Biodegradation and Biosurfactant Production by *Agrobacterium tumefaciens* Utilizing Weathered Mineral base Oil

التفكيك الحيوي وانتاج المستحلبات الحياتية من بكتريا *Agrobacterium tumefaciens* المستهلكة لزيت الاساس المحروق

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Abstract

Optimum condition of biodegradation and biosurfactant production from spilled weathered base oil by *Agrobacterium tumefaciens* was studied in batch culture. Results showed that the optimum conditions for biosurfactant production was at pH7, temperature 30°C, incubation period 72h, and addition of weathered oil in a concentration of 3%, which yielded high biosurfactant production reached 6.6 g/l. The results also showed capability of isolate to degrade 70% of initial concentration of weathered oil 3%. Some characteristics and nature of produced biosurfactant was studied, the results showed that the biosurfactant is white to yellow in color, and viscous at room temperature, and needs little heating to be prepared in aqueous solution, insoluble in water and some organic solvents. The results also indicated higher stability of produced biosurfactant at neutral pH, and the stability decreased at pH less than 5 and up to 9, while the best stability of produced biosurfactant was at 30 and 40˚C. The addition of crude biosurfactant in concentration 20mg/l to the production medium lead to stimulate the isolate for uptake of weathered oil and increase biosurfactant production, while the biomass production did not affected significantly.

المستخلص

تمت دراسة الظروف المثلى للتفكيك الحيوي وانتاج المستحلب الحياتي من بكتريا *Agrobacterium tumefaciens* المستهلكة لزيت الاساس المحروق في مزرعة الدفعة الواحدة . بينت النتائج بأن الظروف المثلى للانتاج كانت عند الرقم الهيدروجيني 7 وبدرجة حرارة 30 م وبفترة حضانة 72 ساعة وعند التركيز الامثل للزيت المحروق 3% . حيث تم الحصول على اعلى انتاج للمستحلب الحياتي بلغت 6,6 (غم/لتر) . كما بينت النتائج قدرة العزلة على تفكيك 70% من التركيز الاولي لزيت الاساس المحروق 3%. درست بعض خصائص وطبيعة المستحلب الحياتي المنتج ، فوجد بان المستحلب ذو لون ابيض مائل الى الصفار ، لزج عند درجة حرارة الغرفة ، يحتاج الى التسخين قليلا عندما يراد تحضيره في الماء ، غير قابل للذوبان في الماء وبعض المذيبات العضوية . كما اكدت النتائج ثباتية عالية للمستحلب الحياتي المنتج عند الرقم الهيدروجيني المتعادل 7 ، وتقل الثباتية بشكل كبير عند الارقام الهيدروجينية الاقل من 5 والاكثر من 9 . في حين لوحظ افضل ثباتية للمستحلب الحياتي المنتج عند درجة حرارة بين (30 ، 40) م . اضافة المستحلب الحياتي (الخام) بتركيز (20) ملغم/لتر الى الوسط الانتاجي ادى الى تحفيز العزلة على استهلاك الزيت المحروق وزيادة انتاج المستحلب الحياتي ، ولكنها لم تؤدي الى زيادة الكتلة الحيوية بشكل كبير .

Key words: Biodegradation, Biosurfactant, diesel oil, surface active properties

Introduction

Biodegradation is generally considered as a phenomenon of biological transformation of organic compounds by living organisms, particularly microbes. Natural process changes in the molecular structure and completely broken of a compounds yielding simpler (mineralization) and comparatively harmless (non-toxic) products like CO_2, H_2O, NH_3, CH_4, H_2s and PO_3. Such changes are brought about by the catabolic activities of bacteria or fungi by their intracellular or extracellular enzymes secreted in the medium [1].

