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# Determining the optimum conditions for bioemulsifier produced by *Rhizobium leguminosarum biovar viciae* isolated from *Vicia faba* root nodules

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**Abstract**

The tested isolate of *Rhizobium leguminosarum bv. viciae* was isolated from root nodules of *vicia faba* plant from Baghdad ,the isolated bacteria was examined for bioemulsifier production when growing in mineral salt medium that containing 1% sunflower oil as sole carbon source . *Rhizobium* isolate was able to produce bioemulsifier with initial emulsification index E24% was 38%. Many of physical and nutritional cultural conditions for optimum emulsifier production was examined including : pH , temperature ,incubation period ,carbon and nitrogen source. The maximum bioemulsifier production form *Rhizobium* isolate was 64.5% when bacteria growing in mineral salt medium with pH 9 ,incubated at temperature 30 ºC , incubation period was 10 days ,the best carbon and nitrogen sources was sesame oil and $NH_4NO_3$ respectively .

**Keywords:** *Rhizobium, Vicia faba* , Root nodules ,Bioemulsifier , Emulsification Index (E24%)

# تحديد الظروف المثلى لانتاج المستحلب الحيوي من بكتريا *Rhizobium leguminosarum biovar viciae* المعزولة من العقد الجذرية لنبات الباقلاء

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**الخلاصة**

تم عزل وتشخيص بكتريا *Rhizobium leguminosarum biovar Viciae* من العقد الجذرية لنبات *الباقلاء Vicia faba* المؤخوذ من احد المزارع في بغداد، ومن ثم اختبار مقدرتها على انتاج المستحلب الحيوي عندما نميت في الوسط الانتاجي له وهو الوسط الملحي الحاوي على 1% زيت زهرة الشمس كمصدر كاربوني وحيد . وجد ان عزلة *Rhizobium* لها القدرة على انتاج المستحلب الحيوي وبمعامل استحلاب اولي emulsification index E24% مقداره 38% . تم اختبار عدد من الظروف الفيزيائية والغذائية لمزارع الانتاج لتحديد الظروف المثلى، والتي تضمنت الاس الهيدروجيني ،الحرارة ، مدة الحضانة ، والمصدر الكاربوني والنيتروجيني. بلغ اعلى انتاجية للمستحلب الحيوي من بكتريا العقد الجذرية *Rhizobium leguminosarum biovar viciae* بمعامل استحلاب emulsification index E24% مقداره 64.5% عندما نميت في الوسط الملحي الذي كان الاس الهيدروجيني له 9 ودرجة الحرارة 30 م° ، وحضن لمدة 10 ايام . وكان افضل مصدر كاربوني وهيدروجيني على التوالي هو زيت السمسم ونترات الامونيوم.

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**Introduction:**

Microbial surface-active compounds are a group of structurally diverse molecules synthesized by different microorganisms and are mainly classified by their chemical structure and their microbial origin. They are consist of a hydrophilic moiety, comprising an acid, peptide cations, or anions, mono, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids [1] . Biosurfactants, synthesized by microorganisms are amphipathic surface active molecules containing hydrophilicand hydrophobic moieties that act by emulsifying hydrocarbons,increasing their solubilization and subsequently rendering them available for microbial degradation They act by forming micelles at the interface of immiscible liquids such as water and oil by minimizing surface and interfacial tension and blocking hydrogen bonding and hydrophilic/hydrophobic interactions . Bioemulsifiers are surface active compounds that do not necessarily reduce surface tension but form stable emulsions between liquid hydrocarbons and water mixtures and are hence also often defined asbiosurfactants [2].

Surfactants and emulsifiers are indispensable components of daily life. They are widely consumed in the pharmaceutical, cosmetic, petroleum and food industries. Most of these compounds are of petroleum origin, which are not easily biodegradable and their manufacturing processes and by-products can be environmentally hazardous. Increased environmental awareness and strict legislation has made environmental compatibility of surfactants an important factor in their applications for various uses [3].

Some investigations proved that surface activity of biosurfactants is comparable with surface activity of synthetic surfactants. Due to their physicochemical properties, low toxicity, excellent surface activity, high specificity, effectiveness under extreme conditions and biodegradability, bioemulsifiers and biosurfactants are widely used in environmental protection techniques, e.g., water and soil remediation, oil spill removal, etc.. These properties of bioemulsifiers and biosurfactants reflect their potential for commercial applications [4].

