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Research article

Production, Optimization and Application of Bioemulsifier Extracted from *Pseudomonas aeruginosa*

Alaa Raheem Kazim*[1], Hussam Mahmood Hassan[1], Suaad Ali Ahmed[1]

ABSTRACT

Pseudomonas aeruginosa was isolated from soil and tested for bioemulsifier production when growing in mineral salt broth medium (production medium) that containing sunflower oil as carbon source. Several nutritional factors, environmental conditions, carbon, nitrogen sources, temperature, incubation period and pH were tested to evaluate the most favorable conditions for bioemulsifier production. The activity of bioemulsifier produced from *P. aeruginosa* as antibacterial substance was examined against many pathogenic bacteria. Maximum production of bioemulsifier was (100%) observed when *P. aeruginosa* grown in mineral salt broth medium with seasam oil and NH_4Cl as carbon and nitrogen sources, incubated at 30 ºC for 3 days, pH 9. Bioemulsifier that extracted from *P. aeruginosa* showed antibacterial activity by reducing growth of several pathogenic bacteria (*Staphylococcus aureus, Bacillus subtilis, Proteus mirabilis* and *Escherichia coli*.

Keywords: Bioemulsifier, Emulsification Index (E24%), *Pseudomonas aeruginosa*.

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INTRODUCTION

Biosurfactants are generally secondary metabolites that form a group of variety compounds synthesized by different microorganisms (bacteria, filamentous fungi and yeasts) [1]. The constriction of these Biosurfactants gave ability to reduce surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases. According to the molecular weight the biosurfactants were classified to low molecular weight compounds called biosurfactants and high molecular weight polymers called bioemulsans or bioemulsifiers [2].

Biosurfactants can be produce as intracellular substances (remain attached to the cell wall) and/or extracellular. The structure of intracellular biosurfactants includes membrane lipids and promotes the transport of insoluble substrates through the membrane; the extracellular biosurfactants help the solubilization of substrate. It's compounded of lipids, proteins and carbohydrates [3]. Bioemulsifier have important applications in the bioremediation of soil and sand or in the spotless of hydrocarbon contaminated ground water and enhanced oil recovery [4]. The filtration properties of bioemulsifier unable microorganisms to grow in contaminated soil and hydrolyzed the hydrocarbon, thus these microorganisms have been reported to produce bioemulsifier [5]. Biosurfactants produced by a strain of *Pseudomonas aeruginosa* SB30 were used to run away the oil from gravel in the oil spill in Alaska [6]. The bioemulsifiers produced by microorganisms get more attention

* Correspondence: Kazim AR.alaa_alkenane@yahoo.com
Department of Biology, College Science, University of Baghdad, Baghdad, Iraq.
Full list of author information is available at the end of the article.

to be used in the application of many fields [7]. Some biosurfactants are known to have important therapeutic applications as antibiotics, antiviral and antifungal agents [4]. Also these compounds have shown antimicrobial activity against bacteria, fungi, algae and viruses. A rhamnolipid produced from *P. aeruginosa* AT10 plays important role as inhibitory substance against *Escherichia coli*, *Micrococcus luteus*, *Alcaligenes faecalis*, *S. marcescens*, *Mycobacterium phlei* and *Staphylococcus epidermidis* and excellent antifungal properties against *Aspergillus niger* [7].

The goal of the present study was to study the effect of some physical and nutritional factors on increasing rate of bioemulsifier from local isolate of *P. aeruginosa* and study the antibacterial activity of this product against different species of bacteria.

