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OPTIMIZATION OF BIOSURFACTANT PRODUCED BY NOVEL THERMOPHILLIC *GEOBACILLUS THERMOLEOVORANS* (JQ 912239) IRAQI STRAIN

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ABSTRACT

Optimization of the locally isolated *Geobacillus thermoleovorans* (JQ 912239) for maximum biosurfactant production was performed depending on emulsification index and surface tension lowering as a two parameters for biosurfactant detection. Results showed that maximum production of biosurfactant was obtained when the medium was supplemented with crude oil 1 %, ammonium chloride 0.3%, incubated at 60 ºC, pH 7, with 200 rpm shaking for 10 days incubation.

KEYWPRDS: *Geobacillus thermoleovorans*, biosurfactant.

INTRODUCTION

Biosurfactant are surface active compounds having both hydrophilic and hydrophobic domains that allows them to exist preferentially at the interface between polar and non-polar media, thereby reducing surface and interface tension (Banat *et al.*, 2010).

Biosurfactant are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi from various substances, including sugars, oils and wastes (Femi-Ola *et al.*, 2015). These molecules comprise complex structures which are grouped either as low (glycolipids and lipopeptide) or high (polymeric biosurfactants) molecular weight compounds (Cameotra *et al.*, 2010). The major classes of biosurfactant include glycolipids, lipopeptide and lipoproteins, phospholipids and

fatty acids, polymeric surfactants and particulate surfactants (Cameotra and Makkar, 2004; Salihu *et al*., 2009).

Recently, much attention have been attributed towards biosurfactant over chemically synthesized surfactants due to their ecological acceptance, low toxicity and biodegradable nature, effectiveness at extreme temperatures or pH values and widespread applicability (Inis and Dhouha, 2015).

During the last decade, biosurfactant have been used as alternatives for synthetic surfactants and are expected to find many industrial and environmental applications such as enhanced oil recovery, crude oil drilling, lubrication, bioremediation of pollutants, foaming, detergency, wetting, dispersing and solubilization. The application of biosurfactant also increased in cosmetic, health care and food processing industries (Dhasayan *et al*., 2014). Biosurfactant display important biological activities including antimicrobial, insecticidal, immune-modulative and antitumoral activities (Cao *et al*., 2009; Liang *et al.,* 2014).

Developments in the optimization area of fermentation conditions have resulte in a significant increase in production yields, making them more commercially attractive (Desai and Banat, 1997). According to those mentioned above, the aim of present work was to determine the optimum conditions for biosurfactant production from novel thermophillic *Geobacillus thermoleovorans* (JQ 912239) Iraqi strain show ability to degrade aromatic compound and isolated from previous study(Al-Jailawi *et al*., 2013).

MATERIAL AND METHODS

Microorganism

Geobacillus thermoleovorans (JQ 912239) was isolated in a previous study (AL-Jailawi *et al.,* 2013). The strain was maintained at 4 ºC on nutrient agar slants, LB broth was used for the activation of this bacterium.

Optimization of biosurfactant production

The optimization experiments was carry out aerobically under aerobic batch cultivation condition on mineral salt medium (g/L): NH_4Cl:4, NaCl:4, KH_2PO_4: 3, Na_2HPO_4: 6, $MgSO_4$: 0.1(Yakimov *et al.*, 1995) and 1 ml of trace element solution. The stock solution of trace elements contained (g/l): (ZnSO4.7H2O: 2.32, $MnSo_4.4H_2O$:1.78, $CuSO_4.5H_2O$:1.0, $H3BO_3$:0.56, EDTA: 1.0, $NiCL_2.6H_2O$:0.004 and KI: 0.66) (Vater *et al*., 2002). Fifty

milliliter of this medium was distributed in 250 ml Erlenmeyer flasks, inoculated with 1% of mid- exponential phase culture of the bacterial strain, then incubated with shaking 180 rpm at 55 ºC for 7 days. After incubation period the emulsification index and surface tension were measured.

