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Elicitation of Biosurfactant Production of *Serratia Marcessens* by Using Biotic and Abiotic Factors

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ABSTRACT

The present study focused on induce the production of biosurfactant from previously locally isolated *Serratia marcessens* using biotic and a biotic factor. The production of biosurfactant was detected by determination of emulsification index (E24%) and emulsification activity (E.A). Two bacterial cells were used as biotic factors for enhancing the production of the biosurfactant, the result showed the ability of these bacteria for enhancement, and the best elicitor concentration was 500 µl for both bacteria. Different types of oils were used in this study as abiotic elicitors, all used oils lead to increase the production, but the maximum increase obtained with the use of soybean and petroleum oils. Two types of nanoparticles in different concentrations were used in this study as abiotic elicitors, the result showed that the used nanoparticles had the ability of enhancing the productive surface active substances and three concentrations lead to increasing the production (5, 10, 20 mg/ml) but the best concentration was 5 mg/ml. Various solvents were used for extraction of biosurfactant, the result showed that methanol: chloroform was the best solvent for extraction with a maximum yield of biosurfactant 8.2 g/l. The result showed that the biosurfactant had antibacterial activity against tested pathogenic bacteria.

Keywords: *Serratia marcessens*, Biosurfactant, Nanoparticles, Edible oils.

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INTRODUCTION

The genus *Serratia marcescens* is a rod – shaped, gram negative, facultative bacterium belonging to the Enterobacteriaceae family, widespread in the environment, it can be differentiated from other enteric bacteria due to its characteristic of red pigmentation prodigiosin (Murray *et al*., 2007; *Mahlen*, 2011). Biosurfactant are produced by microorganisms, mostly by bacterial cells, as an extracellular metabolite which contains hydrophilic and hydrophobic moieties (Lin et al., 1998). Biosurfactant chemical composition is diverse includes glycolipids, lipopeptides, polysaccharide–protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids, neutral lipids. Some biosurfactants are a suitable alternative to synthesized medicines and may be used as safe and effective therapeutic agents (Singh and Cameotra, 2004). The antimicrobial activity of several biosurfactants has been reported against bacteria, fungi, algae and viruses (Cameotra and Makkar, 2004). Biosurfactants are unique amphipathic molecules with properties that have been explored for a variety of industrial and bioremediation applications, pharmaceutical and food processing, and oil recovery industries (Desai and Banat, 1997). The main aim of the present work was to a statement of the effect of different biotic and abiotic elicitors in enhancing the production of surface-active substances by locally isolate of *Serratia marcescens*.

MATERIALS AND METHODS

Microorganisms:

Locally isolated *Serratia marcescens* which produces surface active substances. Pathogenic bacteria which used in the present study as inducers and for an antibacterial activity test of biosurfactant molecules (*Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus subtilis* and *E. coli*) all these bacteria were found in the Department of Biotechnology/ College of Science/ University of Baghdad.

Production of biosurfactants by obtaining *Serratia* isolate.

Serratia marcescens, which was previously isolated from hydrocarbon soil, and identified according to morphological characteristics based on Bergey's classification of determinative Bacteriology (Ghada *et al*., 2018) were conserved in nutrient agar slants and kept at 4ºC in refrigerator. A loopful from the surface of nutrient agar contain this isolate was added to flask contain 5 ml of nutrient broth medium (pH = 7), then the flasks were incubated for 24 h, 121 rpm at 30 ºC in a shaker incubator. After incubation, 1ml from nutrient broth was added to two flasks containing 50 ml of mineral salt media as mentioned by [Arutchelvi *et al*., 2009] (NaCl 4g/l, NH_4Cl 0.5 g/l, KH_2PO_4 0.5 g/l, $MgSO_4.7H_2O$ 0.4 g/l and Na_2HPO_4 1 g/l, then the flasks were incubated for 3 days, 121 rpm at 30 ºC in a shaker incubator. After incubation, samples were taken for determination of emulsification index (E24%) and emulsification activity (E. A) Amiriyan *et al*., 2004 and Macfaddin, J. F., 2000.

Determination of Emulsification Index (E24%)

Two ml of cell free supernatant was added to 2 ml of toluene mixed with vortex for 3 min and left for 24 hours at room temperature, the height of emulsifier layer was measured. The emulsification index was calculated by the following equation (Abouseoud *et al*., 2008).

$$\text{Emulsion Index (E24)} = \frac{\text{Height of emulsion layer}}{\text{Total height of broth}} \times 100$$

Determination of Emulsification Activity (E. A) by measuring optical density

100 ml of inoculated mineral salt broth were centrifugation after an incubation period with cooling centrifuge at 4°C, 8000 rpm for 30 min., the emulsification activity was determined by taking two ml of cell free supernatant and added to 8 ml of Tris-Mg buffer (composed of 20 mM (Tris- HCl) (pH= 7) and 10 mM (MgSO4) [13]) with 0.1 ml of dodecane and mixed with vortex for 2 min. The tubes, then left for 1 hour. and absorbance was measured at 540 nm. Emulsification activity was defined as the measured optical density,

blank was Tris-Mg, dedication and mineral salt broth without income (*Serratia marcescens*) [Sifour, M.*et al*., 2007].

Biotic Enhancement the production of biosurfactant.