MATERIALS and METHODS

Bacterial isolation

Environmental isolate of *P. aeruginosa* that procured from Department of Biology, College Science, University of Baghdad, Baghdad, Iraq was used in current study. This isolates was isolated from soil. The study was carried out by following approval from the academic committee of Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

Production and Detection of Bioemulsifier produced from *P. aeruginosa*

Mineral salts broth medium was used for Bioemulsifier production from *P. aeruginosa*, the medium composed of NH_4Cl 0.5 gm, NaCl 4 gm, KH_2PO_4 0.5 gm, Na_2HPO_4 1 gm, $MgSO_4$. $7H_2O$0.5gm and 10 ml of sunflower oil was added as carbon source. These components were dissolved in 1 liter of distilled water; pH was adjusted to 7.3. After sterilization of media in autoclave inoculated by 10 ml of overnight growth of *P. aeruginosa* and incubated at 37ºC for 5 days [8]**.** The production of bioemulsifier was disclosed by discovering emulsification index (E24%), this was done by adding 1 mL of olive oil to the same volume of culture free cells, vortexes for 2 min and left for 24 h at room temperature [9]. E24% = (he/hT) × 100; where, he is the height of the emulsion layer, and hT is the overall height of the mixture measured by mm [10].

Bioemulsifier extraction

Bioemulsifier was extracted from *P. aeruginosa* by two methods:

Method 1; supernatant of culture was extracted twice with an equal volumes of chloroform / methanol (1:1) by using a separation funnel, the hydrous layer at the bottom of the separation funnel contained bioemulsifier was removed and collected in a glass petri dish and dried in oven at 40 – 45ºC. Bioemulsifier was collected by scrubbing and preserved as dried powder [8]. **Method 2;** similar amount of cell free supernatant and diethyl ether were mixed in separating funnel, the aqueous layer was removed and collected in glass petri dishes, dried and scrubbing and preserved as powder [7].

Effect of Different Conditions on Bioemulsifier Production by *P. aeruginosa*

Effect of Nitrogen Source

Fifty ml of sterilized mineral salts broth containing 1% sun flower oil was prepared in 250 ml flasks by adding 0.05% of different nitrogen sources (NH_4Cl, NH_4NO_3, yeast extract, peptone and gelatine) was inoculated with 0.5 ml of activated bacterial culture broth growing in nutrient broth for overnight, and incubated at 37ºC with shaking for 5 days, after incubation period the emulsification index (E24%) was measured.

Effect of Carbon Source (different kinds of vegetable oils):

Sterilized mineral salts broth (50 ml) containing 0.05% of NH_4CL as nitrogen source and 1% of different kinds of oils (sun flower oil, resinous oil, sesame oil and olive oil) were inoculated with bacterial culture broth and incubated at 37ºC with shaking for 5 days, E24% was measured.

Effect of Temperature

Mineral salts broth medium containing 0.5ml of sesame oil and 0.05% of NH_4Cl as carbon and nitrogen sources, inoculated with 0.5 ml of bacterial culture broth and incubated at different temperatures (25ºC, 30ºC, 35ºC and 40ºC) in shaker incubator for 5 days, after incubation, E24% was determined.

Effect of Incubation Period

Fifty milliliter of mineral salts broth containing 1% sesame oil, 0.05% NH_4Cl was inoculated with activated bacterial culture and incubated at 30ºC at shaking for 3, 5, 7 and 9 days, after that E24% was estimated.

Effect of pH

Fifty ml of mineral salts broth medium containing 1% sesame oil, 0.05% NH_4Cl as carbon and nitrogen sources in 250 ml flasks, each flask was adjusted to different pHs (5, 7 and 9) and inoculated with 0.5 ml of bacterial culture broth, and then incubated at 30ºC (shaking incubater, 15o rpm) for 3 days, after that E24% was measured.

Antibacterial activity

The antibacterial activity of bioemulsifier extracted from *P. aeruginosa* was determined by well diffusion method [11]. Bioemulsifier prepared in two concentrations (100,000 µg/ml and 200,000 µg/ml). *S. aureus, B. subtilis, P. mirabilis* and *E. coli* (clinical isolates), were used to test the antibacterial activity of bioemulsifier. Clinical isolates of bacteria were grown in Muller Hinton broth and incubated over night at 37ºC. The turbidity of broth compared with McFarland tube, 0.1 ml from each bacterial broth spread on Muller Hinton agar, welled was made by cork borer (6mm), 50 µl from bioemulsifier suspension was added into each well. The plates were incubated at 37ºC for 18 h and the diameter of inhibition zones were measured.