Agrobacterium tumefaciens, is a soil-borne Gram-negative rod shaped protobacterium of the family Rhizobiaceae. This bacterium used in different fields such as microbial cellulose production, a new type of polymers. The bacterium was able to grow on agriculture waste materials and synthesized cellulose [2]. In the field of gene and enzyme transformation, [3] mentioned that the haloalkane dehalogenase from *Rhodococcus* sp an enzyme capable of efficient transformation of trihalopropanes to dihalopropanols, under the control of different heterologous promoters. By introduction of these plasmids into *Agrobacterium radiobacter* AD1, the bacterium showed their capability to grow on trihalopropanes. In the field of herbicide degradation, [4] pointed to the biodegradation capabilities of atrazine, one of the herbicides most currently used, by *Agrobacterium radiobacter* J14 and used this strain in bioremediation of contaminated soil and sediments resulted from petroleum pollutants.

According to the literatures, *Agrobacterium* sp exhibited secondary metabolites and biosurfactant production. [5] mentioned that the amino-acid containing biosurfactants like the surfactin produced by *Bacillus subtilis* composed of seven amino-acid ring structure coupled to one molecule of 3-hydroxy-13-methyl tetradecanoic acid. Other biopolymers of this group are the ornithine-containing lipid of *Pseudomonas rubescens* and the lysine-containing lipid of *Agrobacterium tumefaciens.*

Another secondary product of *Agrobacterium* sp is Curdlan a water-insoluble extracellular polysaccharide composed exclusively of 3-1, 3-linked glucose residues. It is synthesized by *Alcaligenes faecalis* var. *myxogenes an*d *Agrobacterium radiobacter* under nitrogen-limiting conditions. The production of curdlan has drawn considerable interest because of its unique rheological and thermal gelling properties. Curdlan is used in food products such as jelly, noodles, edible fiber, and new calorie - reduced products. It is also being used to enhance the fluidity of concrete while increasing its segregation stability. In addition, curdlan might be used as a drug-delivery polymer since curdlan gel can hold and control diffusion of the drug. Furthermore, curdlan sulfate was developed as an antiviral agent able to inhibit infections by the human immunodeficiency virus. Thus, there is a strong interest in reducing the manufacturing cost of curdlan [6].

Diesel oil is an excellent model for studying hydrocarbon biodegradation, since it is constituted of a variety of these molecules, such as paraffin, olefins, and naphtha and aromatic compounds. The molecular weight of the hydrocarbons present in diesel is also variable, with molecules containing from 9 - 20 carbon atoms. There have been

several reports on diesel spills in the environment; besides other pollution problems related to the extensive use of this fuel [7].

A biosurfactant is defined as a surface-active molecule produced by living cells, in the majority of cases by micro-organisms. Biosurfactants comprise a wide range of chemical structures, such as glycolipids, lipopeptides, polysaccharide protein complexes, phospholipids, fatty acids and neutral lipids [8]. Biosurfactants have been tested in environmental applications such as enhanced oil recovery, bioremediation and dispersion of oil spills. They have been used to increase the uptake of organic compounds with limited water solubility during bacterial growth. They can affect the rate of hydrocarbons biodegradation in two ways: by increasing solubilization and dispersion of the hydrocarbons and by changing the affinity between microbial cells and hydrocarbons by inducing increases in cell surface hydrophobicity [9].

The potential use of biosurfactants is in the oil industry with minimum purity specification, so that crude preparation could be used in clean-up of hydrocarbons contaminated sites and for Enhancing Oil Recovery (EOR). It is reported in the literature that the genus *Pseudomonas* and *Serratia* grown on vegetable oils are mainly used in the field of the biosurfactants production [9]. According to the literatures, there are little studies on the use of *Agrobacterium* strains for biosurfactants production with petroleum hydrocarbons. In the present study, an attempts to investigate the ability of *Agrobacterium tumefaciens* in biodegradation and production of biosurfactants from weathered mineral base oil, and study some characteristics of produced buiosurfactant.

Materials and methods

Microorganism

The strain *Agrobacterium tumefaciens* is obtained from Institute of genetic engineering and biotechnology/ Baghdad University, isolated from the rhizospher zone of the soil, used in biodegradation and biosurfactant production. The isolate maintained on nutrient agar medium (Difco, India) at 30 ^{o}C for daily using.