The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10-30% of the overall cost. Most oils and fats synthesized in the world are used in the food industry, which generates great quantities of wastes, tallow, lard, marine oils and free fatty acids from the extraction of seed oils [3].

The objective of the present study was optimizing the bioemulsifier production from local isolate of *Rhizobium* including the effect of temperature, pH, incubation period and different carbon and nitrogen sources on bioemulsifier production.

**Material and methods:**

**Bacterial isolation**

Bacteria *R. leguminosarum bv. viciae* was isolated from root nodules of *Vicia faba* plant which collected from one Iraqi farm lying in Baghdad , by using special medium for *Rhizobium* isolation Mannitol Yeast Extract Agar (MYA).

The active root nodules of plant were selected Figure-1, washed with tap water to remove the soil, sterilized by using ethanol , then washed with distilled water (D.W) to remove the effect of ethanol . The nodules were crashed by using needle and mixed with D.W, from this suspension loopfull was transported to slide for bacterial examination under microscope and another loopfull was streaked on MYA . The plates were incubated at 25 °C for 3-5 days [5] . MYA medium consist of (g/L) $K_2HPO_4$ 0.5g , $MgSO_4$ 0.2g , NaCl 0.1g , Mannitol 10g , Yeast Extract 0.4g , Agar 10g [6] .

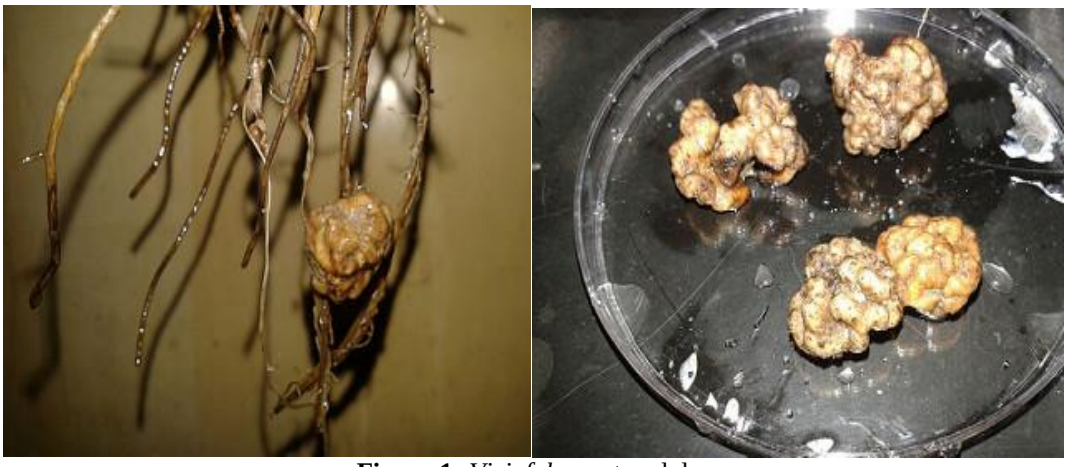

**Figure 1-** *Viciafaba* root nodules

**Bacterial preservation**

**Maintenance in silica gel**

Silica gel powder was put in sterilized vials by autoclaving at 121 ˚C for 30 min , then dried by oven. 0.2 ml from 48h culture was added to the silica gel ,mixed well and maintained at room temperature.[7]

**Bioemulsifier production medium**

**Mineral Salts Broth**

Bioemulsifier production medium consist of (g/L) $NH_4Cl$ 0.5 g , NaCl 4 g , $KH_2PO_4$ 0.5 g , $Na_2HPO_4$ 1 g and $MgSO_4 .7H_2O$ 0.5g. Aliquot of 10 ml of sunflower oil was added as carbon source; pH was adjusted to 7.3 and incubated at 30ºC for 5 days [8]

**Detection of Bioemulsifier Production by determination of Emulsification Index (E24%)**

E24% of culture samples was determined by adding 1 mL of olive oil to the same amount of culture free cells and vortexed for 2 min, leaving to stand for 24 h at room temperature [1] , E24 = (he/hT) × 100; where he (mm) is the height of the emulsion layer, and hT (mm) is the overall height of the mixture.[4]