RESULTS

Bioemulsifier Production

For evaluation of level of bioemulsifier production by *P. aeruginosa*, E24% was detected. Result showed that the bacteria was able to produce bioemulsifier and the E24% was 45%.

Extraction methods

In this study, two extraction methods were used to extract bioemulsifier from *P. aeruginosa*. The results showed that the best method of bioemulsifier extraction was by using chloroform/methanol (1:1) the emulsification index was 44.5%, while E24% of the another method that used diethyl ether was 39%.

Effect of Different Cultural Conditions on Bioemulsifier Production

Nitrogen sources

In present study, *P. aeruginosa* was grown in mineral salts broth medium with different nitrogenous sources (NH_4Cl, NH_4NO_3, Yeast extract, Peptone and Gelatin). Maximum biemulsifier production was found in medium containing NH_4Cl and the E24% was 46% **Fig 1**.

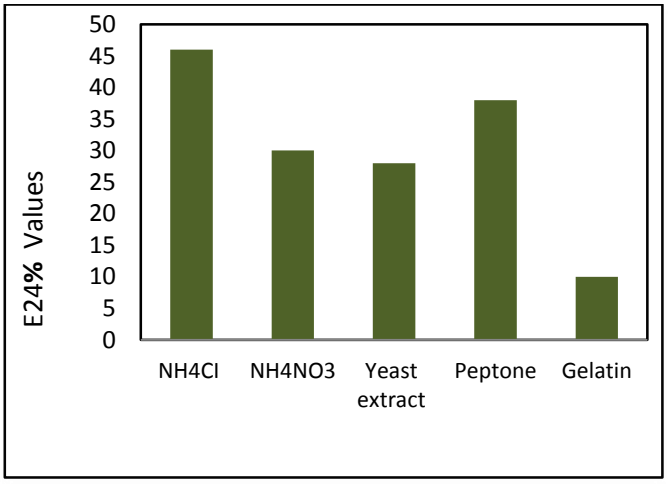

Fig 1. Effect of Different nitrogen sources (organic and inorganic sources) on bioemulsifier production by *P. aeruginosa*.

Carbon Sources (vegetable oils)

The production of bioemulsifier from *P. aeruginosa* was evaluated by adding different carbon source (vegetable oils). The highest production of bioemulsifier was observed when seasam oil used as a carbon source, E24% was 48% (**Fig 2**).

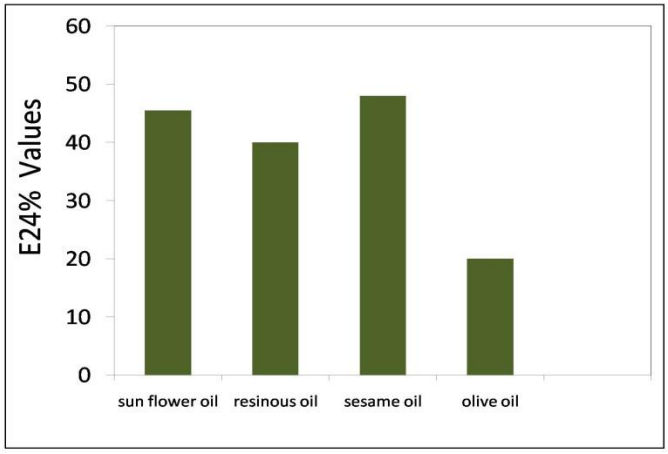

Fig 2. Effect of carbon source (different vegetable oils) on bioemulsifier production by *P. aeruginosa.*

Temperature

Effect of incubation at different temperatures (25, 30, 35, 40˚C) on bioemulsifier production by *P. aeruginosa* was studied. The results showed that the highest production bioemulsifier was observed at 30 ºC, the E24% at this temperature was 50% (**Fig 3**).