Media and cultivation conditions

The isolate is grown in nutrient broth for (16–18) h at 30˚C. This culture was used as stock culture inoculums at concentration 1% (v/v). To determine the capability of the isolate for biosurfactant production, a mineral salt medium (MSM) with the following composition (g/L) was used: K_2HPO_4 (1), KH_2PO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.6), $FeSO_2 \cdot 7H_2O$ (0.01), NaCl (0.05), $CaCl_2$ (0.02), yeast extract (0.5) and 0.1 mL of trace element solution containing (g/L): 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g $H3BO_3$, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. The pH of the medium was adjusted to 7.0 [10]. Carbon source was added separately. Cultivations were performed in 250 mL flasks containing 50 mL mineral salt medium at 30˚C, and stirred in a rotary shaker incubator (Basal Switzerland) at 150 rpm.

Medium optimization for biosurfactant production

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at a specific set of conditions. Four factors were chosen aiming to obtain higher productivity of the biosurfactant: pH,

temperature, and incubation period and carbon source. The pH used was (4-9), temperature used were (25-45)˚C. For incubation period *Agrobacterium tumefaciens* was grow at different incubation period (1-6) days at pH7 and 30˚C in shaker incubator 150 rpm. For appropriate concentration of carbon source as energy for biosurfactant production, different concentration of weathered mineral base oil (1, 2, 3, 4, 5, 6) % (w/v) were used, at optimized condition; pH7, incubated at 30˚C, and 150 rpm for 72h. At the end of each experiment the dry weight, biosurfactant production, emulsification index (E24%) and surface tension was measured.

Preparation of carbon source

Weathered mineral base oil was used as the sole source of carbon and energy. The source of oil is (Al- Dora refinery- Iraq). The refinery produces three type of mineral base oil (mineral oil 40, 60, and 150). Mineral oil 60 and 150 are used mainly as diesel oil. The spilled diesel oil (weathered oil), was taken from one of the oil replacement station in Baghdad and used in the present study. Weathered diesel oil used as suspension (after mixing with silica gel in the ratio 1:1 v/w) in the culture medium before autoclaving. The importance of this procedure is to increase the surface area for microbial attack to the substrate [11].

Biomass measurement

The dry weight technique was used to quantify microbial growth as bacterial dry weigh. Biomass obtained after centrifugation of the culture at 10.000 rpm, for 15 min at 4˚C, then the precipitate cells transferred to weighted container and dried overnight at 105˚C and reweighed.

Extraction of Crude byproduct

A crude biosurfactant was extracted from the cell-free culture supernatant of *Agrobacterium tumefaciens* grown on weathered base oil, using the technique described in [12]; briefly, at the end of the cultivation in weathered diesel oil, the hydrophobic layer located at the surface has been removed, the culture medium was centrifuged at 10. 000 rpm for 15 min at 4˚C. The pH of cultures was adjusted to approximately 2.0 with 1N HCl and the biosurfactant extracted for 15 min with an equal volume of chloroform: methanol (2:1 v/v). The extract was concentrated and dried at room temperature and weighted; resulting crude extract.

Surface tension measurement

The surface tension measurement of cell free supernatant was determined according to the method described by [12] in a K6 tensiometer (Krüss GmbH, Hamburg, Germany), using the du Nouy ring method. The values reported are the mean of two measurements. All measurements were made on cell-free broth obtained by centrifuging the cultures at 10,000 rpm for 15 min.

Emulsification index (E24%)

Emulsification index of culture samples was determined by adding 2 mL of a hydrocarbon (kerosene) to the same amount of culture or culture free cell (supernatant), mixing by a vortex for 2 min, and leaving to stand for 24 h. The E24% is given as percentage of height of emulsified layer (mm) divided by the height of the hydrocarbon phase (mm) and multiplying to 100 [13].