Effect of carbon source

Different carbon sources (fructose, manitol, sucrose, diesel, date extract, crude oil, sun flower and glucose) were used to determine the optimum source for biosurfactant production, each of these sources was added separately to the mineral salt medium at a concentration 1%, pH was adjusted to 7.0, and after inoculated with the bacterium, the flasks incubated in shaker incubator (180 rpm) at 55ºC for 7 days.

Emulsification index and surface tension were measured and the optimal carbon source was employed later on.

Effect of carbon source concentration

Different concentrations (0.25%, 0.5%,0.75%, 1%, 2%,3%,4 %) of crude oil were used to grow the *Geobacillus thermoleovorans* (Ir1), in order to determine the optimum concentration for biosurfactant production. After the pH adjustment to 7.0, flasks with mineral salt medium were incubated in shaker incubator with180 rpm at 55 ºC for 7 days.

Effect of temperature

In order to determine the optimum temperature for biosurfactant production, mineral salt medium after pH adjustment to 7.0 inoculated with bacteria, flasks were incubated in shaker incubator (180 rpm) at different temperatures such (40, 50, 55, 60, 65 and 70 ºC) for 7 days, then the optimal temperature was subsequently employed depending on the result.

Effect of different nitrogen sources

Different nitrogen sources (NH_3SO_4, NH_4NO_3, NH_4Cl, KNO_3, urea, and yeast extract) were used to determine the optimum condition for biosurfactant production by the bacterial isolates. These nitrogen sources were added to the mineral salt medium in a concentration 0.4 %, and pH was adjusted to 7.0, then flasks were incubated in shaker incubator with 180 rpm at 60 ºC for 7 days. Optimal nitrogen source was selected and employed later on.

Effect of nitrogen sources concentration

The optimal nitrogen source which was shown the better results than other, so it was added in gradual concentration (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%) to the mineral salt medium. And pH was adjusted to 7.0 then incubated in a shaker incubator with 180 rpm at 60 ºC for 7days. Then the optimal concentration was employed.

Effect of pH: To enhance the activity of biosurfactant, mineral salts medium adjusted with different pH values (5, 6, 6.5, 7, 8 and 9) to determine the suitable value. The flasks were incubated in shaker incubator with 180 rpm at 60 º C for 7 days. And the better pH value was employed in later experiment.

Effect of aeration

Different rpm value (120, 150, 180, 200, 220 rpm) were examined to determine the optimum aeration required to obtain the high biosurfactant activity, incubated the mineral salt medium with 1% crude oil, 0.3% ammonium chloride at 60 ºC for 7 days and the optimal aeration was used in latter experiment.

Effect of incubation period

The optimum period required for biosurfactant production, under the previously monitored culture conditions was determined by incubated bacterial cultured in mineral salt medium containing 1% crude oil, 0.3% ammonium chloride with pH 7 in shaker incubator (200 rpm) at 60 ºC for (1–11) days, and after each incubation period the activity was determine and the optimum incubated period was choose.

Surface tension measurement

Geobacillus thermoleovorans (Ir1) has been grown in mineral salt medium and the surface tension was measured with a DuNouy tensiometer. All measurements were made with culture supernatant obtained after centrifugation at 8000 rpm for 15 min. at 4 ºC. The surface tension of the mineral salt medium (68 mN/m) and water (72mN/m).

Emulsification index measurement

The emulsification activity of the biosurfactant was determined through measured the emulsification index by mixing one ml of cell free supernatant and 1ml of sun flower oil (equal volumes v/v), mixing with vortex for 2 min., and left for 24 hrs. at room temperature, the height of emulsifier layer was measured. The emulsification index is given as percentage

of height of emulsified layer (mm) to the total height of the liquid column (mm) multiplying by 100 (Tabatabaee *et al*, 2005).

