

Antimicrobial Activity of a Bioemulsifier Produced by *Serratia marcescens* S10

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Abstract

This study was designed to evaluate the ability of bioemulsifier to inhibit the growth of some pathogenic microorganisms. Fourteen isolates belonged to *Serratia* sp. were collected and tested for their ability to produce bioemulsifier. Results showed that *Serratia marcescens* S10 (isolated from the gut of the American cockroach) had the highest ability to produce bioemulsifier, among 14 isolates belong to *Serratia* spp. and it had the ability to inhibit the growth of some microorganisms. The production of bioemulsifier was detected by determination of emulsification index (E24%), qualitative drop-collapse test, emulsification activity (E.A) and measuring the surface tension (S.T). The results of bioemulsifier produced by *Serratia marcescens* S10 cultivated in mineral salts broth containing olive oil were: E24%= 87%, E.A= 0.31 and reducing of surface tension from 65mN/m to 41.5mN/m. The bioemulsifier was extracted by organic solvent (chloroform-methanol and diethylether) and antimicrobial activity (antibacterial and antifungal) of bioemulsifier was tested against some pathogenic microorganisms. Results revealed that bioemulsifier reduced the growth of bacteria (*Lesteria* spp., *Salmonella* spp., *Klebsiella* spp. and *Staphylococcus aureus*) and inhibited the growth of fungi (*Candida albicans*, *Aspergillus niger* and *Geotricum* spp.).

Keywords: Bioemulsifier, drop-collapse, emulsification index (E24%), emulsification activity (E.A), surface tension (S.T), antimicrobial activity.

Introduction

The microbial surfactants called as bioemulsifier are microbial compounds with a distinct surface activity that exhibit a broad diversity of chemical structures such as: glycolipids, lipopeptides and lipoproteins, lipopolysaccharides, phospholipids, fatty acids and polymeric lipids. Therefore, it is reasonable to expect diverse properties and physiological functions of bioemulsifier such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis. A host of interesting features of bioemulsifier have led to a wide range of potential applications in the medical field. They are useful as antibacterial, antifungal and antiviral agents [1].

The bioemulsifiers produced by microorganism get more attention and more likely to be used in the application of many field (agriculture, food, cosmetics, medical, industrial, waste utilization, and environmental pollution control such as in degradation of hydrocarbons present in soil) in spite of the presence of chemical emulsifier but these

chemicals may cause toxic side effects as well as its benefits so microbial bioemulsifier more safe to be used in life [2].

Several biosurfactants have shown antimicrobial activity against bacteria, fungi, algae and viruses. A rhamnolipid mixture obtained from *Pseudomonas aeruginosa* AT10 showed inhibitory activity against *Escherichia coli*, *Micrococcus luteus*, *Alcaligenes faecalis* (32mg/ml), *S. marcescens*, *Mycobacterium phlei* (16mg/ml) and *Staphylococcus epidermidis* (8mg/ml) and excellent antifungal properties against *Aspergillus niger* (16 mg/ml) [3].

The mechanism of bioemulsifier antimicrobial activity: It is generally accepted that bioemulsifier act on the integrity of cell membranes, which leads to cell lysis. However, the ways in which the bioemulsifier affect the membrane integrity differ, the lipopeptide, iturin A, is thought to disrupt plasma membranes of yeast cells by accumulation of intramembranous particles in the cells and by increasing the electrical conductance of the membrane. The lipopeptide, surfactine has been shown to

increase membrane permeability through interaction with cell membrane phospholipids. A similar mechanism has been proposed for rhamnolipid, a glycolipid which are thought to act on the lipid part of cell membranes or outer proteins, causing structural fluctuations in the membranes. Rhamnolipid antifungal activity has been attributed to zoospore lysis [4].

The aim of this study is production, extraction of bioemulsifier from *Serratia marcescens* S10 and investigation the bioemulsifier potential antimicrobial activity as reduced microbial growth and inhibition zones.