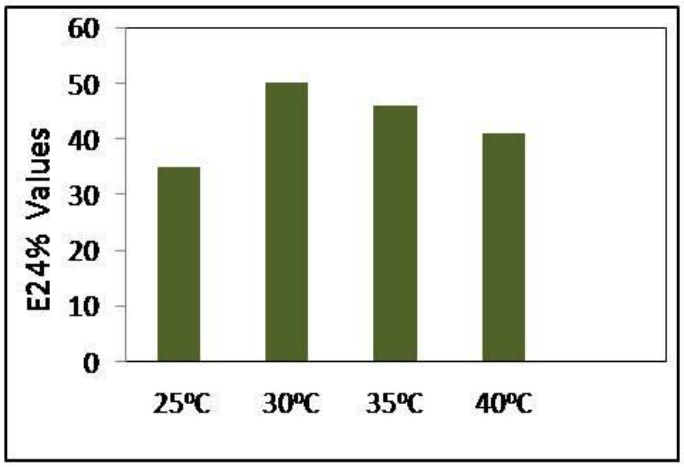

Fig 3. Effect of temperature on bioemulsifier production by *P. aeruginosa.*

Incubation time

The bacterial isolate, *P. aeruginosa* was cultured in mineral salt broth medium and incubated at different time intervals (3, 5, 7, 9 days). The results revealed that incubated the bacteria for 3 days gave the highest cellular mass and bioemulsifier production, E24% was 75%**.** The yield of growth and emulsifier production decreased with increasing the time of incubation (**Fig 4).**

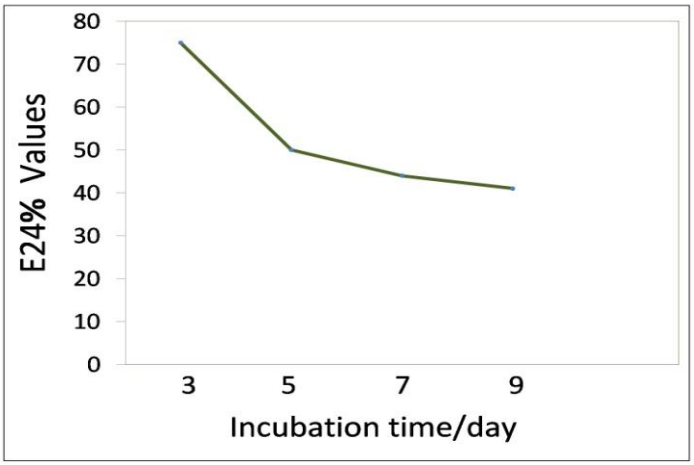

Fig 4. Effect of incubation time on bioemulsifier production by *P. aeruginosa*

pH

The effect of different pH values on bioemulsifier production by *P. aeruginosa* was studied. The bacteria isolate was grown in mineral salt broth medium with different pH values (5, 7 and 9). The maximum production of bioemulsifier was found in the alkaloid medium (pH 9), E24% index was 100% (**Fig 5**).

Antibacterial activity of bioemulsifier

The antibacterial activity of bioemulsifier that produced from *P. aeruginosa* was chwcked against different pathogenic bacteria *S. aureus, B. subtilis, P. mirabilis* and *E. coli*. The results revealed that the bioemulsifier reduced the growth of these bacteria (**Fig 6**) but in different level. The diameters of inhibition zones were showed in **Table 1**.

