**Evaluation of various nutritional and** **environmental factors effect on biosurfactant production by *Staphylococcus epidermidis***

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**Abstract**

Biosurfactants are biological surface-active compounds synthesized mainly by hydrocarbon-utilizing microorganisms. The properties of biosurfactants make them promising compounds for the application in various fields. The current study was toevaluate of various nutritional and environmental factors effect on biosurfactant produced by *Staphylococcus epidermidis*. This bacterium for first time in world was isolated from oilfield reservoir in current study and identified by 16S rDNA sequencing and considered biosurfactant producer according to screening tests. Several factors were evaluated in the current study to assess the optimal conditions for producing the biosurfactant. The results demonstrated that the best source of carbon was olive oil with 2 % concentration and the glutamic acid was the best source of nitrogen with 0.2 % concentration. The study demonstrated highest biosurfactant production was recorded at incubation period of 5 days and 3% inoculum size. Further study is required to determine the chemical structure of produced biosurfactant and evaluate the potential application of biosurfactant in various fields, therefore constitute a stimulus for future studies.

**Keywords:** *Staphylococcus* *epidermidis*, Biosurfactant, Carbon and Nitrogen sources, environmental factors.

**Introduction**

Biosurfactants (BSs) are biological surface-active compounds synthesized mainly by hydrocarbon-utilizing microorganisms which either released into the environment or adhered to the producer cell's plasma membrane (Ndlovu *et al.* 2016; Alyousif *et al.* 2020a).

BSs are amphiphilic compounds that have both hydrophilic and hydrophobic groups that solubilize two separate phases in a heterogeneous solution by interacting with the interface between them (Mani *et al.* 2016). According to chemical structures, they exhibit a broad diversity such as glycolipids, phospholipids, lipoproteins, fatty acids, particulate and polymeric biosurfactants (Maier and Soberon-Chaez 2000).

BSs have different functional properties including wetting, cleansing, emulsification, foaming, surface activity and anti-microbial activity in addition, as well as the ability to function in extreme conditions such as high pH, temperature and salt concentration, making them suitable for use in pharmaceuticals, cosmetics, environmental remediation agriculture, food industries, and enhanced oil recovery (Sachdev and Cameotra 2013; Elazzazy *et al.* 2015).

The natural environments contaminated with hydrocarbons such as soil, water and production water of oilfields are abundant in biosurfactant producing bacteria (Sohail and Jamil 2020). Different genera of bacteria are capable of producing different types of biosurfactants. *Pseudomonas* sp., *Rhodococcus* sp., *Lactobacillus* sp., *Bacillus* sp., *Acinetobacter sp., Arthobacter* sp. and *Candida* sp. that are most genera utilized for biosurfactant production (San Keskin *et al.* 2015).

*Staphylococcus* genus is a known for its pathogenic effect to human and animal, but some *Staphylococcus* strains isolated from natural environments and could be produced secondary metabolites that exhibited biotechnological significance (Eddouaouda *et al.* 2011).

Several studies demonstrated the ability of biosurfactant production by *Staphylococcus species* such as *S. aureus, S.s hominis, S. sp. strain 1E, S. saprophyticus, S. xylosus xylosus* (Mariano *et al.* 2008; Eddouaouda *et al.* 2011; Hamed *et al.* 2012; San Keskin *et al.* 2015; Mani *et al.* 2016)*. S. epidermidis is* a normal and an abundant human skin inhabitant and reported by Hamed *et al.* (2012) as biosurfactant producer.

The current study was toevaluate of various nutritional and environmental factors effect including different carbon sources, nitrogen sources, inoculum size and incubation period on biosurfactant production by *Staphylococcus epidermidis*, which isolated from crude oil of oilfield.

**Materials and methods**

**Sample and** **molecular identification**

The bacterium utilized in present study was isolated in previous study (Alyousif *et al.* 2020a) from crude oil of Al-Garraf oilfield in Thi-Qar province (31°14′N 46°19′E). The bacterium was identified by molecular technique using sequence analysis of 16S ribosomal DNA gene. The bacterial DNA was isolated by using bacterial DNA isolation kit from the Geneaid company. The 16S ribosomal DNA gene was amplified by the PCR technique using the bacterial universal primers 27F (5AGAGTTTGA TCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The PCR were prepared in a total volume of 50 μl. The first step is denaturation at 96 °C for 3 min followed by 27 cycles including of 96 °C for 30s, 56°C for 25s and 72°C for 15s and final extension step at 72°C for 10 min (Miyoshi *et al.* 2005). The PCR products were purified and sequenced by Macrogen Company (South Korea). The 16S ribosomal DNA gene sequence was proofread and compared with bacterial 16S rDNA sequences available at NCBI using BLAST tools “http://www.ncbi.nlm.nih.gov“ to find the sequence similarity and identification of the isolated bacterium.