Materials and Methods

Bacterial Isolates

Isolation of bacteria on: Nutrient, MacConkey and DNase agar and identification was carried out according to morphological and biochemical tests like: Oxidase, Catalase, Urease, Motility test and also identification by API 20 E System [5, 6].

Bioemulsifier Production Medium

Mineral Salts Broth

Bioemulsifier production medium contained: A quantity of 0.5 g NH_4Cl , 4 g NaCl , 0.5 g KH_2PO_4 , 1 g Na_2HPO_4 and 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 liter distilled water. Aliquot of 10 ml of olive oil (or any other oils) was added as carbon source; pH was adjusted to 7.3 and incubated at 30°C for 5 days [7, 8].

Extraction of Bioemulsifier

The bioemulsifier was produced by culturing *S. marcescens* S10 in two liters of optimum medium which divided into 8 flasks of (500ml) volume each flask contained 250 ml of the medium (mineral salt broth containing sesame oil as carbon source, ammonium sulphate as nitrogen source with pH8 incubated at 37°C in shaker incubator for 9 days). After that the extraction of bioemulsifier was performed by 2 methods:

Method (1): extraction with diethyl ether: equal volumes of cell free supernatant from cultured mineral salts medium and diethyl ether were mixed in separating funnel (250ml) very well by shaking and allowed to stand, the aqueous layer was removed and the emulsifier layer was collected in a sterilized and measured glass Petri dishes and dried in oven

at (40–45) °C, the dried emulsifier was measured [9].

Method (2): extraction with chloroform – methanol (1:1):

Bioemulsifier from the supernatant was extracted twice with an equal volumes of chloroform–methanol (1:1), and mixed in separation funnel, the aqueous layer at the bottom of the separation funnel was removed and the emulsifier layer was collected in a glass petri dish and dried in oven at 40–45 °C, the emulsifier was collected by scrubbing and preserved in a clean screwed glass vials as dried powder [10].

Detection of Bioemulsifier Production

Qualitative Drop – Collapse Test

A rapid method (drop–collapse) was used for screening the biosurfactant production by bacterial isolates. This method was used in order to detect the surfactant in culture broth. It was performed by placing 20 μl drop of cell free culture supernatant on a hydrophobic surface such as the lid of a Petri dish. The drop is either remains globular or collapses rapidly while increasing the wet area on the surface; this result indicates the presence of a surfactant capable of lowering the surface tension of the liquid [11].

Determination of Emulsification Index (E24%)

One ml of cell free supernatant was added to 1 ml of kerosene or (oil) hydrocarbon (equal volumes V:V), mixed with vortex for 2 min., and left for 24 hrs. at room temperature, the height of emulsifier layer was measured. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) multiplying by 100 [12].

Determination of Emulsification Activity (E.A) by Optical Density

Cells were separated from 50ml cultured mineral salts broth (containing 0.5 ml olive oil) by centrifugation with cooling centrifuge at 4°C and 5000 – 10000 rpm for 30 min., the emulsification activity was determined by taking 0.5 ml of cell free supernatant and added to 7.5 ml of Tris-Mg buffer { composed of 20 mM (Tris- HCl) (pH= 7) and 10 mM (MgSO_4) [13] } and 0.1 ml of dodecane and

mixed with vortex for 2 min. the tubes were left for 1 hr. and absorbency was measured at 540 nm. Emulsification activity was defined as the measured optical density; blank was Tris-Mg, dodecane and mineral salt broth without culture [14, 15].

Surface Tension Measurement (ST)

The surface tension measurement of cell free supernatant was determined in a K6 tensiometer, using the du Nouy ring method. All measurements were made on cell-free broth obtained by centrifuging the cultures at 10000 rpm for 15 min [16].

Determination of Antimicrobial Activity for The Bioemulsifier

The antimicrobial activity of bioemulsifier was applied against some bacteria and fungi:

The Antibacterial Activity

The antibacterial activity of bioemulsifier was evaluated by agar disc diffusion method [12, 1]. The following bacteria were used for determination of the antibacterial activity for the bioemulsifier: *Lesteria* spp. (isolated from clinical sample), *Salmonella* spp. (isolated from food), *Klebsiella* spp. and *Staphylococcus aureus* (from nasal sample), these isolates were obtained from Biology Department, College of Science, Baghdad University.