**Bioemulsifier extraction**

Culture supernatant obtained was extracted twice with an equal volume chloroform/methanol (1:1). The combined extracts were concentrated to dryness using a separation funnel, the aqueous layer at the bottom of the separation funnel were removed and the emulsifier layer was collected in a glass petri dish and left in oven at ( 40 – 45 ) ºC till dryness , the emulsifier was collected by scrubbing and preserved in a clean screwed glass vials as dried powder [8, 9]

**Fourier Transform Infrared (FT-IR) analysis**

The powder of extracted bioemulsifier was made into a pellet for IR analysis . The relative intensity of transmitted light energy was measured against the wavelength of absorption on the region 400-4000 $cm^{-1}$ using FTIR spectrometer.

**Effect of different physical and cultural conditions on bioemulsifier production:**

**1-Effect of pH:**

Fifty ml of mineral salts broth containing 0.5ml sunflower oil in (250 ml flasks) each flask were prepared at different pHs from 5 to 9 which was inoculated with 0.5 ml of bacterial culture broth growing in (MYA broth) for 24h, and incubated at 25ºC with shaking for 5 days, after incubation period emulsification index (E24%) was estimated for the supernatant.

**2- Effect of temperature**

Fifty ml of sterilized mineral salts broth containing 0.5ml of sunflower oil , pH was 9 inoculated with 0.5 ml of bacterial culture broth, then incubated at different temperatures (25ºC, 30ºC, 37ºC and 40ºC) in shaker incubator for 5 days, after incubation period: (E24%) was determined.

**3- Effect of incubation period:**

Prepared 50 ml of mineral salts broth containing 1% sunflower oil with PH 9 was inoculated with 0.5 ml of activated bacterial culture and incubated at 30ºC with shaking for (3,5,7,9,10) days, after each incubation period E24% was estimated.

**4- Effect of different kinds of oils:**

Fifty ml of sterilized mineral salts broth containing 1% of different kinds of oils (sunflower oil, olive oil, sesame oil and resinous oil) were inoculated with 0.5 ml of bacterial culture broth, and incubated at 30ºC with shaking for (10) days, pH was 9 , after incubation period E24% was measured.

**5- Effect of different nitrogen sources:**

Sterilized mineral salts broth (50 ml) was prepared containing 1% of sesame oil as sole carbon source and 0.05% of different nitrogen sources including ($NH_4CL$ ,$NH_4NO_3$ ,Gelatine , Peptone , Trypton and Yeast extract) were inoculated with 0.5 ml of activated bacterial culture broth and incubated at 30ºC with shaking for (10) days. pH was 9 , After incubation period E24% was determined.

**Results and Discussion**

Amongst the soil bacteria a unique group called Rhizobia has a beneficial effect on the growth of plants. It can live either in the soil or within the root nodules of host legumes.The bacteria live and colonize within root nodules, where it converts atmospheric nitrogen to ammonia and provides the plants wirh organic nitrogenous compounds. [10] .*Rhizobium* has been known with itś high specialization to the host legumes ,*R.leguminosarum biovar viciae* domestic specialized to *Vicia faba* plant [6,11].

After incubation for 5 days at 30°C on (MYA) medium ; the growth colonies were rounded convex, smooth, white ,mucoid and glistening [12] Figure-2. Microscopic examination of bacterial cells showed that they were gram negative, bacilli in their shape.

**Figure 2-** Rhizobium growth

Graham, P. H.(1969) reported that the strains of fast-growing rhizobia incubated onYMAA for 3 to 5 days at 28°C produce smooth,white, glistening colonies 1 to 3 mm in diameter, while Altaee,M.I.andAlhasso,M.Z. (2008) reported that the isolated *Rhizobium leguminosarum bv. viciae* was appeared under light microscope by using Gram staining technique as pink colored Gram negative rods[10 ,12].

**Bioemulsifier production**

Biosurfactants are microbial surface active agents synthesized by microorganisms such as bacteria, yeasts and fungi. They may be extracellular or intracellular in nature. Substrates for biosurfactant production are sugars, oils, alkanes and waste materials [1].