RESULTS AND DISCUSSION

Effect of carbon source

Effect of carbon source on the production of biosurfactant was elucidated in figure (1) with emulsification index 68% and surface tension 53mN/m was achieved when crude oil was used as sole source of carbon and energy, followed by sunflower with E24% and surface tension (50%, 59mN/m) respectively. While the lowest activity was obtained when date extract was used.

Depending on the obtained results of emulsification index and surface tension, biosurfactant production come from the growth on water-insoluble organic substrates (crude oil, sunflower) were better than the growth on obtained results from water-soluble organic substrates (fructose, sucrose, glucose, manitol). The biosurfactant production come from growth on water soluble substrate was low due to the simplicity and bioavailability of these substrates (Banat *et al*., 2000).

Also the crude oil was more stable at high temperatures, while the low alkane was highly volatile. This result were in agreement with Wang *et al.* (2006) they demonstrated the thermophilic nature of *Geobacillus thermodenitrificans*, which grows between 45 and 73°C, and the volatility property of light fraction n-alkanes, which evaporates rapidly at elevated temperatures, may explain the lack of a mechanism for short-chain n-alkanes degradation in *Geobacillus thermodenitrificans.*

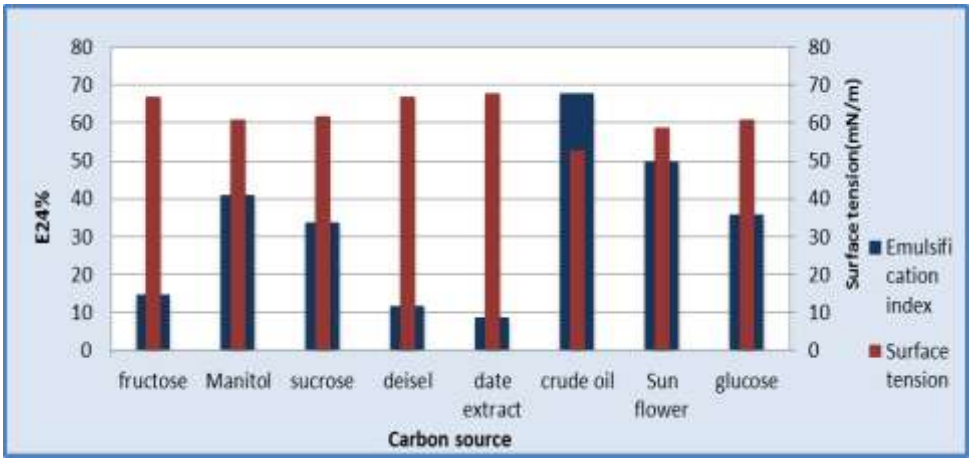


Figure (1): Effect of different carbon source on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7) at 55 °C in shaker incubator (180 rpm) for 7 days.

Effect of carbon source concentration

Different concentrations (0.25, 0.5%, 0.75, 1%, 2%, 3% and 4%) of crude oil were used to grow the *G. thermoleovorans* (Ir1) in order to determine the optimum concentration.

Results shown in figure (2) indicates that the gradual increasing of crude oil carbon source concentration accompanied by increasing in emulsification index and dropping of surface tension, which was in turn an indicator of biosurfactant production, till optimum carbon concentration, these dramatic changes in emulsification index and surface tension reached to its better values 68% and 53 mN m^{-1} respectively at a concentration of 1%. Then after, the activity was dropped perhaps the concentration increase. The low concentration of crude oil may be support the growth of the bacteria and induced it to produce biosurfactant, while the higher concentration of crude oil may be have a toxic effect on bacterial growth and/or not induce bacterium to produce biosurfactant. According to those results, the optimal concentration of crude oil (1%) was used in the next experiments.