These bacteria were grown in Muller Hinton broth and incubated overnight at 37°C. Serial dilutions (10^{-1} – 10^{-6}) were prepared, 0.1 ml from the last dilution was spread on Muller Hinton agar.

Sterile filter paper discs (0.6 cm surface diameter) were soaked with bioemulsifier at a concentration (100) mg/ml in 0.1M (Tris-HCl) and distributed on the cultured medium. The plates were incubated at 37°C for 18 hrs. and the inhibition or reducing in bacterial growth were observed around the discs.

The Antifungal Activity

The antifungal activity of bioemulsifier was determined against:

Aspergillus niger, *Geotricum* spp. and *Candida albicans* (isolated from vagina)

(obtained from Biology Department, College of Science, Baghdad University).

These fungi were cultivated in Muller–Hinton broth and incubated at 28°C for about 4 hrs; 0.1 ml of these cultures was spread on Muller–Hinton agar plates. Filter paper discs were soaked with bioemulsifier and applied on medium surface, incubated at 28°C for 5 days. The inhibition zone was observed and recorded after incubation period [1].

Results and Discussion

Bacterial Isolate

Fourteen isolates belonged to *Serratia* spp. were collected and tested for their ability to produce bioemulsifier. One isolate was selected due to its good production for bioemulsifier, this isolate was confirmed as *Serratia marcescens* S10 (isolated from the gut of the insect American cockroach) which showed the highest ability to degrade oils, hydrocarbons and production of bioemulsifier and it is non-spore forming, Gram negative and short rod. Results of biochemical characteristics showed that it was negative for oxidase, urease and positive for catalase, motility and DNase production and gave negative result for arabinose fermentation [17].

Screening of Bioemulsifier Producing Isolates

Drop-Collapse Test

In the case of bioemulsifier production by the bacterial isolates, the surface tension of the mineral salts medium was reduced and this could be observed by drop-collapse and spread that change the shape of the droplets. If no changes occurred on the shape of the drop and remain beaded it means that there is no reduction in surface tension like in control drop (Fig.(1 A) and (B)).

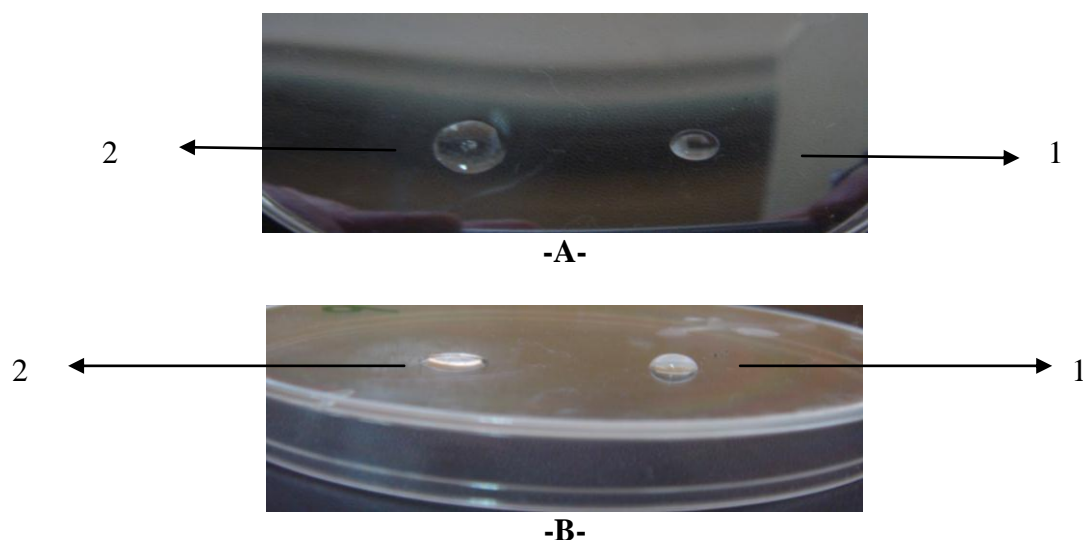


Fig.(1) A-drop-collapse test on the lid of Petri dish (top view)
1- Control, 2- with bioemulsifier of *Serratia marcescens*
B- drop-collapse on the lid of Petri dish (lateral view)
(1- Control, 2- The drop containing bioemulsifier from *Serratia marcescens*).