Live cells of the bacteria *E. coli* and *Staphylococcus aureus* were used in the current study as a biotic catalyst for the production of biosurfactant by *Serratia marcescens*. Inocula of these bacteria were Equipped as follows: a few loopfuls of every bacterial growing of an overnight culture on nutrient agar were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of mineral salt media and incubated at 37°C for 24 hours. After the incubation, a McFarland standard tube was used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria cells was adjusted to be nearly 1×10^8 cells/ml by adding fresh sterile normal saline if necessary. Then, the inoculum of each elicitor was centrifuged at 10000 rpm for 15 min. Bacterial cells were then washed and re-suspended by adding 15 ml of sterile normal saline. The number of *Serratia marcescens* cells was adjusted to 1×10^8 cells/ml by using the same strategy as mentioned above. Then the mineral salt medium was inoculated with prepared *Serratia* inoculum at a level of 1% (v/v). At zero-time, catalyst inoculums were added to the culture media, separately, at a level of (250, 500, 750 and 1000 µl), one flask containing 50 ml mineral salt medium was inoculated with 1ml of *Serratia spp*, which was used as a control in this experiment. Then flasks were incubated at 30 C° for 72 h. 10 ml samples were taken at (Zero time, 24h, 48 h and 72 h) for determination of Emulsification Index (E 24 %) and emulsification activity (E. A).

A biotic Enhancement by using various oil sources.

To determine the effect of using different oils as inducers for enhancing the production of surface-active substances, 1% of various oils (Sesame oil, Castor oil, Olive oil, Soybean oil and Petroleum oil) was added in duplicate to ten (250ml) Erlenmeyer flasks, each containing 50 ml of mineral salt medium at PH 7. After sterilization, each flask was inoculated with 1ml of broth containing *Serratia* inoculum, and incubated in a rotary shaker (120 rpm) at 30 ºC for 72 h. After the incubation, The Emulsification Index (E 24 %) and emulsification activity (E. A) were calculated.

A biotic Enhancement by using different concentrations of ZnO_2 and Fe_3O_2 nanoparticles.

Different concentrations (5, 10, 20, 30 and 40 mg/ml) of ZnO_2 and Fe_3O_2 nanoparticles were prepared using deionized distilled water and Sonication technique. One ml of each concentration was added separately to ten flasks containing sterile mineral salt medium at pH 7 inoculated with 1ml of broth containing *Serratia* inoculum, then the flasks were incubated in a rotating shaker (120 rpm) at 30 ºC for 72 hours. After the incubation, supernatant was taken for the determination of Emulsification Index (E 24 %) and emulsification activity (E. A).

Extraction and partial purification of biosurfactant

One ml of the isolated *Serratia marcescens* broth was added to the flask containing 50 ml of mineral salt medium at pH 7 after sterilization, then it incubated in a rotating shaker (120 rpm) at 30 ºC for 72 hours. The culture medium was first centrifuged for 15 min at 10000 rpm. The supernatant containing biosurfactant was conveyed to a separation funnel and extracted by using diverse solvent systems: chloroform: methanol (2:1), chloroform, methanol and DI ethyl ether. The aqueous layer at the bottom of the separation funnel was removed and the emulsion layer was collected in a glass petri dish and dried at room temperature, the emulsifier was collected by scrubbing and stored in a clean glass vial as dried powder [Maneerat, S. and Dikit, P.,2007]. The resulting powder was weighted to determine the best extraction method.

Tested the antimicrobial activity of the produced biosurfactant.

A stock solution of biosurfactant was prepared by weighting 0.04 g of biosurfactant powder and dissolved in 1 ml of DMSO. The antibacterial activity of biosurfactant was determined against *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneumonia*, using well diffusion method (Yalçin, E. and Ergene, A. (2009). 100 µl of Overnight growth (24 hours) culture of the test bacterium containing approximately $1.5x10^8$ cells/ml was streaked on the surface of the sterile Muller Hinton Agar and left for 10 minutes to settle down the bacteria, 150 µl of the biosurfactant stock solution was added to the prepared wells, 150 µl of DMSO was used in each plate as control. The plates were incubated at 37°C for 24 hrs, then the diameter of the zones was measured, and the results were recorded.

RESULT AND DISCUSSION

Production of biosurfactant by *Serratia marcescens*.

The result showed the locally isolated *Serratia marcescens* had the ability to produce the surface-active substances (biosurfactant) in mineral salt medium, after incubated at 30 °C for 72 hours, as demonstrated in the figure (1).

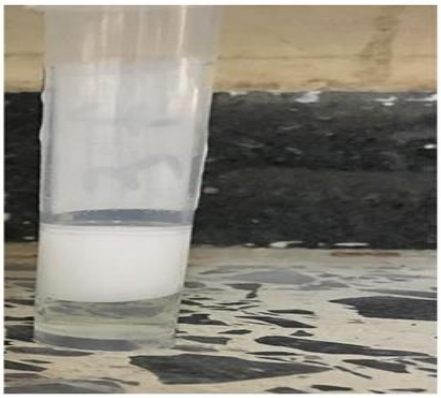

Figure 1. Surface active substances produce by *Serratia marcescens* isolate (30 °C for 72 h).

Many studies improved the ability of *Serratia marcescens* to produce various types of the surface-active substances such as the study of Rajkumar Bidlan and his co-workers (2007) Helvia W. Casullo Araújo1 and co-workers (2017).