For screening the ability of bacteria for bioemulsifier production , the emulsification index E24% was detected, the result showed that the bacteria was able to produce bioemulsifier and the E24% was 38%.

Lopes,E.M.et.al. (2014) reported that the culture medium and substantially cell-free medium from wild-type cell culture and mutant strains of *Bradyrhizobium elkanii* SEMIA 587 exhibited potential emulsifying properties for biotechnology applications that have promise for bioremediation. As biosurfactants are known to increase bioavailability and carry out biodegradation of hydrophobic compounds, different technologies, such as soil washing technology, employ biosurfactants for the effective removal of hydrocarbons and metals, respectively. These biosurfactants can be widely applied in agricultural areas for the enhancement of biodegradation of pollutants to improve the

quality of agriculture soil, also for indirect plant growth promotion through antimicrobial activity and to increase the plant-microbe interaction that is very beneficial for plants. [4].

**FTIR result**

The infrared spectrum of the bioemulsifier that extracted from *R. leguminosarum bv. viciae* showed broad absorption at 3267–3454 $cm^{-1}$ due to N–H stretching , implicating the presence of peptide bonds. C-O stretching vibrations in 1024-1454 $cm^{-1}$ and strong absorption bands of aliphatic carbonyl (ester bond) C=O in 1635 $cm^{-1}$ and 1745 $cm^{-1}$ , 2854-2927 $cm^{-1}$ indicated C–H stretching of aliphatic chains Figure-3.