Biodegradability test

Biodegradability test performed in 250 ml Elementary flasks containing 3% of weathered mineral base oil at optimized condition pH7, tempearature 30˚C. The flasks incubated in shaker incubator at 150 rpm for 72 h. After end of incubation period, the culture media centrifuged at 10000 rpm for 15 min to remove the cells. The filtrate transformed in to the 100 ml separating funnel, and extracted by addition 50 ml of n-hexane [13]. The mixture was mixed thoroughly and left to stand for 30 minutes. Then, it was filtered into a pre-weighted conical flask and immersed in a warm water bath at approximately 80˚C for 1 hour to evaporate the hexane. The final weight of flask and weathered oil remains in it was noted and the difference between the final and the tare weight represent the mass of weathered oil recovered in the sample.

Effect of physical factors on emulsification activity of produced biosurfactant

Effect of temperatures and pH on emulsification activity was performed in 250 ml Elementary flasks containing 50 ml of mineral salt medium at optimized conditions pH7, temperature 30˚C, weathered base oil 3% (w/v) and then incubated in shaker incubator (150 rpm) for 72 hr. Then 3 ml from each culture and supernatant cell free taken and exposed to different temperatures (20-100˚C) for 30 min, E24% was estimated .The same condition above were used but in different pH after adjusting the culture and supernatant cell free to pH (4-10), and E24% was estimated.

The role of biosurfactant addition in enhancing biodegradation and biosurfactant production

The test was performed in 250 ml Elementary flasks containing 50 ml of mineral salt medium at optimized conditions pH7, temperature 30˚C, and weathered base oil 3% (w/v). Then 1 mg (20 µg/ml) of crud biosurfactant was added for each flask, and then the flasks incubated in shaker incubator (150 rpm) for 72 hr. control in duplicate also conducted as the same condition above but without addition of biosurfactant. Dry weight, biosurfactant production and E24% estimated at the end of the incubation.

Results and Discussion

Effect of cultural and environmental conditions on growth and biosurfactant production:

The effects of pH, temperature, incubation period, and weathered diesel oil concentration on biomass yield and biosurfactant production of *Agrobacterium tumefaciens* were studied. The results in Figure (1) showed that the maximum biomass yield was obtained at pH8, while biosurfactant production increased with increase in pH. At pH7 maximum surfactant production was obtained and the E24% value of the culture free cell increased to 65%. Any change in pH to lower or higher level caused drop in the biosurfactant production. At the optimum level of pH7, the maximum biomass and biosurfactant production obtained with increase the E24% of the culture free cell to maximum value at temperature 30 ˚C Figure (2). Any increase in the temperature resulted in decreasing in the growth and biosurfactant production and decrease E24% value. This result indicates that the isolate favored the mesophilic condition.

Growth and biosurfactant production increased with incubation period and reached maximum 3.13 g/l at 96h, for biomass production, while maximum biosurfactant

production 6.5 g/l obtained at 72h of incubation which causes to increase E24% of the culture free cell to 70%, beyond which biomass, biosurfactant production and E24% were decreased Figure (3).

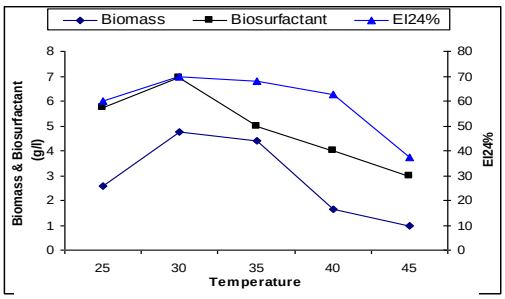

Fig (2): Effect of temperature on growth rate and biosurfactant production of *Agrobacterium*

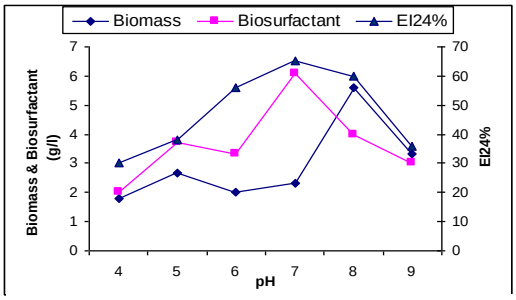

Fig (1): Effect of pH on growth rate, biosurfactant production and E24% of *Agrobacterium tumefaciens* at 30 °C in