Enhancement by using live cells of the bacteria *E. coli* and *Staphylococcus aureus.*

The addition of live cells of *E. coli* and S. *aureaus* of *Serratia marcescens* culture had a great influence on the creation of surface-active substances in which the production of this substance was significantly increased. In the culture of *Serratia* spp. without elicitor (control), the values of E24% for biosurfactant on the first day of incubation was 27 which up to 42 on the second day and increased to 57 on day three. As shown in Fig. (2 and 3). Several concentrations of the elicitor cells were tested (250, 500, 750 and 1000 µl) the results showed that, tow concentrations (250 and 500 µl) had a great effect on increasing the production of these compounds as the E 24% of biosurfactant reached at 76 and 82 respectively on the day three of incubation in comparison with pure culture (control) with the using of *E. coli* (fig 2) and 100 to 155 with bacteria *S. aureaus* (fig 3). At the same time, the Emulsification Activity (E. A) values of surface-active substances increased with the addition of *E. coli* and S. *aureaus* as compared with the production medium free of these bacteria figs (4 and 5).

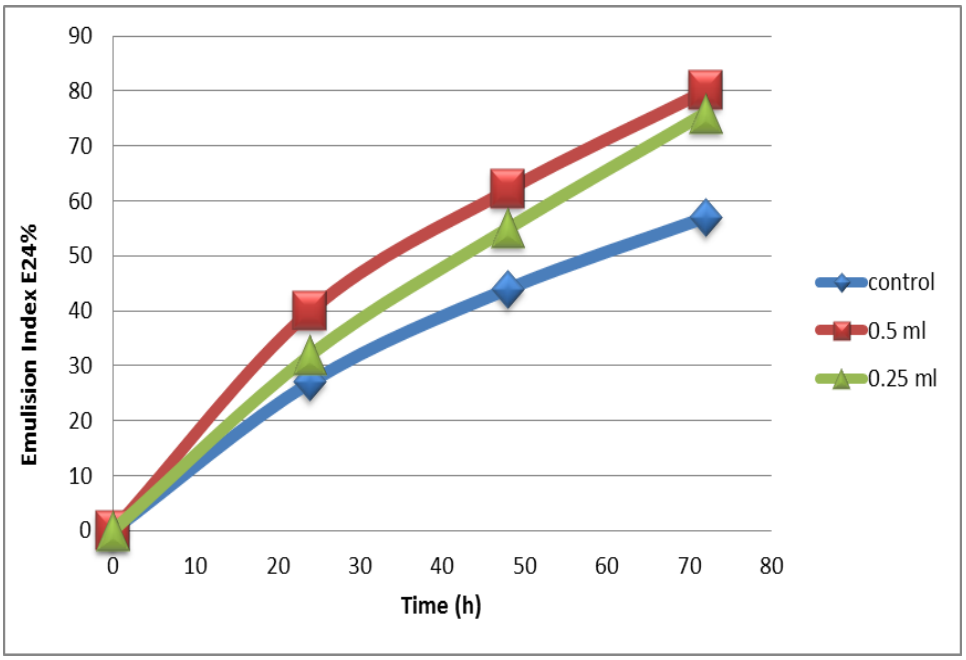

Figure 2. E 24% index of biosurfactant produced by *Serratia marcescens* enhancing with live cells of *E. coli*.

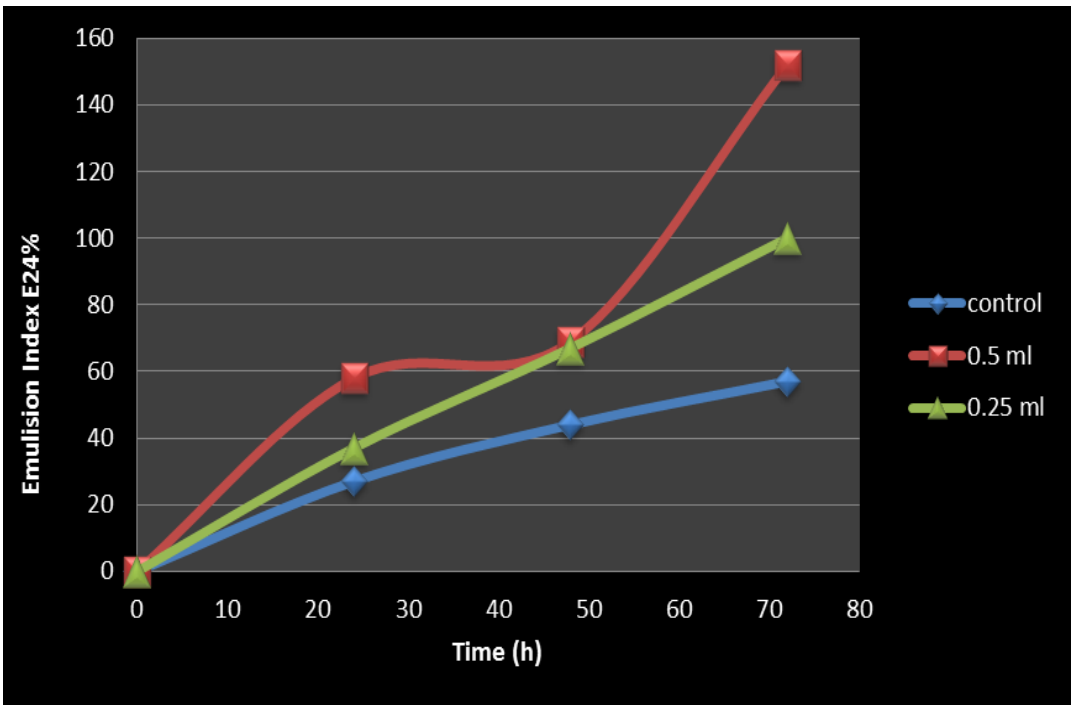

Figure 3. E 24% values of biosurfactant produced by *Serratia marcescens* enhancing with live cells of *S. aureaus*.