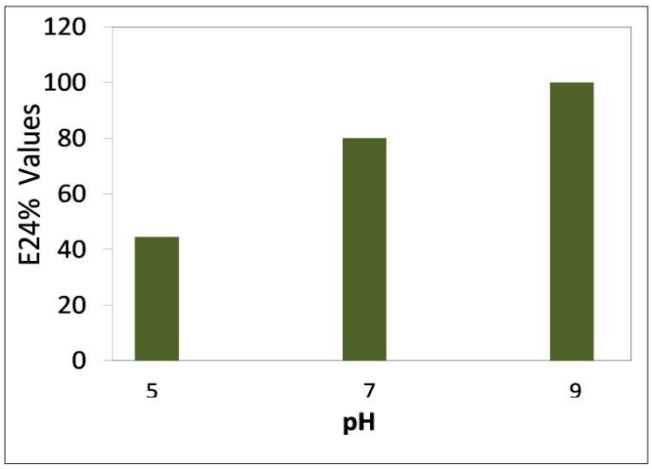

Fig 5. Effect of different pH values on bioemulsifier production by *P. aeruginosa*.

DISCUSSION

Production of biosurfactant has been proven to be important and quite a challenge in industry, but there are many problems such as low yield and high production cost of biosurfactant are important factor and cannot be ignored. These problems could be overcome by searching for more effective and higher yield of biosurfactant from microorganisms. Therefore, become necessary production of biosurfactant for industrial application [2].

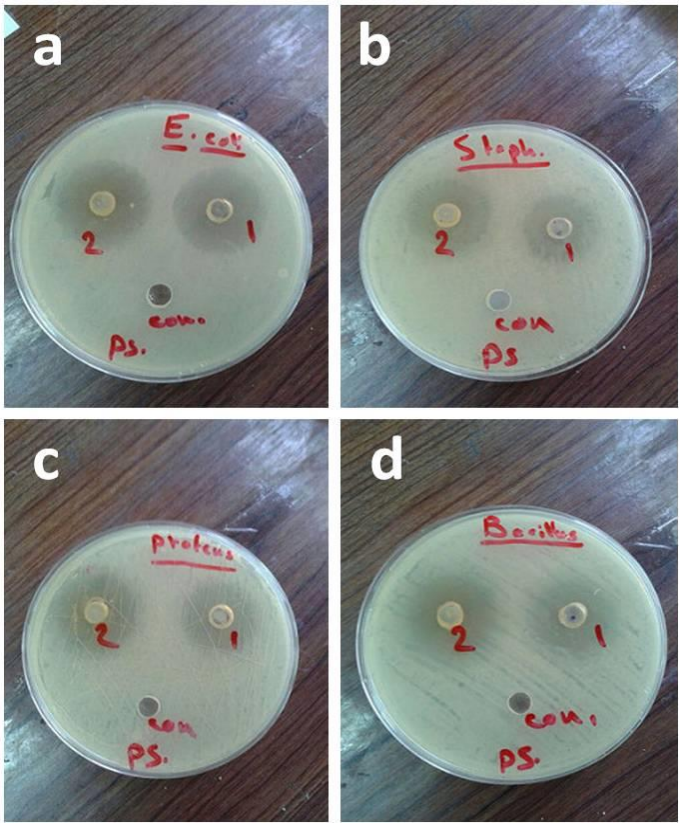

Fig 6. Antibacterial activity of bioemulsifier produced by *P. aeruginosa* against different bacteria species (a, *E. coli;* b, *S. aureus;* c, *P. mirabilis*; d, *B. subtilis*). Con, ontrol; 1, 100,000 µg/ml; 2, 200,000 µg/ml.

Table 1. Diameters of inhibition zones (mm) that formed by the effect of bioemulsifier produced by *P. aeruginosa* against different species of pathogenic bacteria *(E. coli, S. aureus, P. mirabilis, B. subtilis*), 1, 100,000 µg/ml; 2, 200,000 µg/ml.