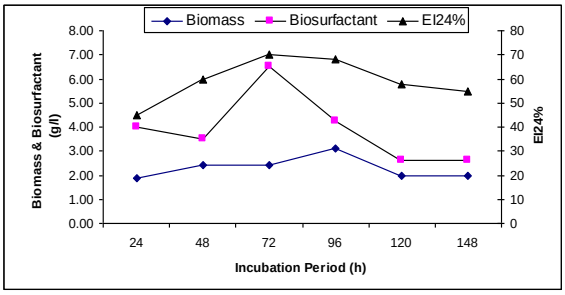

Fig (3): Effect of incubation period on growth rate and biosurfactant production of *Agrobacterium tumefaciens* at 30 °C, pH 7 in shaker incubator at 150 rpm for 7 days

When applying the optimum conditions for biosurfactant production (pH7, temperature 30°C, and incubation period 72h) maximum yield of biosurfactant 6.6 g/l and biomass 5.25 g/l was obtained at a substrate (weathered oil) concentration of 3% (w/v), which causes to increase the E24% value to 74% and decreased the surface tension value of the culture to minimum value 39 mN/m Figure (4). The results obtained indicated that the surfactant production is a linear function of growth and substrate concentration.

Types of carbon sources play a major role in biosurfactant production. Different carbon sources such as cheap materials (molasses, corn steep liquor, way wastes) and n- alkanes, were used in biosurfactant production due to their low cost and availability. Agricultural and food industry wastes such as rice, starch waste liquors, whey, and domestic waste also used in biosurfactant production [14].

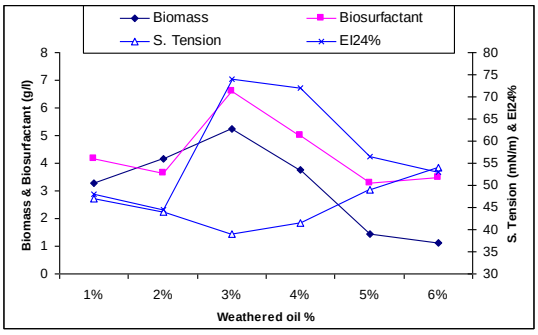

Fig (4): Effect of Weathered oil concentration on growth rate and biosurfactant production of *Agrobacterium tumefaciens* at 30 °C, pH 7 in shaker incubator at 150 rpm for 72 h

Municipal sludge waste has also been proposed to be utilized for biosurfactant production. The type of carbon source used has been shown to play a key role in the type of biosurfactant produced. When n- alkane was used as a carbon source, a raminolipid was produced by *Pseudomonas* sp [15]. In a similar investigation the strain *Pseudomonas aeruginosa* LBI was capable to produce the raminolipid biosurfactant using diesel oil, crude oil, oily sludge, kerosene and glycerol [16]. Also in the study of [11] mentioned the ability of the isolate *Serratia marcescens* to produce glycolipid biosurfactant, maximum biosurfactant (10.5 g/l) produced at the optimum condition (pH8, temperature 30˚C, incubation period 96h and weathered diesel oil concentration 6% v/v) which reduced the surface tension value to 41 mN/m. [16] also obtained the biodegradation of commercial diesel oil by *Pseudomonas aeruginosa* at concentration 10% with higher biosurfactant production, which causes to decrease the surface tension value to 45 mN/m. [5] pointed out to the capability of *Agrobacterium tumefaciens* to produce biopolymer biosurfactant containing lysine-lipid at the optimum condition of pH7, and temperature 30˚C. [6] observed the syntheses of water- insoluble extracellular polysaccharide composed exclusively to 3-1, 3- linked glucose residues from *Agrobacterium radiobacter*.