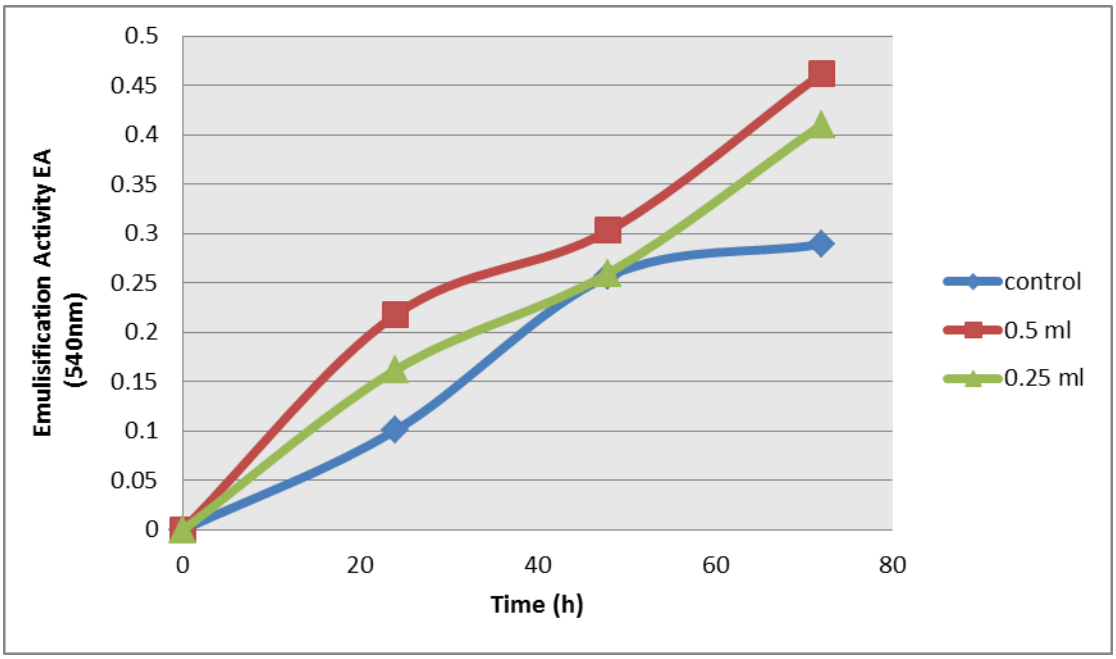

Figure 4. The Emulsification Activity (E. A) of biosurfactant produced by *Serratia marcescens* enhancing with live cells of *E. coli*.

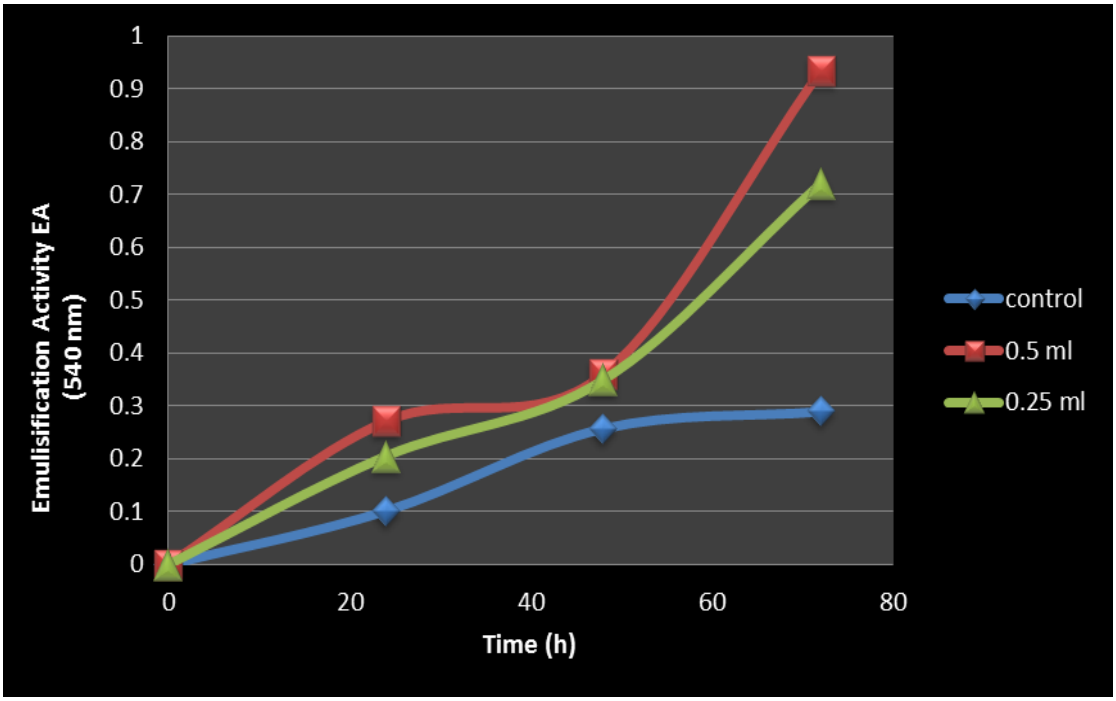

Figure 5. The Emulsification Activity (E. A) of biosurfactant produced by *Serratia marcescens* enhancing with live cells of *S. aureaus*.

