتعددة وفى العديد من المجالات الطبية والصيدلانية.

وتعتبر مركبات مخفضات التوتر السطحى المنتجة بيولوجيا كمجموعة متنوعة من الجزيئات النشطة فى خفض التوتر السطحى للسوائل مما يجعلها مرشحا فعالا فى استخلاص الزيت الخام. وتتفوق هذه المركبات عن مثيلاتها المصنعة كيميائيا بدرجة أقل فى السمية ،ولها قدرة عالية على الهدم الحيوى الى جانب تفوقها فى احداث الرغوة، ولها درجة حساسيه عالية ومتخصصة عند درجات حرارة العالية وأس هيدروجينى غير عادى ودرجات ملوحة مختلفة كما أنها تتميز بامكانية انتاجها من مخلفات الزراعية والصناعية. ولذا فقد استهدفت هذه الدراسة عزل سلالات بكتيرية تتميز بكفاءتها فى انتاج مخفضات التوتر السطحى ثم إختيار أفضلها ودراسة المواد الغذائية اللازمة لتحفيزها على انتاج مركب ذو نشاط استحلابى عالى.

وتتلخص النتائج التى تم التوصل إليها فى هذا البحث فى النقاط التالية:

1- عزل واختيار 15سلالة بكتيرية مختلفة مورفولوجيا ودراسة قدراتها على انتاج مخفضات التوتر السطحى وأوضحت النتائج أن جميع العزلات ذات نشاط استحلابى عالى بالرغم من ان البعض منها لم يتمكن من خفض التوتر السطحى بدرجة عالية. وتميزت السلالات بقدرتها على تحليل الدم فى أطباق بترى محتوية على أجار الدم.

2- ومن النتائج تم اختيار عزلتين (3C.A – C1) تميزتا بأعلى نشاط استحلابى ومنها تم عزل الحمض النووى الريبوزومى وتعريضه لتقنية تفاعل سلسلة البوليمرات ثم تحليل التتابع النيوكليوتيدى لكل عزله. وتم ايداع التتابعات النيوكليوتيدية فى البنك الجينى بأرقامJF806950.1GU247131 وباستخدام برامج حاسب آلى متخصصة فى انشاء شجرة التطور النوعى لمحاولة التعرف على العزلتين ومنها تبين أن العزلة الأولى 3C.A تنتمى الى الجنس باسيلاس بنسبة تشابه 99% والعزلة الثانية تنتمى الى الجنس بسودوموناس بنسبة 99%. وبناء على ذلك اختيرت العزلة الأولى التى تتبع للجنس باسيلس لإستكمال الدراسة.

3- أجريت عملية التخمر للبكتيريا *Bacillus subtilis* Eg1 فى وسط غذائى معدنى بعد استبدال الهكساديكان بأحد المركبات الكربوهيدراتية سهلة الإمتصاص واجرى التحضين فى مزارع مغلقة عند 30درجة مئوية ولمدة 24ساعة وبعد فصل الخلايا تبين ان الراشح الناتج من المزارع محتوى على الجلوكوز (50 جرام/ لتر) و قد أعطى اعلى نشاط إستحلابى (98.2 %).

4- تم إختبار مدى تأثير النشاط الإستحلابى للراشح البكتيرى بالمادة المستخدمة كمصدر للنيتروجين وتبين تفوق كبريتات الألمونيوم

بتركيز (4جم/ لتر) عن باقى المركبات المستخدمة وذلك فى وجود الجلوكوز (50جم /لتر).

5- أجريت دراسة لتحديد كفاءة المركب الناتج من *Bacillus subtilis* Eg1 فى خفض التوتر السطحى ونشاطه الإستحلابى عند درجات حرارة مختلفة وأس هيدروجينى متنوع. وقد تبين نشاط المركب فى خفض التوتر السطحى والنشاط الإستحلابى عند مدى واسع من الأس الهيدروجينى ودرجات حرارة تتراوح ما بين 70 – 100درجة مئوية.