Note: Controls (filter paper discs) were performed in which pure solvent (0.1M Tris-HCl) was used without bioemulsifier.

When bioemulsifier from *S. liquefaciens* MG1 is dissolved in water at 1 µg/ml and higher, rapid drop collapse was occurred [11]. Jain *et al.* [18] mentioned that this assay relies on the destabilization of liquid droplets by surfactants, therefore, if the liquid dose not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable, but if the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops depend on surfactant concentration and correlates with surface and interfacial tension.

Determination of Emulsification Index (E24%)

Bacterial isolates of *S. marcescens* S10 was cultured in mineral salts medium containing 1% olive oil as a sole source of carbon and energy to induce the *Serratia marcescens* S10 isolate to produce bioemulsifier. The results of emulsifier index (E24%) showed that the isolate was able to produce emulsification layer, the isolate S10 achieved the highest E24% in olive oil containing medium (E24%= 87%) Fig.((2) and (3)).

The results showed an increasing in the bacterial growth in mineral salts medium supplemented with oils. That was observed by increasing the turbidity of the media, and by breaking up the oil drops to small droplets to facilitate its uptake that assist the bacterial growth.



Fig. (2) The emulsification index (E24%) for S10 (the supernatant of culture media were added with kerosene and left at room temperature for 24 hrs.).

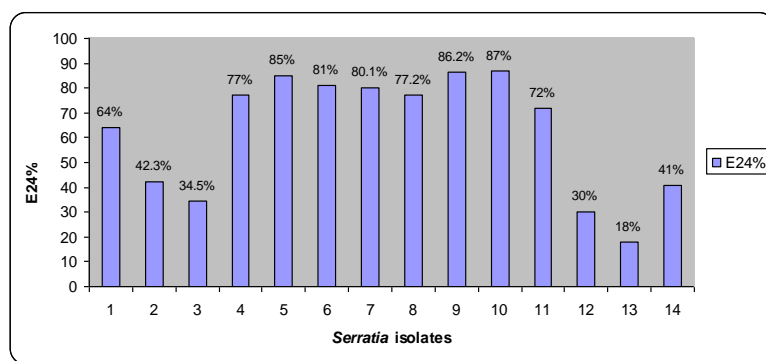


Fig.(3) Emulsification index (E24%) for *Serratia* isolates grown in media containing olive oil as carbon source with pH7.3 incubated for 5 days at 30 °C.

Bacteria generally prefer to metabolize substrates present in the aqueous phase; they also can take up the substrate if they are in close contact with the insoluble phase of the hydrocarbons [19].

Emulsification Activity (E.A)

The emulsification activity (E.A) for the supernatant of *Serratia* cultures was

determined, E.A value ranged between 0.075-0.31. The maximum E.A value was (0.31) recorded in bacterial strain S10 (Fig.(4)).

The measurement of E.A is an indicator for production of bioemulsifier, and can discriminate the more active bacteria.