These results were compatible with the results obtained by Samer and his work group [2015] who reported that introducing microbial elicitor cells to Serratia marcescens culture media lead to enhance the production of prodigiosin pigment by this bacterium isolate. Khalid and Reem [2013] follow the same strategy and they also found that the addition of live and destroyed cells of E. coli, Bacillus subtilis and Saccharomyces cerevisiae were enhanced phenazine pigment production by the bacteria *Pseudomonas aeruginosa*.

Enhance the production of biosurfactant by using abiotic factors:

Enhancing by using various oils:

Mineral salt media containing edible and heavy oils were used for enhancing the production of biosurfactant from *Serratia marcescens*. The the edible oils and petroleum oil were found to be more suitable for the production of these substances. Out of the different oils used, Soybean oil and petroleum oil were found the best sources for enhancing the production of biosurfactant which led to increasing the value of E 24% and Emulsification Activity (E. A) respectively in comparison with the value of E 24% in the culture free of oil sources (control) as shown in (fig 6 and 7).

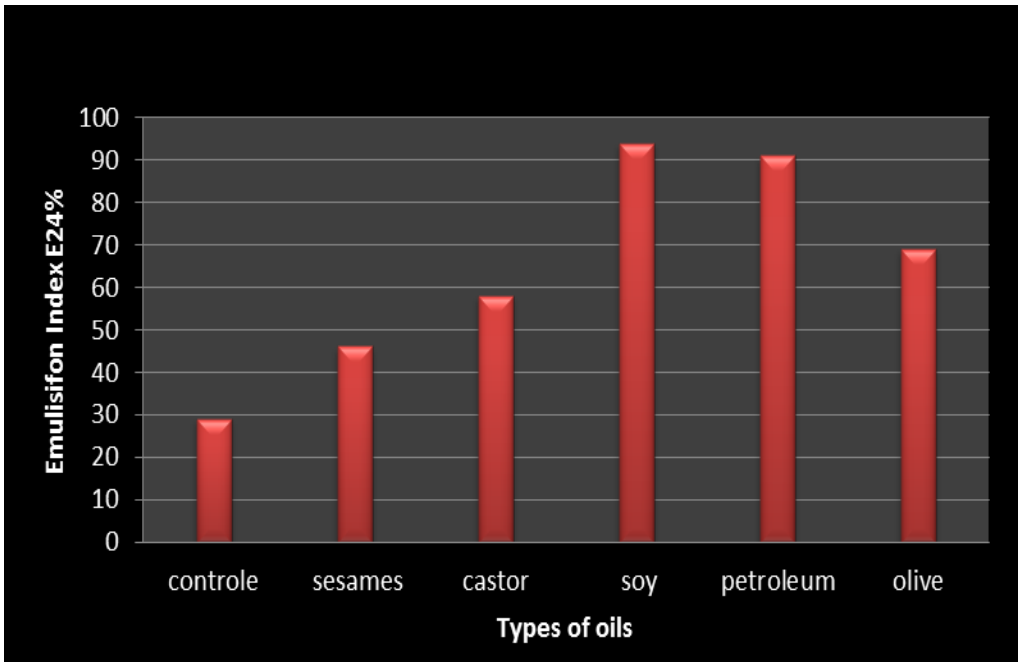

Figure 6. E 24% index of biosurfactant produced by *Serratia marcescens* enhancing with different oils.

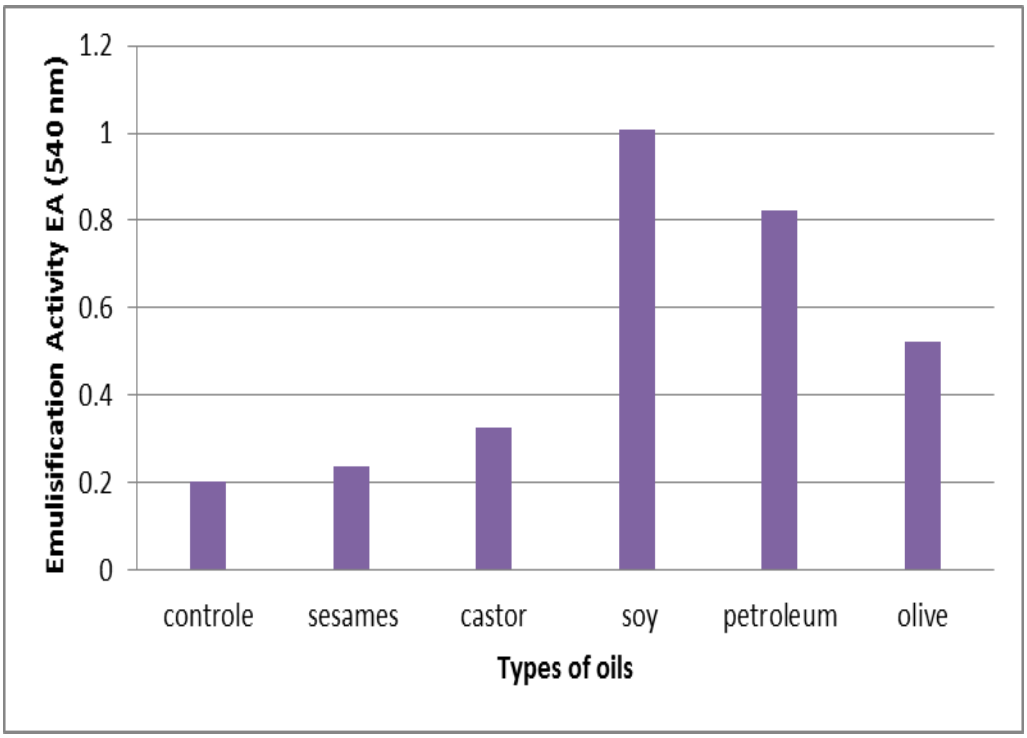

Figure 7. The Emulsification Activity (E. A) of biosurfactant produced by *Serratia marcescens* enhancing with different oils.