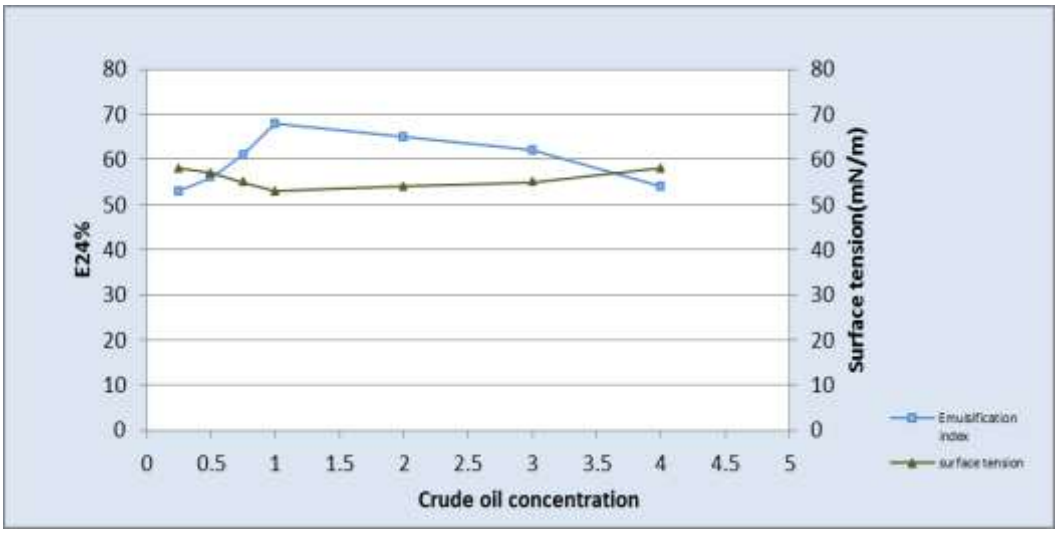


Figure (2): Effect of different concentration of crude oil on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7), at 55 ºC in shaker incubator (180 rpm) for 7 days.

Effect of temperature

The temperature is one of the most important parameter affected the production of biosurfactant, so different temperatures (40, 50, 55, 60, 65, 70 ºC) were used. Results showed in figure (3) pointed out that the better emulsification index (70%) and minimum surface tension (52 mN m^{-1}) was noticed at 60 ºC, it was consider as the optimum temperature for biosurfactant production. While Mahdi (2013) pointed that the 55ºC was optimum temperture for growth of this bacterium. These results were in accordance with

Zheng *et al.* (2011) who demonstrated that the 60°C was optimum temperature for biosurfactant production by *G. pallidus.*

Saharan *et al*. (2011) observed that the growth of *C. bombicola* reaches a maximum at a temperature of 30°C, while 27°C was the best temperature for the production of its biosurfactant. While Kitamoto *et al.* (2001) were observed the highest mannosylerythritol lipid production at 25°C and this temperature was used for both growing and resting cells.