**Preparation of the bacterial inoculum**

The inoculum of the bacterium was prepared in 250 ml flask containing 50 ml of autoclaved nutrient broth (Himedia, India) and incubated for 24 h at 30 °C. The MSM was inoculated with 5% (v/v) of prepared inoculum.

**Biosurfactant production**

The mineral salt medium was taken from (Deng *et al.* 2014) and composed of the following ingredients (g/l) NaCl (5), KH2PO4 (2), NH4NO3 (1), Na2HPO4 (3), MgSO4.7H2O (0.7) and 1 ml /l trace salt solution. The trace salt solution containing (mg/l) CaCl2 (20), CuSO4 (0.5), FeCl3 (30), MnSO4.H2O (0.5), and ZnSO4.7H2O (10). One hundred ml of MSM prepared in 250 ml flask was autoclaved at 121 °C for 15 min and inoculated with 5 ml (106 CFU/ml) of an activated inoculum. The flask was incubated at 30 °C with agitation (150 rpm/min) in a shaking incubator.

**Screening for producing the biosurfactant**

After the end of fermentation, the cultures broths were centrifuged at 5000 rpm at 4 C° for 20 min to eliminate the bacterial cells (Xiangsheng *et al.* 2010). The bacterial cells-free supernatants were subjected to the following tests to select the optimum factors for producing of biosurfactant.

**Hemolytic assay**

The hemolytic assay was used to examined the ability of bacterium to produce biosurfactant according to the method described by Youssef  
*et al.* (2009). The bacterium was streaked on the plate of 5% human blood agar and incubated at 30 °C for 24 h, then the hemolysis zone was observed around the spot.

**Oil spreading assay**

The oil spreading assay was carried out by pouring 40 ml of distilled water into a 15 cm diameter Petri dish, then pouring 20 l of crude oil onto the water surface to form a thin layer. 10 µl of supernatant was poured onto the oil surface. The diameter of the clearing zone was measured and compared with distilled water as a negative control (Satpute *et al.* 2010).

**The** **emulsification index**

Two ml bacterial cell free supernatant was mixed with two ml kerosene and vortexed for 2 min. The emulsification index was calculated by measuring the height of the emulsion layer after a 24 h incubation period at room temperature (Viramontes-Ramos *et al.* 2010). The emulsification index (E24 %) is calculated using the following equation:

E24 (%) = The height of emulsion layer x 100%  
 The height of total solution

**Emulsification activity**

The supernatant (0.5 ml) was poured to a screw tube containing 7.5 ml of Tris-Mg [20mM Tris HCl (pH 7.0) and 10 mM MgSO4] and 0.1 ml of kerosene. The screw tubes were vortexed for 2 min and allowed to sit for 1 h. The samples' absorbance was measured at 540 nm, and the measured optical density was used to calculate emulsification activity (EA) (Sifour *et al.* 2005).

**Biomass determination** The biomass of the sample was separated by centrifuging 10 ml sample at 5000 rpm for 15 min at 5 °C and the bacterial cell pellets were dried in an oven at 105°C for 24 h (Santos *et al.* 2018).