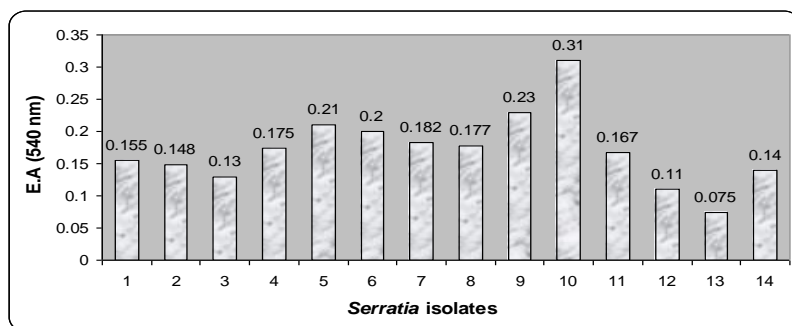


Fig.(4) The emulsification activities (E.A) at (540 nm) for the *Serratia* isolates cultured in mineral salt medium containing 1% olive oil, pH 7.3 and incubated at 30°C for 5 days.

Sifour *et al.* [15] reported that the bioemulsifier produced from *P. aeruginosa* with E.A about 0.43 for olive oil, and when n-alkanes was used E.A increased with the increasing of the number of carbon atoms.

Reducing of Surface Tension

The production of bioemulsifier by the bacterial isolates was further detected by the reduction of surface tension, the highest reduction for surface tension belonged to the isolate S10 (41.5 mN/m) compared with the surface tension of control (65 mN/m) and water (72 mN/m) Fig.(5).

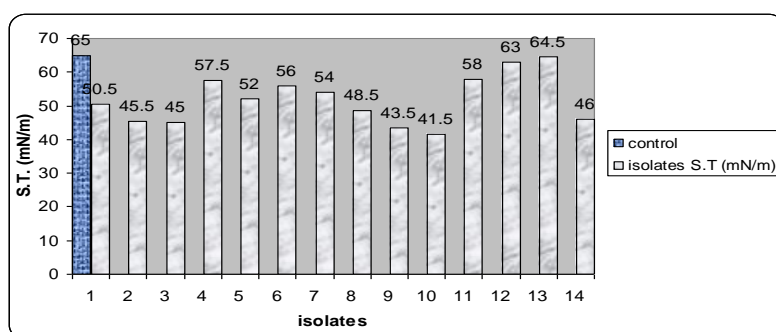


Fig.(5) The reduction in surface tension (S.T mN/m) for *Serratia* isolates, in supernatant of mineral salt medium contain 1% olive oil in shaker incubator at 30°C for 5 days.

Wei *et al* [20] reported that *S. marcescens* SS-1 and its mutant SMΔR produce extracellular surfactant able to decrease surface tension of water from 72 to 37 mN/m for (SMΔR strain) and to 45 mN/m for (SS-1 strain) because its lipopeptide surfactants (serrawettins) lighten the surface-tension burden working at the colony periphery.

The ability to synthesize compounds with surface tension-reducing properties in *Serratia liquefaciens* MG1 revealed a correlation between swarming motility and surfactant production, many produced detectable amounts of acylated homoserine lactone, this indicates that surfactant production in *Serratia* in general is responsive to cell density by

quorum-sensing mechanisms that may control the expression of biosurfactant synthetases [21].

The Antimicrobial Activity of *Serratia* Bioemulsifier

The Antibacterial Activity

The antimicrobial activity of bioemulsifier was tested against some pathogenic bacteria included: *Lesteria* spp., *Salmonella* spp., *Klebsiella* spp. and *Staphylococcus aureus*.

It was observed that the bioemulsifier reduced the growth of these bacteria, the highest effect was observed on the growth of *Staphylococcus aureus*, *Salmonella* and *Lesteria* respectively Fig.(6).