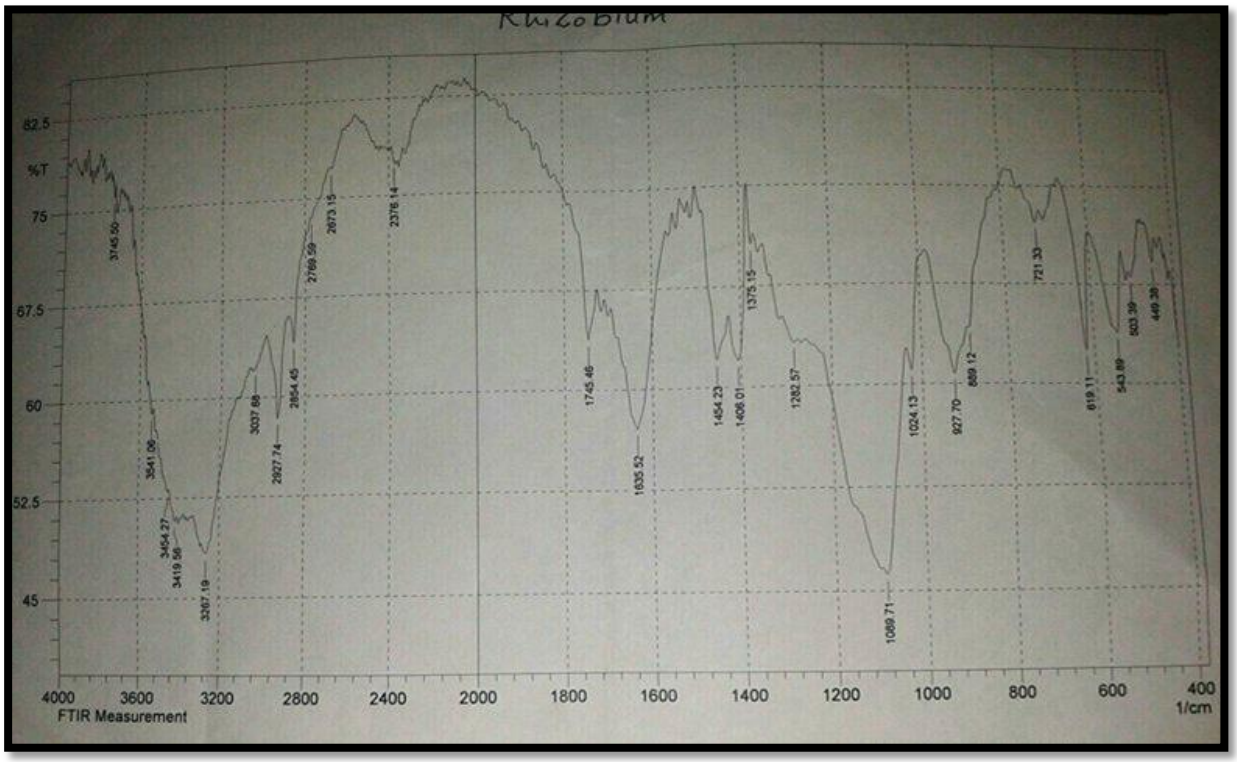


**Figure 3-** FTIR of bioemulsifier produced by *R.leguminosarum bv.viciae*

Sousa, T. d.and Bhosle, S.(2012) [2] reported that broad absorption at 3000–3535 $cm^{-1}$ due to N–H Stretching .methyl and methylene deformation of aliphatic compounds and C-O stretch vibrations in 1000-1465 $cm^{-1}$ [13] . Vibrations observed at 1074.35 $cm^{-1}$ -1726.29 $cm^{-1}$ were characteristic of ester carbonyl groups [2]. 2854-2923 is the stretching of C-H group [14].

The result of infrared spectrum for bioemulsifier produced by *R.leguminosarum bv.viciae* similar to the infrared spectrum of lipopeptide produced by *Pseudomonas nitroreducensTSB.MJ10* [2] .Lopes, E. M.(2014) reported that in general *Bradyrhizobium. elkanii* bacteria are able to produce low molecular weight molecules such as glycolipids, lipopeptides associated to efficient reduction of surface and interfacial tensions, as well as high molecular weight polymers that are efficient emulsifiers [4].

**Effect of different culture conditions on bioemulsifier production**

**1-pH**

The pH of the medium is important characteristics for cell growth and metabolites production. The isolated bacteria was grown in mineral salt broth medium with different pH values (5 - 9), the maximum bioemulsifier production revealed in the medium with pH 9, E24% index was 40%.
Figure-4.

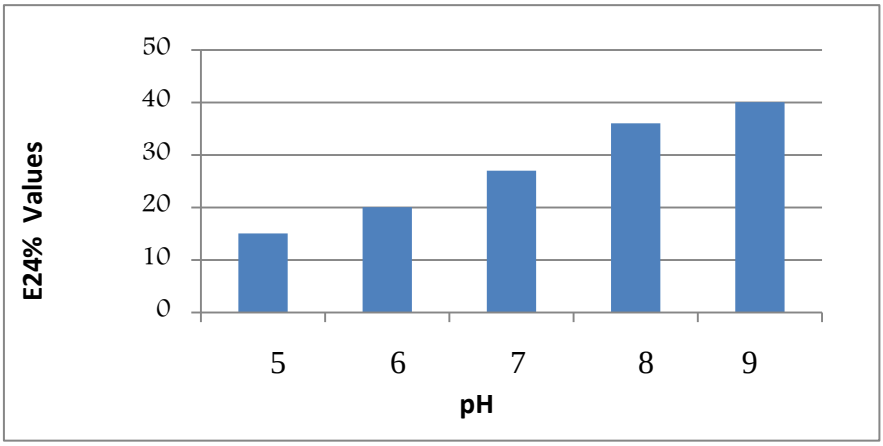


**Figure 4-** The effect of different pH values on bioemulsifier production by *R.leguminosarum*

The bioemulsifier production was affected by initial pH of culture medium. Maximum growth and bioemulsifier production by isolate *Streptomyces* sp. SS 20 was at pH 7 (E24%= 100% and EA=0.30) [1]. Also at pH 7 was the optimum one for production of biosurfactant by three *Candida* strains; *C. famata* No. 11, *C. albicans* No. 13 and *C. albicans* No. 25 [15] .Sudha et al. ( 2010 ) observed that pH 3.5 was the optimum for the production of sophorolipid from *Candida tropicalis* [16] ,while the maximum biosurfactant production from *S.marcescens* was in pH 8 [17].