**Selection of factors affecting biosurfactant production**

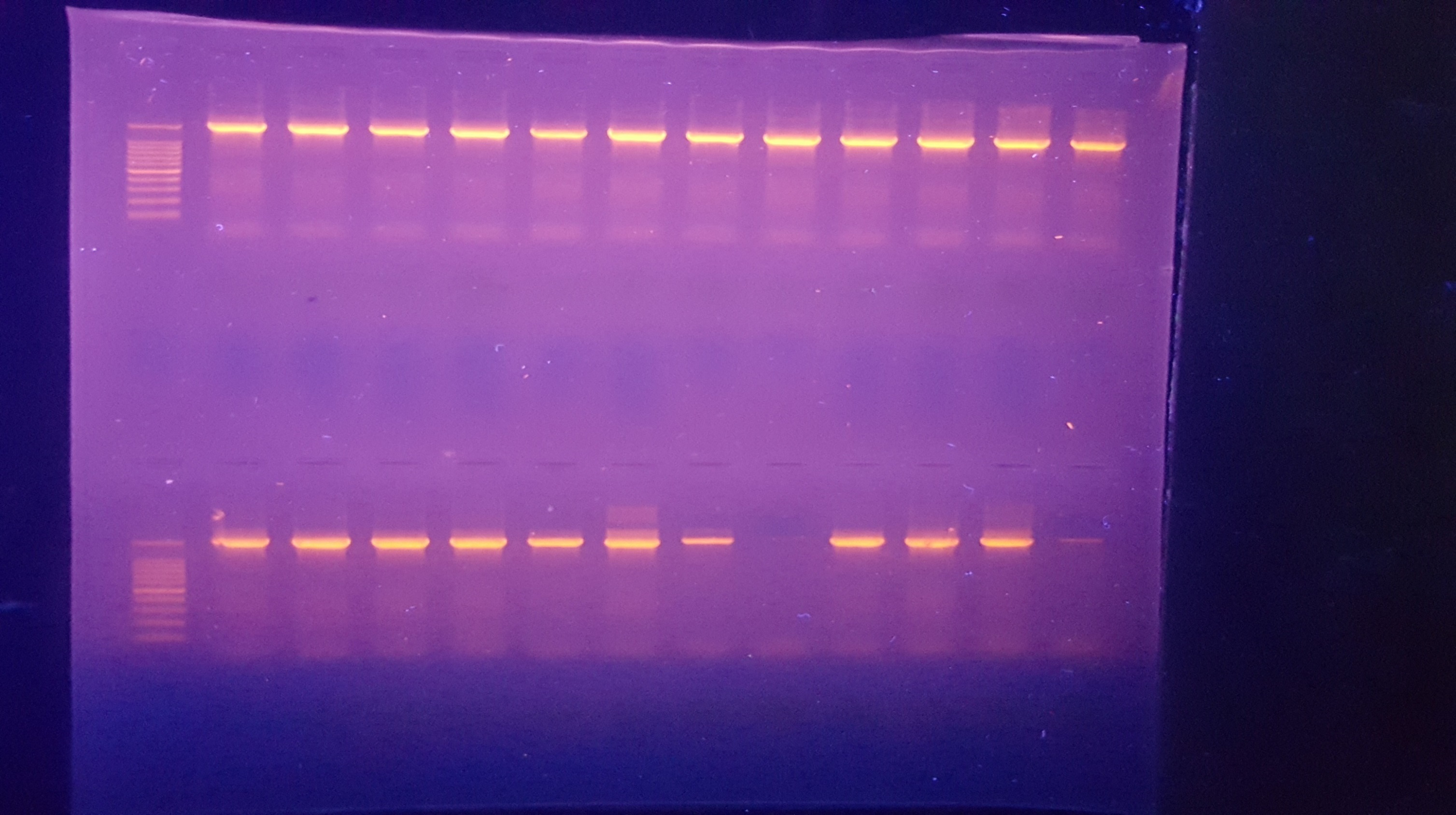
The culture media incubated at differentnutritional and environmental conditions to choose the best nutritional and environmental factors for producing the biosurfactant which included the following factors: (source of carbon, concentration of selected carbon, source of nitrogen, concentration of selected nitrogen, incubation period, and inoculum size), after that the production of biosurfactant was assessed by testes ofemulsification index,oil spreading and emulsification activity. One hundred ml of production medium supplemented with 1% of one of the following sources of carbon including glucose, corn oil, olive oil, glycerol, lactose, mannitol, and sunflower oil were examined to choose the best source of carbon. The medium was incubated at 30 °C for 7 days in a shaking incubator. Five concentrations of olive oil were tested which included the following concentrations (1, 1.5, 2, 2.5 and 3 %) to select the best concentration of carbon source. Four nitrogen sources (glutamic acid, KNO3, NH4NO3 and urea) were tested to choose the optimum nitrogen source in production medium. Four increasing concentrations of glutamic acid (0.1 %, 0.2 %, 0.4 % and 0.6) were tested to determine the optimum concentration of nitrogen. The inoculum volume was evaluated by using different inoculum volumes (1, 2, 3, 4 and 5 %) of the bacterium. The incubation period for producing the biosurfactant was evaluated by incubated the medium at various incubation periods including 1, 2, 3, 4, 5, 6 and 7 days.