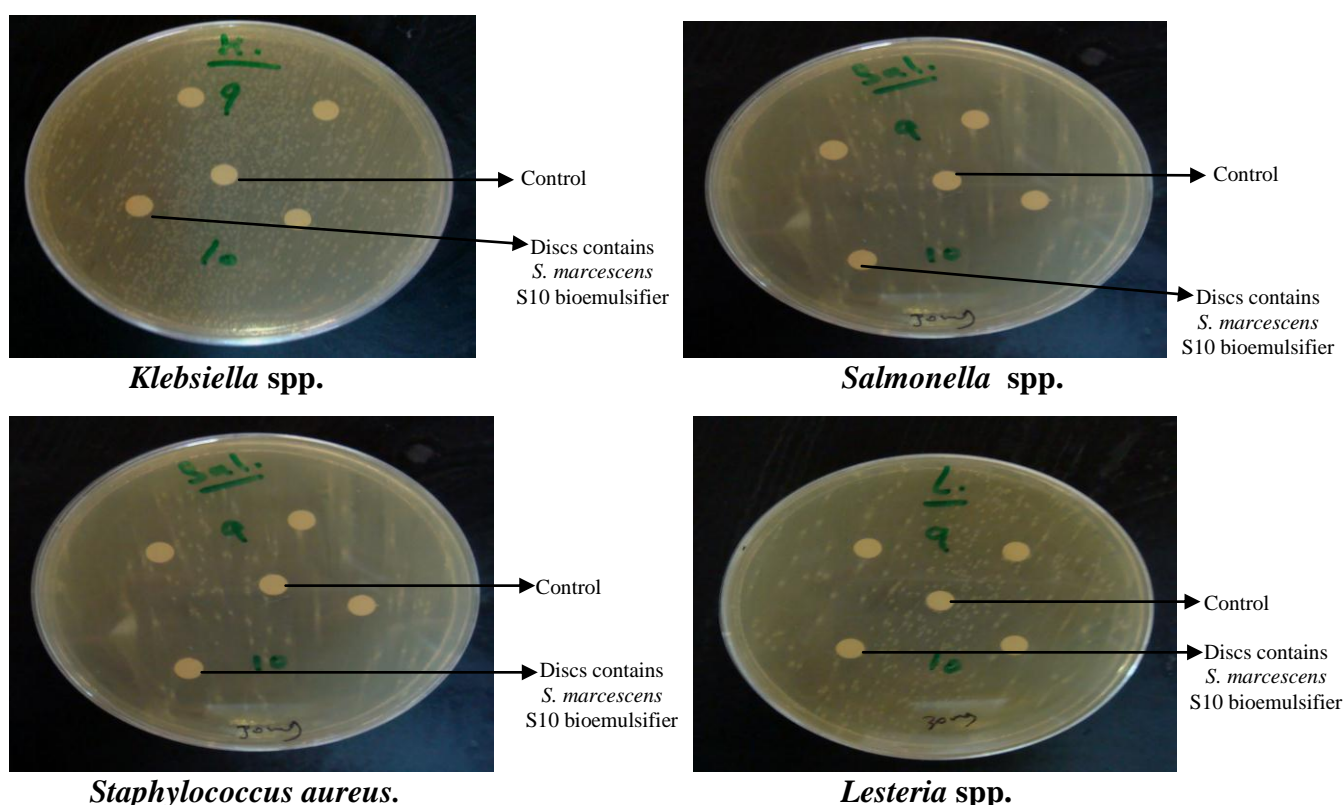


Fig.(6) Antibacterial activity of *Serratia* bioemulsifier against: *Lesteria* sp., *Salmonella* sp., *Klebsiella* sp. and *Staphylococcus aureus*
(Figure showed the heavy growth of bacteria in the middle around the control disc While the growth decreased around the bioemulsifier disc).

Serratia bioemulsifier may effects on the cell wall of the bacteria especially on the lipid in the bacterial cells by emulsifying the lipids. Serrawettins produced from *Serratia* are nonionic surfactants; serrawettins seem to have physiological roles. Since some cyclic lipopeptides have antibiotic activities, serrawettin W2 was examined for this type of activity. The antibacterial activity of serrawettin W2 and serrawettin W1

(serratomolide) against *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 *B. subtilis* ATCC 6633, and *S. aureus* ATCC 25923 was shown to be not so strong [21].

The biosurfactants surfactin, a cyclic lipopeptide, is produced by *B. subtilis* strains and has well-known antimicrobial properties. It has been reported to interact with artificial and biomembrane systems, for example bacterial protoplasts [22].