**2- Temperature**

Temperature played an important role in the growth and bioemulsifier production. The effect of different temperatures (25 , 30 , 37 , 40 ˚C) on bioemulsifier production by *Rhizobium* bacteria were determined , the results showed that maximum emulsification index E24% by this bacteria was 56.3% at 30 ˚C Figure-5

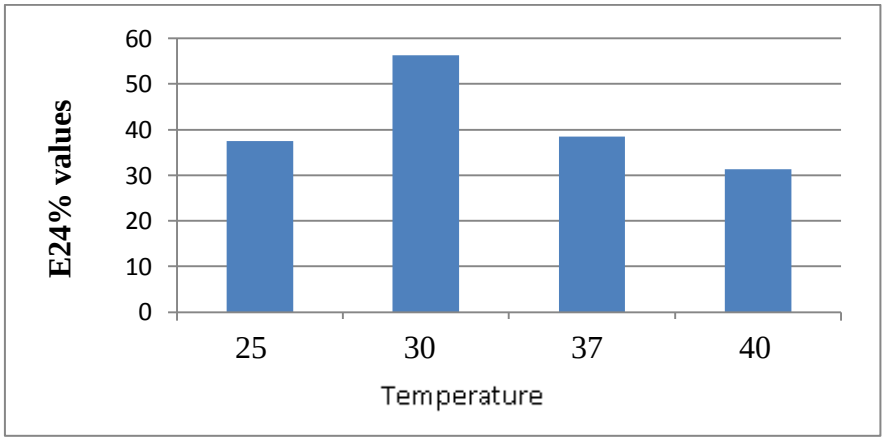


**Figure 5-** The effect of different temperature values on bioemulsifier production by *R.leguminosarum*

The highest value of bioemulsifier activity from *Streptomyces* sp. SS 20 reached when the isolate was grown at 30 °C (E24%= 100% and EA= 0.44) [1], also the optimum temperature for production of sophorolipid from *Candida tropicalis* was 30 °C [16] ,Desphande and Daniels (1995) observed at 27˚C *C. bombicola* was gave maximum bioemulsifier production [18]. While the optimum temperature for production of biosurfactant by three *Candida* strains was mostly 20 °C. [15]

**3-Incubation periods**

The bacteria of this study was cultured in mineral salt broth medium and incubated for (3,5,7,9,10) days to determine the optimum time for bioemulsifier production. Maximum bioemulsifier production was obtained after 10 days of growth and E24% index was 58.8% Figure-6**.** Mahdy,HM. et.al (2012) reported that incubation period 8 days was a significant and optimum to produce biosurfactant by three tested *Candida* strains [15], also the optimum incubation period for sophorolipid production from *Candida tropicalis* was 8 days [16].

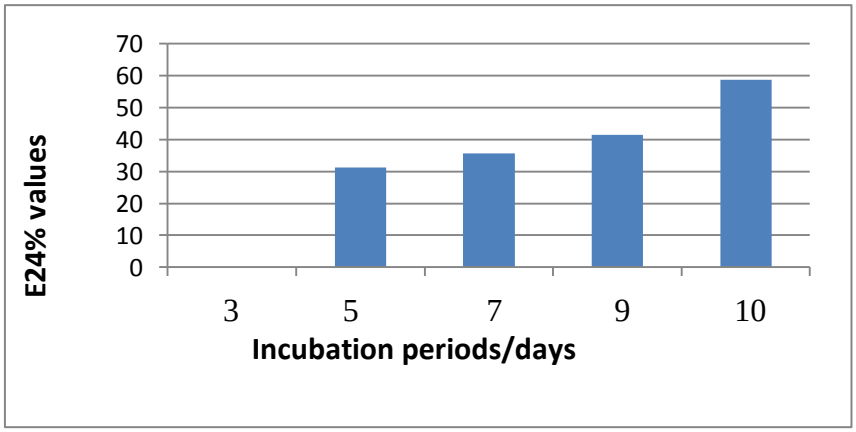


**Figure 6-** The effect of different incubation periods on bioemulsifier production by *R.leguminosarum*

**4 -Types of oil**

Media components and precursors are reported to affect the process of bioemulsifier production and the final quantity and quality of product [15]. The growth of bacteria was carried out with different types of oil (sunflower oil, olive oil, sesame oil and resinous oil). The production of bioemulsifier from *Rhizobium leguminosarum bv. viciae* was induced by addition of oils , among the four oils tested as substrate for bioemulsifier production, sesame oil was the best with maximum emulsification index E24%= 61% Figure-7.