Type of bacteria	control	1	2
S. aureus	No inhibition zone	23	30
B. subtilis	No inhibition zone	25	35
P. mirabilis	No inhibition zone	28	33
E. coli	No inhibition zone	27	29

Bioemulsifiers are biodegradable products and can be produced in large amounts by different microorganisms, also they are not dependent on petroleum derived products, so they might well be able to replace the traditional synthetic surfactants [12]. Because of the characteristics of some bioemulsifiers that produced by microorganisms such as chemical diversity and excellent functional properties, these compounds used in broad spectrum of potential applications such as agriculture, cosmetics, environmental, food, leather, paper, pharmaceutical, textile industries, oil and petroleum industries, water and soil bioremediation, metal treatment and processing [13].

Among three extraction methods, the mixture of chloroform-methanol (1:1, v/v) was the most efficient in extracting the cell-associated bioemulsifier from cell suspension of *Myroides* sp [8], also Gudiña *et al.* (2015) used chloroform/methanol mixture to extract bioemulsifier from *Paenibacillus* [14]. Desai and Banat (1997) reported there are many factors affect on production of bioemulsifier, the most important factors are carbon and nitrogen sources in addition to other cultural or environmental conditions, ammonium salts and urea were the preferred nitrogen sources for the best production of bioemulsifier by *Athrobacter paraffineus* [15]. Mahdy *et al.* (2012) reported that ammonium ions considered the optimum nitrogen sources for highest production of bioemulsifier [1].

When carbohydrates and vegetable oils used as substrate (carbon source), the bioemulsifier was obtained in optimal yields [12]. Wild type of *B. elkanii* SEMIA 587 and mutant strains produced different bioemulsifiers with different oils and hydrocarbons. Maximum E24% (95%) was obtained from bacterial cell culture of wild type strain SEMIA 587 when used sunflower oil as carbon source [10]. Using sesame oil as carbon source for production of bioemulsifier from *S. marcescens* S10 gave the maximum E24% (92%) [16].

Productivity of the bioemulsifier from *Candida glabrata* was carried out in Erlenmeyer's flasks containing 100 ml of the production medium at 27ºC [12]. While highest production of emulsifier from *C. guilliermondii* S9 was achieved at 30ºC, E24% was (77%) [17], the best value of E24% was 100% obtained from *Streptomyces* sp. SS 20 at 30 °C [2]. Maximum production of biosurfactant from *C. guilliermondii* was 76% at the 7th day of incubation [17]. Hyder. (2015) observed that bioemulsifier produced from *Acinetobacter baumanii* AC5 was associated with bacterial growth. The productivity increased with cell biomass up to 72 h, and maximum bioemulsifier production was gained at the end of the logarithmic phase [18]. Important characteristics of medium for cell growth and metabolites were been at the initial pH [2]. Maximum production of biosurfactant from *S. marcescens* was achieved at pH 8 [19]. While at pH 4 *C. guilliermondii S9* produced maximum amount of bioemulsifier, E24% was 72% [17].

Mechanism of antibacterial activity of bioemulsifier is bioemulsifier act on the integrity of cell membranes, which

leads to cell lysis. There are different ways in which the bioemulsifier affect on the membrane integrity. The lipopeptide, surfactine increased membrane permeability through interaction with cell membrane phospholipids, while rhamnolipid, a glycolipid which are thought to act on the lipid part of cell membranes or outer proteins, causing structural fluctuations in the membranes [20]. Hyder, (2015) reported that bioemulsifier produced by *A. baumanii* AC5 reduced the growth of *E. coli, Salmonella sp., S. aureus and P. aeruginosa.* , the highest effect observed on the growth of *S. aureus* [18]. Bioemulsifier produced by *S. marcescens* S10 had also antimicrobial effect against some pathogenic bacteria included; *Lesteria* spp., *Salmonella* spp., *Klebsiella* spp. and *S. aureus*, largest inhibition zone was observed around the growth of *S. aureus* [7]. While rhamnolipids biosurfactant extracted from *P. aeruginosa* showed antimicrobial activity against different bacteria and fungi strains [11].

Conflict of interest

The authors declare that they have no conflict of interests.

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Author affiliation

1. *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*