Antifungal Activity of Bioemulsifier

The antifungal activity of *Serratia* bioemulsifier was detected against *Candida albicans*, *Aspergillus niger* and *Geotricum* spp. Inhibition zones were observed around the disks containing bioemulsifier, the inhibition zones of *Serratia marcescens* (S10) bioemulsifier against *Aspergillus* and *Geotricum* sp. were 12mm and 10 mm respectively. Also the bioemulsifier showed reducing in the growth of *Candida albicans* the inhibition zone of S10 was 9 mm Fig.(7).

The fungi possess cell wall composed largely of carbohydrate layers, long chains of polysaccharides, as well as glycoproteins and lipids [23]. Bioemulsifier can acts on the lipids in the cell, this interaction alters the membrane fluidity and perhaps produces pores in the membrane through which ions and small molecules are lost.

Ballot [4] observed that mono-rhamnolipid of *P. aeruginosa* ATCC 9027 possibly dose not have antimicrobial activity against fungal growth of *Botrytis cinerea*, no zones of clearing were observed when rhamnolipid solution was added in wells of plates with fungal growth .

Biosurfactants produced by *Pseudomonas* spp. displayed antifungal activity against yeasts (*Candida albicans* FMC 17 and *Candida krusei* ATCC 6258), with diameters of zone inhibition ranging between 12 and 17 mm [1].

Iturin (A) kind of surfactant produced by *Bacillus subtilis* has been proposed as an effective antifungal agent for profound mycosis. Other members of the iturin group, including bacillomycin D and bacillomycin Lc, were also found to have antimicrobial activity against *Aspergillus flavus*, but the different lipid chain length apparently affected the activity of the lipopeptide against other fungi [24].