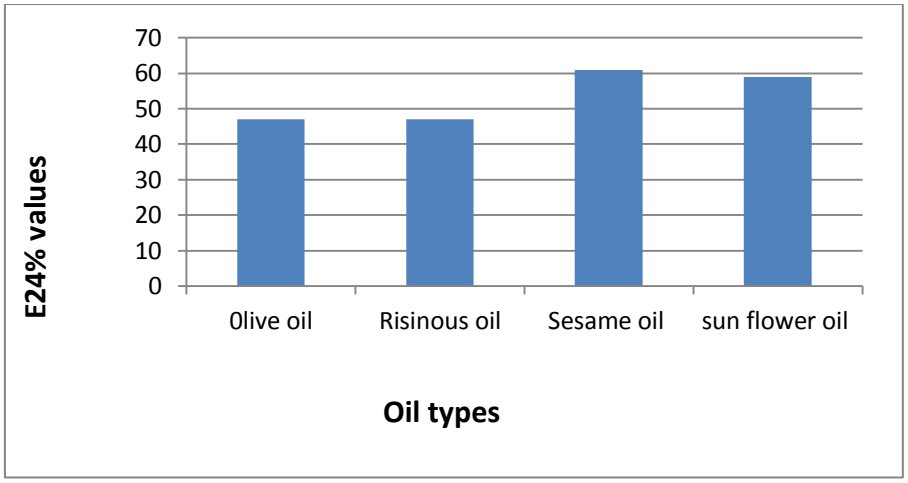


**Figure 7-** The effect of different types of oils on bioemulsifier production by *R.leguminosarum*

The production of biosurfactant from *Streptomyces* sp. SS 20 was induced by addition of oils and hydrocarbons. Among the six oils tested for bioemulsifier production, corn oil was the best with maximum growth and emulsification activity (E24%= 100%) [1] , while soybean oil was the best for bioemulsifier production from *Bradyrhizobium elkanii* SEMIA 587 , the emulsification index (E24%) was higher than 50%, with values of 65.22% [4] ,olive oil was the best carbon source for biosurfactant synthesis from *Pseudomonas florescens* ,the E24% was 49% [19].

**5- Nitrogen source**

One of the important factors that affect the growth and production of bioemulsifier from *Rhizobium* bacteria is the nitrogen Source. Nitrogen sources also effect the production of bioemulsifier by different microorganisms. In the present study among different organic and inorganic nitrogen sources were tested, $NH_4NO_3$ was found to be the best source of nitrogen for growth and bioemulsifier production. Maximum E24% (64.5%) were obtained in media contains $NH_4NO_3$ Figure-8.

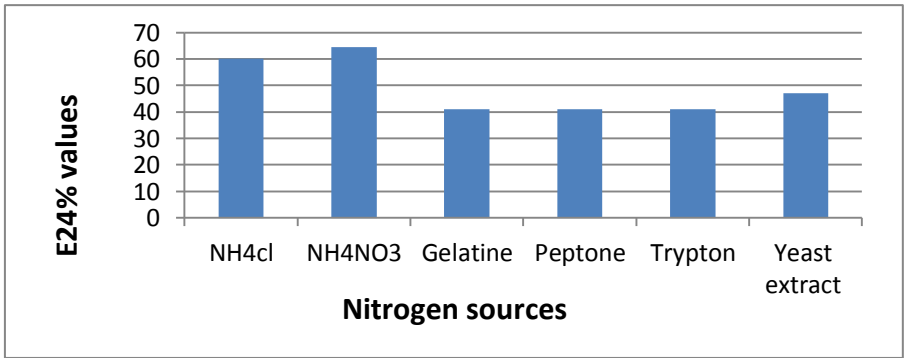


**Figure 8-** The effect of different types of nitrogen sources on bioemulsifier production by *R.leguminosarum*

The optimum concentration of $(NH_4)_2SO_4$ was 12 % that recorded 33, 36 and 31 mm of oil displacement and 33.8, 28.2 and 39.4 mN/m of surface tension for *C. famata No. 11, C. albicans No. 13 and C.so albicans No. 25*, respectively [15] . Maximum growth and emulsifying activity E24% (80%) from *Streptomyces* sp. SS 20 were obtained in media contains yeast extract [1], sodium nitrate was the best nitrogen source for rhaminolipid production from *P.aeruginosa* [20].

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