**Statistical analysis**

The differences among the factors were assessed using one-way ANOVA of SPSS software (version 20). P value less than0.05 was considered as statistically significant. The average of emulsification activity and biomass values were calculated using two replications and expressed as mean standard deviation.

**Results**

**Molecular identification of bacterial isolate**

 The isolate was identified by PCR technique to amplify 16S ribosomal DNA gene, on an agarose gel, the PCR product was as shown in (Figure 1). The sequence analysis of 16S ribosomal DNA gene of the isolate was identified this isolate as *S. epidermidis.*

1500bp

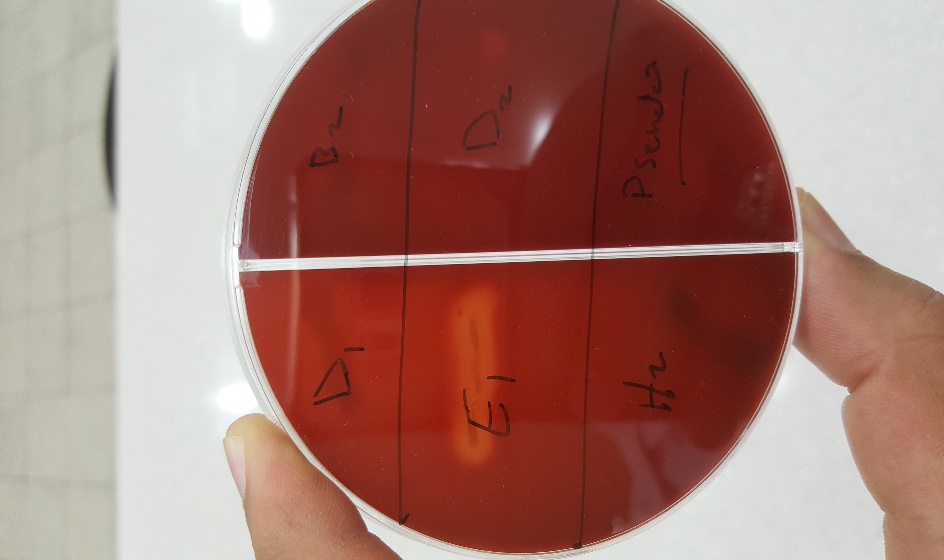
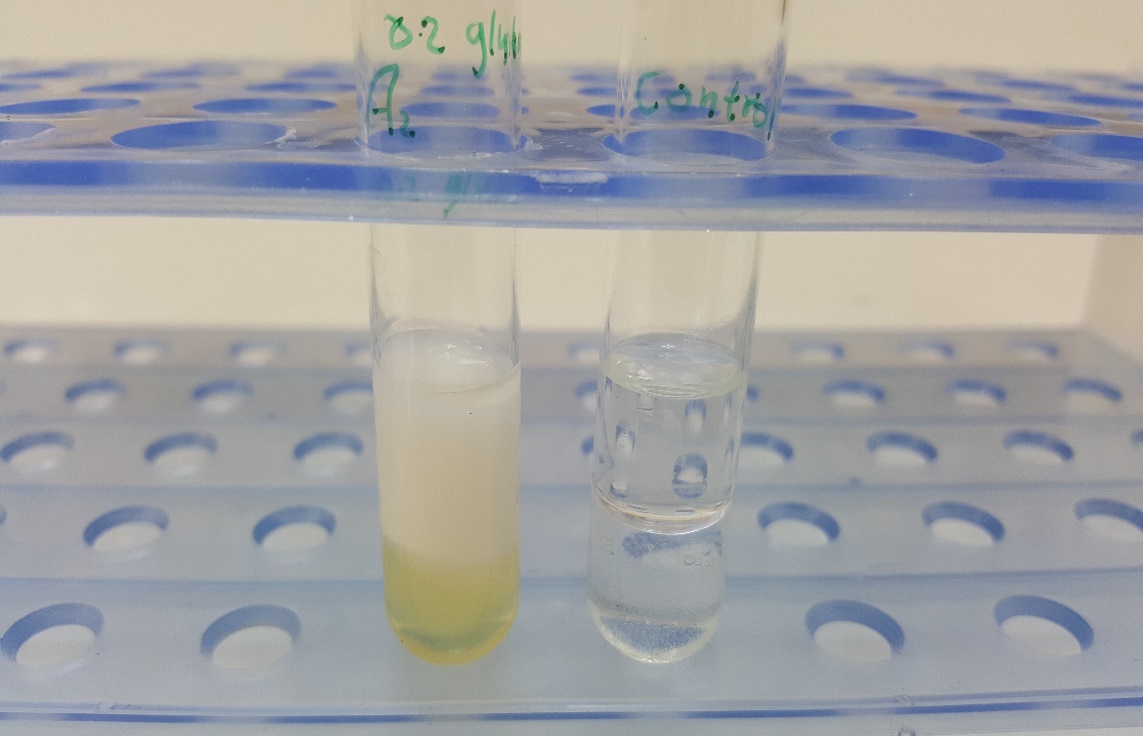
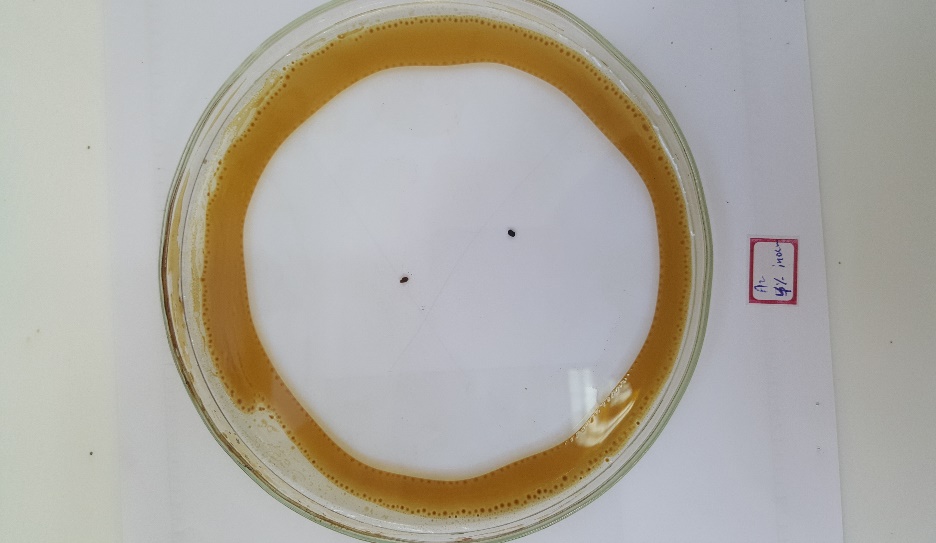
1000bp