Weathered Diesel Oil Degradation by *Agrobacterium tumefaciens*

At first, we evaluated the degradation capabilities of the diesel oil by *Agrobacterium tumefaciens* by cultivating it on MSM supplemented with diesel oil as the sole carbon source with the concentration 3% (w/v) at optimized condition. The evolution of the growth distinguished by brake down large molecules of oil in to small droplets in medium culture, this indicates the production of bioemulsifier during the degradation process. After 72h of incubation the results showed that 70% of the initial concentration of weathered diesel oil (3%) was degraded. It appears that *Agrobacterium* tumefaciens assimilated the diesel oil according to the mode of direct contact between the diesel oil droplets and biosurfactant micelle molecules. The process can have acted according to two ways: either making cellular surface more hydrophobic or the biosurfactant enhanced the aqueous solubilization and dispersion of the weathered diesel oil. Such hydrocarbons uptake mechanism has been extensively reported by [9]. It should be noted that diesel oil is a complex hydrocarbons mixtures that are highly variable in structure and in susceptibility to biodegradation; in addition, fresh diesel oil contains a light hydrocarbon fraction, rich in aromatic compounds that may be toxic to some strains. In the study of [9], the same experiment with weathered diesel oil showed much higher degradation and surfactant production levels, with a rhamnolipid-producing strain of *Pseudomonas aeruginosa*. [16] in their experiment demonstrated that low concentrations of pure diesel oil (2% or 5%) enhance more significantly the biodegradation by single culture of *Pseudomonas aeruginosa* than mixed culture consortia. [17] shown decreasing in degradative activity from 40.46% to 15.05% when waste lubricating oil concentration increased from 1% to 10% by gram negative, rod shaped *Agrobacterium tumefaciens*.

Characterization of the produced bioemusifier

Physical characteristics

The results in Table (1) showed physical properties and the nature of produced biosurfactant by the isolate Agrobacterium *tumefaciens*. The produced biosurfactant is white to yellow in color, and with the nature of the resin after hardening, as well as forms viscous solution when it melts in the water at room temperature, and needs little heating to be prepared in aqueous solution. It is insoluble in pentane, heptane, hexane, benzene and acetone. These results are agreement with the findings of [18], they stated that a high proportion of the components of crude oil or its derivatives turn to the substance can not be extracted with benzene. The results also indicate the solubility of biosurfactant produced in diethyl ether, methanol, ethanol, and chloroform.

Effect of pH and Temperature on Emulsification activity of biosurfactant

The effect of different pH (4-10) on the stability of produced biosurfactant was studied. The results in Figure 5 revealed that the produced biosurfactant is more stable at neutral pH, and biosurfactant activity decreased at pH less than 5 and up to 9. The results also showed that the emulsification stability in the basal environment affected gradually with a high pH value. These results are agreed with those found by [19] they found that the biosurfactant produced by *Pseudomonas aeruginosa* has higher emulsification stability to wide range of pH 'between' 4 to 10, with decrease the surface tension values of the media from 31 to 34 mN/m. Also [11] found that the rhamnolipid produced by *Pseudomonas aeruginosa* was more effective and stabile at neutral pH.

Table(1): Some physical characteristics and the nature of produced biosurfactant by *Agrobacterium tumefaciens*

No.	Some physical characteristics and nature	Results
1.	Color	White to yellow
2.	Nature at room temperature	Viscous after hardening
3.	Solubility in water	Water-insoluble by product
4.	Solubility in solvents:	
	- Pentane	Insoluble
	- Heptane	Insoluble
	- Hexane	Insoluble
	- Benzene	Insoluble
	- Acetone	Insoluble
	- diethyl either	Soluble
	- Methanol	Soluble
	- Ethanol	Soluble
	- Chloroform	Soluble

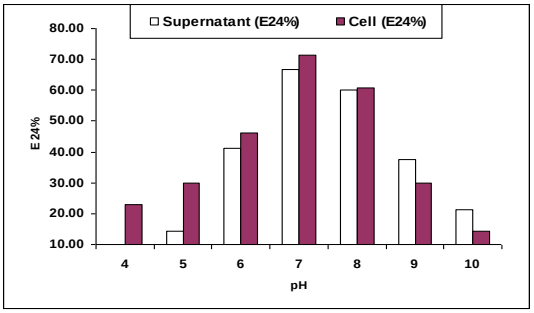

Fig (5): The effect of pH on emulsification activity of biosurfactant produced by *Agrobacterium tumefaciens*