Many studies showed that addition of oils to culture media lead to supports the production of microbial secondary metabolites. S. Shahitha and K. Poornima [2012] reported that addition of different oil sources in culture medium enhanced the production of prodigiosin pigment by *Serratia marcescens*. As well as in the study of Swaadoun and his work group the result revealed that, addition of different oil sources for cultures of *Streptomyces* sp. 6621 enhanced the production of Cephamycin C antibiotic by this bacterium [1999].

Enhancing by using different concentrations of ZnO_2 and Fe_3O_2 Nanoparticles.

Different concentrations of ZnO_2 and Fe_3O_2 nanoparticles (5, 10, 20, 30 and 40 mg/ml) were added to *S. marcescens* cultures for increasing the production of surface-active substances from this bacterial isolate. The results demonstrate that three concentrations of ZnO_2 and Fe_3O_2 nanoparticle (5, 10 and 20 mg/ml) had an important effect on the production of these substances as the values of Emulsification Index (E, 24 %) and Emulsification Activity (E. A) of biosurfactant has increased significantly compared to the cultures free of these stimuli, while the 30 and 40 mg/ ml concentrations of ZnO_2 and Fe_3O_2 nanoparticle had a negative effect on the biosurfactant production as shown in the figures (8,9,10 and 11).

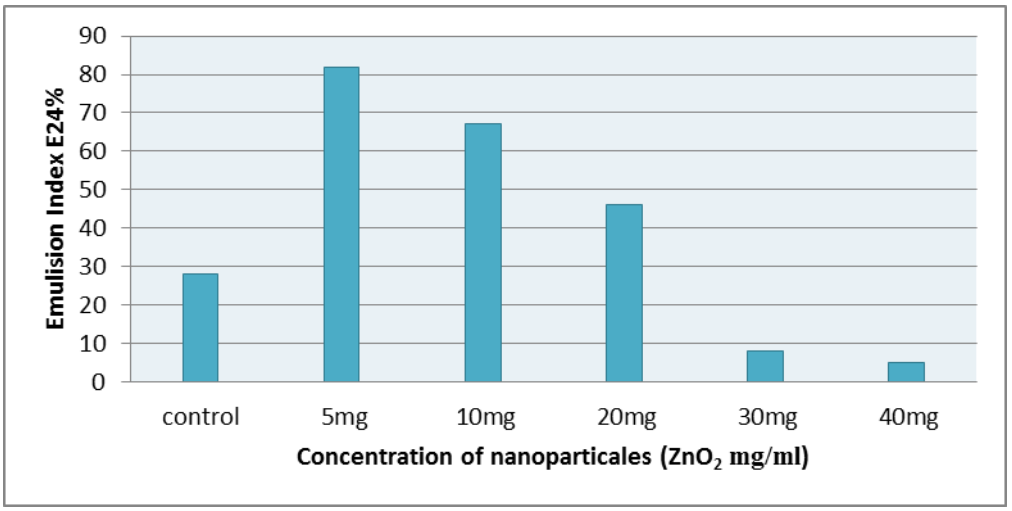

Figure8. E24% values of biosurfactant production by *S. marcescens using* different concentrations of ZnO_2 nanoparticle

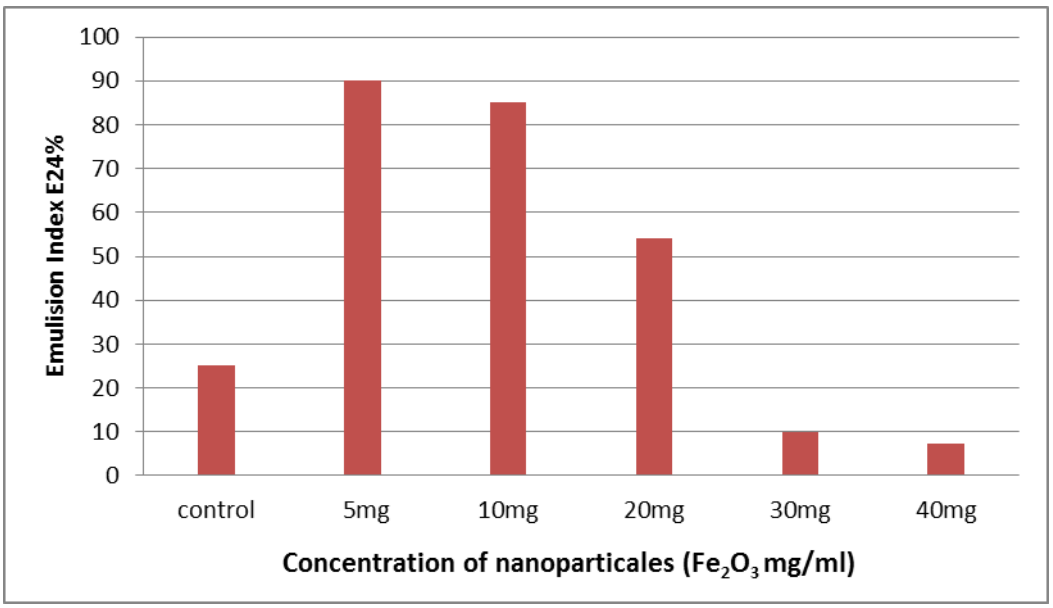

Figure 9. E24% values of biosurfactant production by *S. marcescens* using different concentrations of Fe_3O_2 nanoparticle.