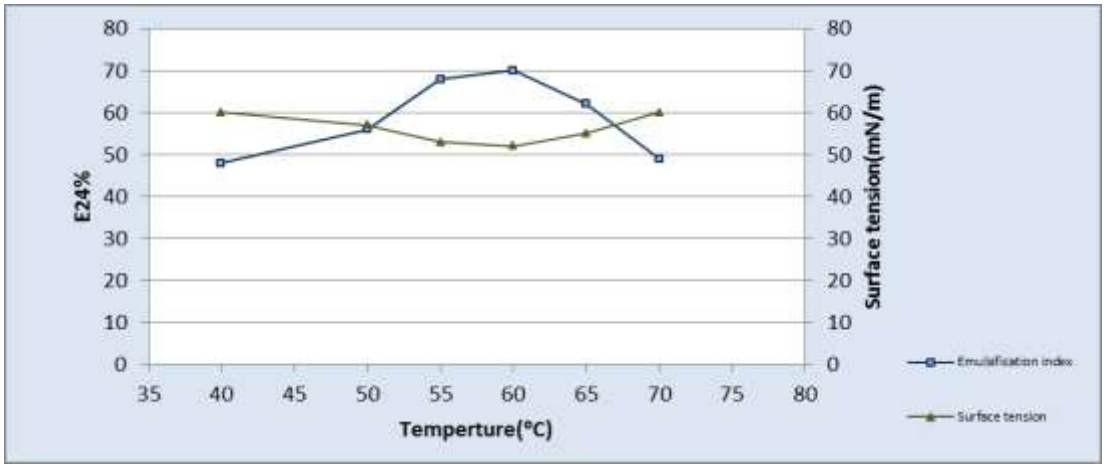


Figure (3): Effect of different temperature on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7) containing 1% crude oil, in shaker incubator (180 rpm) for 7 days.

Effect of nitrogen source

The obtained results mentioned in figure (4) indicated that the efficient emulsification index 70% and lowest surface tension 52 mN m-1 were obtained by using ammonium chloride as a nitrogen source in production medium, therefore it has chosen in the subsequent experiments. And this may attributed to effect of nitrogen source on the metabolism of cell and that in accordance with Okoliegbe and Agarry (2012) who mention that the bacteria require nitrogen to complete its metabolic pathways and essential for microbial growth as protein and enzyme syntheses depend on it. According to these results, NH_4Cl was the best among the other nitrogen sources; this may be because NH_4Cl is a simple nitrogen and easy uptake by bacteria. And this result was in agreement with Abu-Rawaida *et al*. (1991) and Guerra-Santos *et al*. (1986) who detected that the ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *A. paraffineus*, whereas nitrate supported maximum surfactant production by *P. aeruginosa* and *Rhodococcus* sp. In comparism with Johnson *et al.* (1992) who used the potassium nitrate for maximum production of biosurfactant by the yeast *R. glutinis* IIP30.

Effect of nitrogen source (NH_4Cl) concentration

The production medium was supplemented with different concentration of ammonium chloride (NH_4Cl) ranging between 0.05 and 0.5% to determine the optimum concentration required from nitrogen compounds. Results in figure (5) showed that using 0.3% (W/V) of ammonium chloride gave a better production was obtained at this concentration, as indicated by the maximum emulsification index 77% and minimum surface tension 49 mN m^{-1}. Dastgheib *et al.* (2008) referred that the 2% sodium nitrate is the best nitrogen source for emulsifier production by *B. licheniformis.*

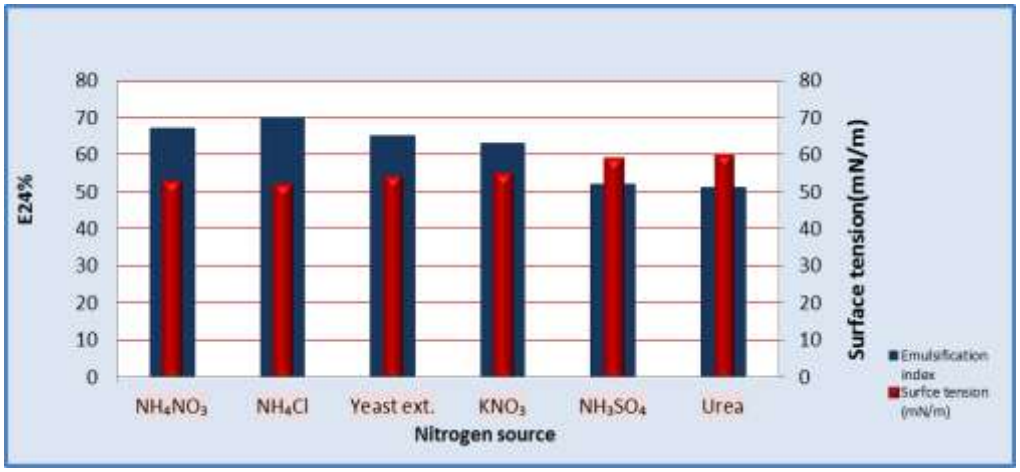


Figure (4): Effect of nitrogen source on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7) containing 1% crude oil, at 60°C in shaker incubator (180 rpm) for 7 days.