Effect of different temperature on emulsification activity also studied. The results in Figure 6 showed the stability of produced biosurfactant at high temperature up to 60°C. The best emulsification activity was at temperature between 30 and 40 °C, and reached maximum at 35°C, causes to increase E24% value of culture free cell (supernatant) and the culture to (68, 70)% respectively. The results also showed decrease in E24% values with increase temperatures until it reached (38, 42)% respectively at a temperature of 70°C. The decrease in emulsification activity at elevate temperature may be due to, the effect of temperature in the composition of biosurfactant, resulting an immiscible of aqueous phase with hydrocarbon phase. Also in the study of [12] they found stability of biosurfactant produced by *Pseudomonas* sp at high temperatures reached 80°C and maximum E24% with the cells reached 63% at temperatures (60-70)°C. [20] found the activity of the biosurfactant alasan increased by (2.5 - 3) times after heated to 100 °C.

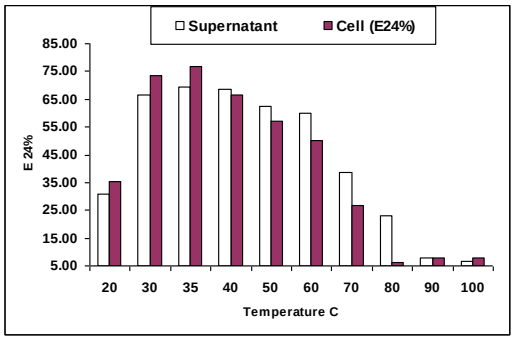

Fig (6): The effect of temperature on emulsification activity of biosurfactant produced by *Agrobacterium tumefaciens*

The role of produced biosurfactant in emulsification and biodegradation of weathered oil

The effects of biosurfactant addition on biodegradation and biosurfactant production are shown in Table (2). The results showed that the addition of crud biosurfactant to the production media in concentration of (20 mg/l) stimulate the ability of the isolate to emulsify and up take of weathered diesel oil. As noted in the results, the addition of biosurfactant in to the production media, did not lead to increased biomass significantly compared to the treatment (control)-free biosurfactant, while it was observed that the quantity of production of the biosurfactant increased to 7.6 g/l when crud biosurfactant added compared with control 5.5 g/l, which causes to increase E24% from 65% to 79%. From the results obtained we conclude that the addition of crude biosurfactant facilitate: mixing of hydrocarbon molecules with liquid phase in the media, reduced the lag phase of microbe to substrate, and working to increase hydrophobicity of microbial cell, and increase affinity between the surface of cells and hydrocarbons molecules, which resulted an increase in the solubility and availability of hydrocarbons molecules by microorganisms [21]. [22] observed complete emulsification of hexadecane when glycolipid at concentration of 100 mg/l added to the culture media. Also [23] noticed that the addition of 0.6 M of rhamnolipid lead to increase the biodegradation rate of octadecan and hydrophobicity

of microbial cells from (15 - 75)%, while addition of 6 M of rhamnolipid reduced the biodegradation rate and hydrophobicity of cells from (70% - 30)%. This decrease may be due to inhibitory effect of these compounds at high concentrations.

Table (2): The effect of biosurfactant addition on biomass and biosurfactant production by *Agrobacterium tumefaciens*

Source of carbon	Biomass (g/l)	Biosurfactant (g/l)	E24%
Weathered oil 3%	3.5	5.5	65
Weathered oil 3% + 20 mg/l crude biosurfactant	4	7.6	79

Conclusion

We can conclude from the results obtained in the present study that the isolate *Agrobacterium tumefaciens* is able to grow in the presence of weathered diesel oil and can be used to reduce wastes generated by using diesel oil in different applications. Also to convert weathered oil (a cheap renewable material) in to higher valuable products. So that crude preparation of produced biosurfactant could be applied for: enhanced oil recovery, cleaning oil storage tanks, in a variety of biotechnological applications including bioremediation, biodegradation and bioadsorption of heavy metals. In biological control as antimicrobial and antifungal, in food industries and in medicine.

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