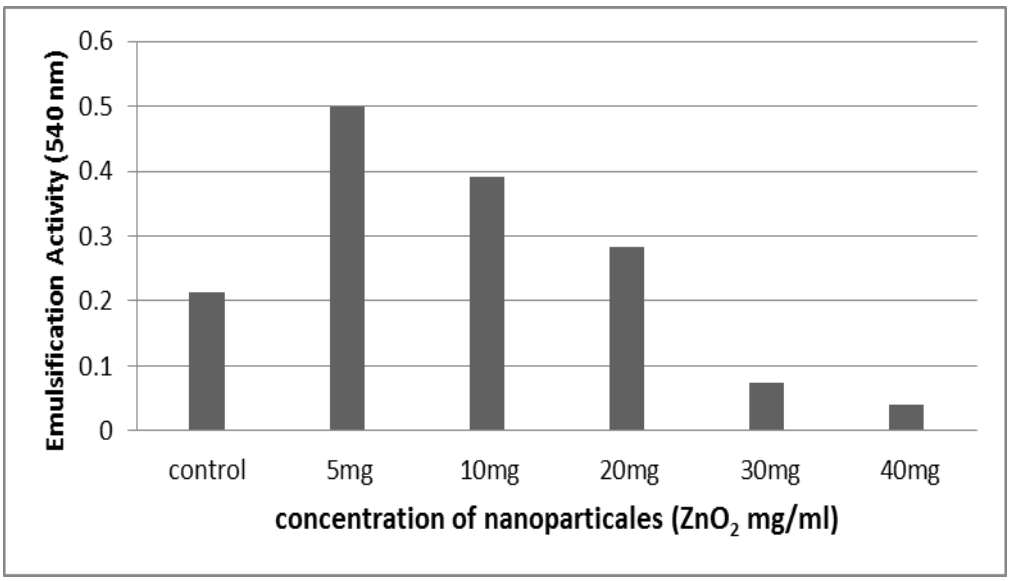

Figure 10. The Emulsification Activity (E. A) of biosurfactant produced by *Serratia marcescens* enhancing with ZnO_2 nanoparticle

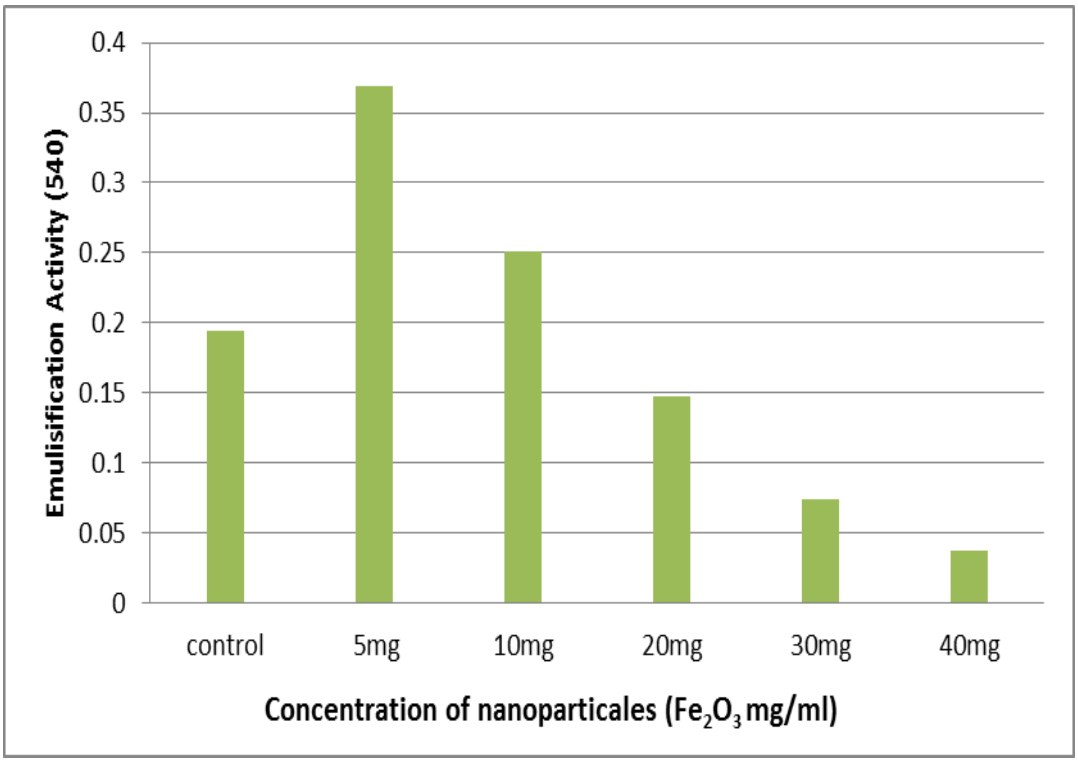

Figure 11. The Emulsification Activity (E. A) of biosurfactant produced by *Serratia marcescens* enhancing with Fe_3O_2 nanoparticle.

There are no previous studies documented using the nanoparticles as elicitors for enhancing the production of secondary metabolites by microorganisms. This represents the first study in this field.

Extraction and partial purification of biosurfactant.