Saharan *et al*. (2011) detected that the production of biosurfactant often occurs when the nitrogen source is depleted in the culture medium, during the stationary phase of cell growth, as example the biosurfactant production increased by *P. aeruginosa*, *C. tropicalis* IIP-4 and *Nocardia* strain SFC-D due to the nitrogen limitation (Kosaric *et al*., 1990; Singh *et al*., 1990).

Effect of pH

The production medium was prepared at different pH values, ranged between 5 to 9 , in an attempt, to determine the optimum pH required for biosurfactant production *G. thermoleovorans*. The obtained results as shown in figure (6) elucidated that an efficient production occurred at pH 7 which was indicated by the maximum emulsifying index (77%) and minimum surface tension (49 mN m^{-1}).

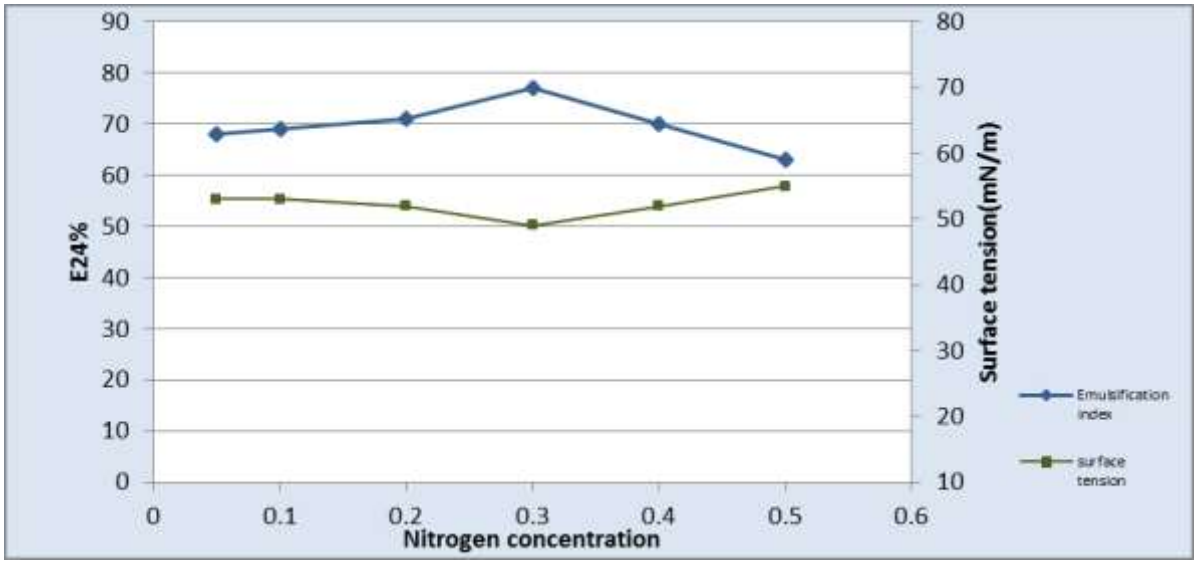
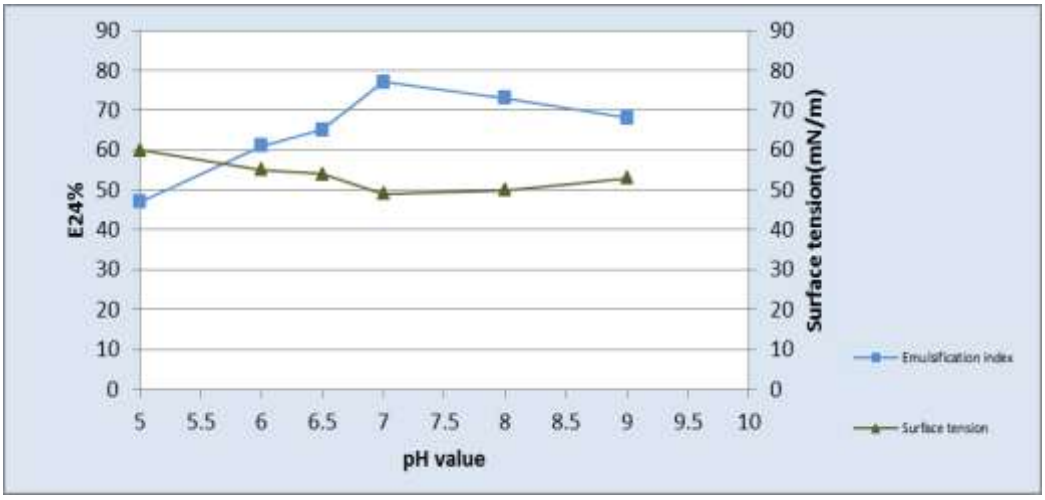