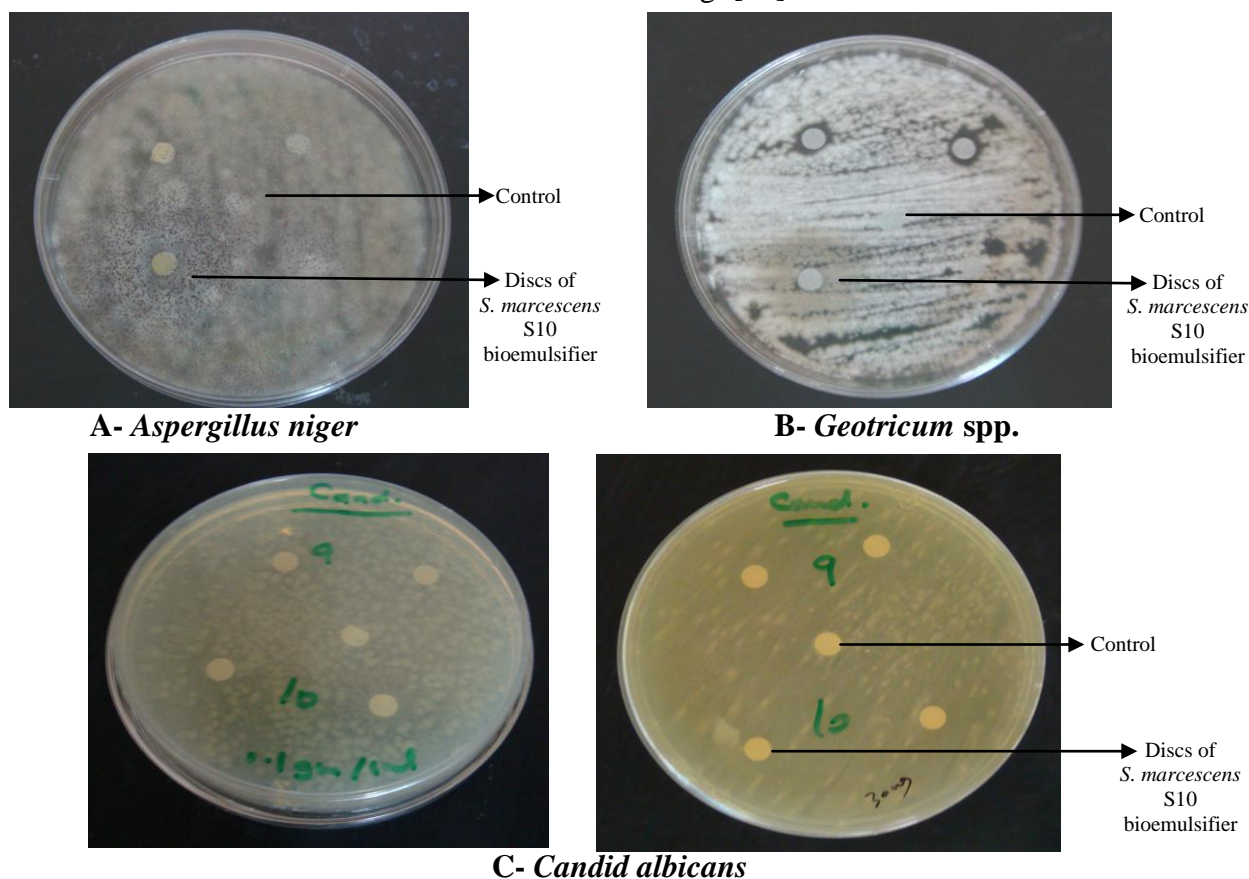


Fig.(7) Antifungal activity of *Serratia* bioemulsifier against: A-*Aspergillus niger*, B-*Geotricum* sp. and C-*Candid albicans* (A and B showed that the disk of control was disappeared because it was covered with the molds growth while there was clear zones surrounded the bioemulsifier disks, C-showed the heavy growth of yeast in the middle around the control disc While the growth decreased around the bioemulsifier disc).

Conclusions

- 1- Locally isolated *Serratia* spp. has the ability to produce bioemulsifier, and the *Serratia marcescens* (S10) isolated from insects has been shown the best production for bioemulsifier.
- 2- The bioemulsifier has antimicrobial activity, since it showed antibacterial activity against *Klebsiella* spp., *Salmonella* spp., *Staphylococcus aureus* and *Lesteria* spp., also has antifungal activity against *Aspergillus niger*, *Geotricum* spp. and *Candida albicans*, and the antifungal activity was highest than antibacterial activity.

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الخلاصة

هذه الدراسة صممت لتقييم قابلية المستحلب الحياتي لتثبيط نمو بعض الاحياء المجهرية الممرضة، اربعة عشر سلالة تابعة لبكتريا *Serratia* spp كانت قد جمعت واختبرت قابليتها لانتاج المستحلب الحياتي.

ان بكتريا *Serratia marcescens* S10 (المعزولة من امعاء الصرصر الامريكي) لها اعلى قابلية لانتاج المستحلب الحياتي من بين 14 عزلة تابعة لبكتريا *Serratia* spp.

تم الكشف عن انتاج المستحلب الحياتي بتحديد النسبة المئوية لدليل الاستحلاب (E24%)، الكشف النوعي لانتهاء القطرة، فعالية الاستحلاب وقياس الشد السطحي، كانت نتائج المستحلب الحياتي المنتج من قبل *Serratia marcescens* S10 المنمأة في وسط الاملاح المعدنية السائل الحاوي على زيت الزيتون كالاتي:

E24%= 87 % ، E.A = 0.31 وخفض الشد السطحي من 65 ملي نيوتن /متر الى 41.5 ملي نيوتن/متر .

استخلص المستحلب الحياتي بالمذيبات العضوية (كلوروفورم - ميثانول و داي اثيل اثير) وتم اختبار الفعالية ضد مايكروبية (ضدالبكتريا وضد الفطريات) للمستحلب الحياتي ضد بعض الاحياء المجهرية المرضية، حيث اظهرت النتائج اختزال المستحلب الحياتي النمو لبكتريا:

(*Salmonella* spp., *Klebsiella* spp., *Lesteria* spp. و *Staphylococcus aureus*)

وثبط نمو الفطريات: (*Aspergillus* *Candida albicans*) و *Geotricum* spp. و *niger* .