Different solvents (methanol, chloroform, chloroform: methanol (2:1 v/v) and Di ethyl ether) were used for extraction of the biosurfactant produced by *S. marcescens*, the results showed that chloroform: methanol (2:1) was the best solvent system as the yield of the substances obtained by this solvent system was 9.8 g/l as shown in table 1, fig. 12 and figs. 13.

Table 1. The yield of biosurfactant produced by *S. marcescens* for different solvents used.

Solvent	Yield g/l
Chloroform: Methanol (2:1)	9. 8
Methanol	5.1
Chloroform	4. 3
Di ethyl ether	3.6

Figure11. Biosurfactant produced by *S. marcescens* during the extraction with chloroform: methanol (2: 1 v/v)

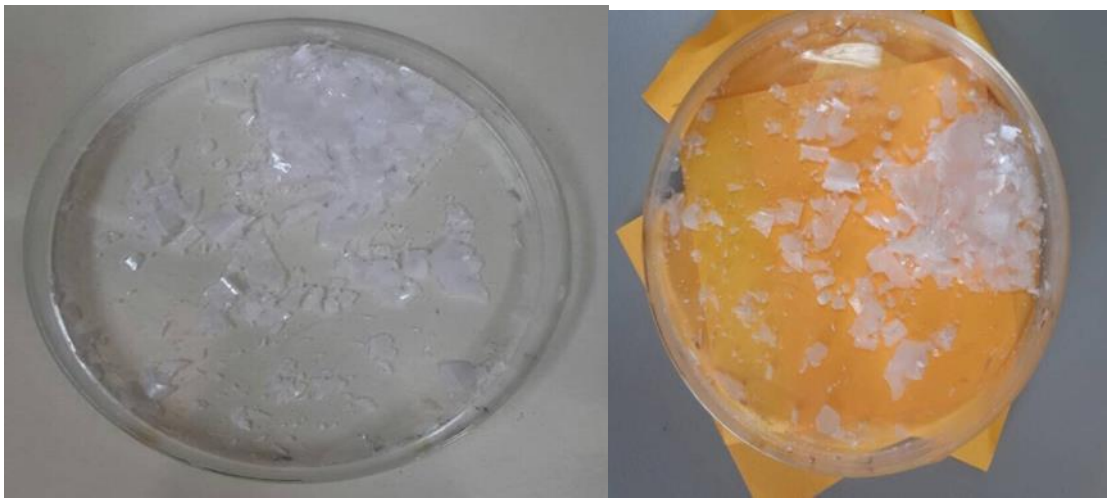

Figure12. Biosurfactant produced by *S. Marcescens* after extracting using chloroform: methanol (2:1 v/v) and dried at room temperature.

Several studies have confirmed that chloroform: methanol is the best solvent to extract surfactants as studied of Entissar and Shatha (2013), and Ghada and her work group (2018).

Antibacterial activity of biosurfactant:

The antibacterial activity of biosurfactant was tested against *E. coli*, *S. aureus*, *B. subtilis* and *K. pneumonia*. It can be observed from the figure (13), these substances were effective against all tested pathogenic bacteria with inhibition zone reached to 15.8 mm with bacteria *B. subtilis*, 16.3 mm against *K. pneumonia*. 14.2 mm against *S. aureus* and 14.1 mm with *E. coli.*

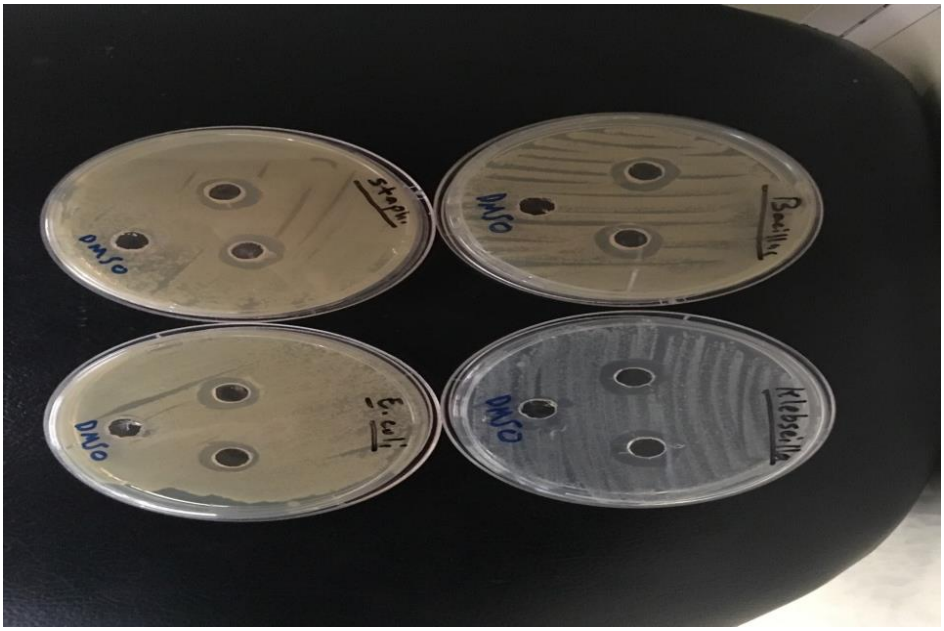

Figure13. The antibacterial activity of biosurfactant produced by *S. marcescens*.

The antimicrobial activity of biosurfactant was recorded in many previous studies as in the study of Ghada et al., (2018); Entissar and Shatha (2013) and Lapenda J. C. *et al*., (2015).

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