Figure (5): Effect of ammonium chloride concentration on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7) containing 1% crude oil, at 60 °C in shaker incubator (180 rpm) for 7 days.

The synthesis of the biosurfactant decreased without the pH control indicating the importance of maintaining it throughout the fermentation process (Bednarski *et al.*, 2004). This result pointed that pH 7 was optimum for bacterial growth and for biosurfactant production.Mahdi, 2013 was found that this pH value was optimum for *G. thermoleovorans* growth.

Effect of aeration

The aeration represents another important factor influencing the biosurfactant production by *G. thermoleovorans*. To evaluate the effect of the aeration, the cultures were incubated at different agitation speed (rpm) value ranging between 120-220rpm.



Figure(6):Effect of pH value on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium containing 1% crude oil, and (0.3%) ammonium chloride at 60°C in shaker incubator (180 rpm) for 7 days.

Results illustrated in figure (7) indicate that maximum E24% was 80% with reduction in surface tension to 47mNm ˉ1 obtained at 200rpm.

This result was in agreement with Zheng *et al*. (2011) who noticed that the optimum agitation speed was 200 rpm when biosurfactant produced by thermophillic *G. pallidus*. Priya and Usharani (2009) declared that biosurfactant production by *B. subtilis* and *P. aeruginosa* was optimized in a shaker operating at 120 rpm.

While Ghayyomi *et al*. (2012) detected the optimum rpm for biosurfactant production by *Bacillus sp*. was150 rpm.

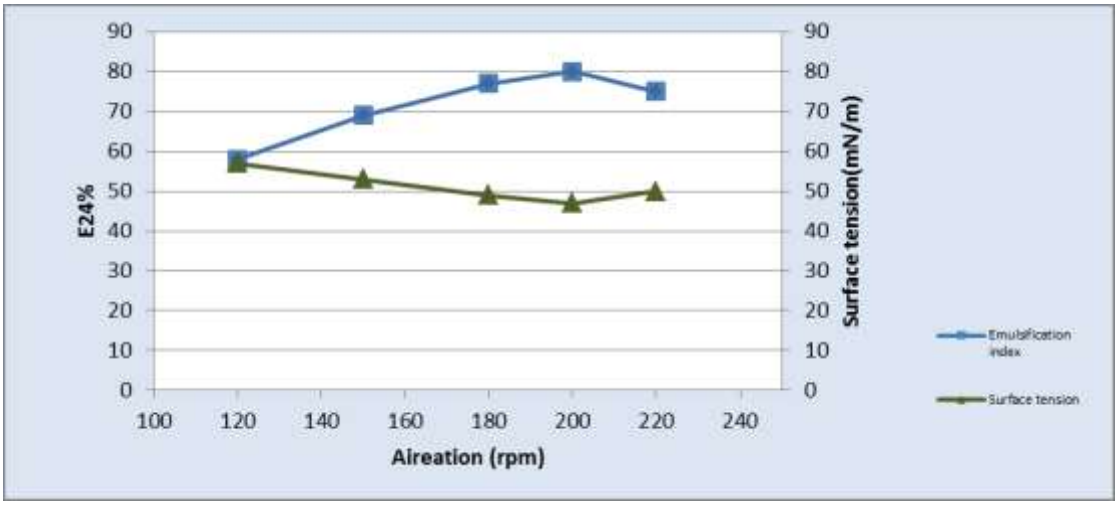
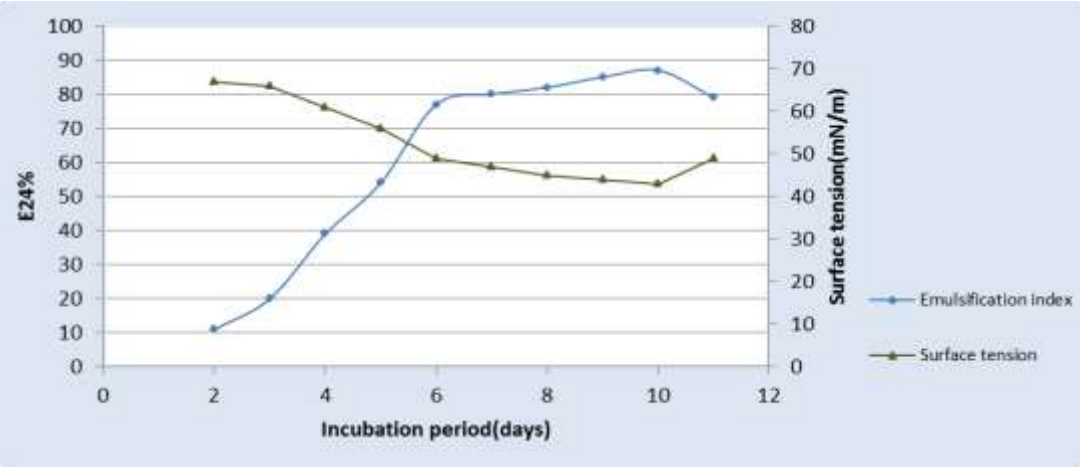


Figure (7) Effect of rpm value on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7) containing 1% crude oil, and (0.3%) ammonium chloride at 60°C for 7 days

Effect of Incubation Period

The optimum period required for biosurfactant production under the previously monitored culture conditions was determined as represented in figure (8). Results showed that the maximum production of biosurfactant was obtained at 10 days of incubation, at this period the emulsification index and surface tension of cell-free broth was 87%, 43 mN m^{-1} respectively. After ten days of incubation the E24% was dropped and this may be due to mix of metabolites. Bonilla *et al.* (2005) mentioned that biosurfactant biosynthesis stopped, probably due to the production of secondary metabolites which could interfere with emulsion formation and the adsorption of surfactant molecules at the oil–water interface. Rosenberg *et al.* (1979) was also reported maximum emulsan production by *A. calcoaceticus* RAG-1 during the stationary growth phase.



Figure(8): Effect of incubation period on biosurfactant production from *G. thermoleovorans*, grown in mineral salt medium (pH 7) containing 1% crude oil, and (0.3%) ammonium chloride at 60ºC in shaker incubator (180 rpm).

CONCLUSIONS

The results concluded that the efficient production of biosurfactant through optimization the *Geobacillus thermoleovorans* (JQ912239), by grown it in mineral salt medium (pH7) containing 1% crude oil as sole carbon source and 0.3% ammonium chloride as a nitrogen source, at 60 ºC, with 200 rpm for 10 days to achieve maximum emulsification index (87%) and lower surface tension reduction (43 mNm^{-1}).

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