L N

**Figure 1.** The PCR product of 16S rDNA gene. Lane L: 100 bp DNA ladder. Lane N: 16s RDNA gene

**Screening for biosurfactants production**

*S. epidermidis* was screened to assess an ability of isolate to produce biosurfactant by using screening tests. For the hemolytic test, the bacterium *S. epidermidis* showed a clear zone around the colony of bacterium on blood agar and considered positive result as shown in (Figure 2A). The result showed 53.3% value for emulsification index test (Figure 2B), 10 cm diameter of clear zone for the oil spreading test (Figure 2C) and 0.896±0.082 for emulsification activity therefore, they considered positive result for producing biosurfactant. The biomass concentration obtained from *S. epidermidis* was 2.237±0.080 gm.



**A B C**

**Figure 2.** screening tests of biosurfactant production **A**: Hemolytic assay **B:** emulsification index, **C**: oil spreading test

**Screening of nutritional and environmental conditions for enhanced the production of biosurfactant**

Several factors were evaluated in the current study to determine the best conditions for producing biosurfactant by *S. epidermidis*. Various sources of carbon were evaluated for producing the biosurfactant. The results gave in (Table 1) demonstrated that the olive oil was the best source of carbon for producing the biosurfactant with emulsification activity (0.896± 0.055), E24% (53.3 %), oil spreading fourteen cm and biomass 2.237± 0.035 gm. Mannitol was found to be the weakest source of carbon for producing the biosurfactant with emulsification activity (0.043± 0.121), no value recorded for E24 %, oil spreading 0.3 cm and biomass 1.971± 0.072 gm.

**Table 1.** Effect of various carbon sources on biosurfactant production by *S. epidermidis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Carbon sources** | **Emulsification activity/ 540 nm** | **Emulsification index (E24%)** | **Oil spreading/cm** | **Biomass gm/l** |
| **Olive oil** | 0.896± 0.055\* | 53.3 | 14 | 2.237± 0.035 |
| **Sunflower oil** | 0.497± 0.036 | 36 | 9.5 | 1.326± 0.063 |
| **Corn oil** | 0.111± 0.073 | 3.3 | 5 | 1.413± 0.321 |
| **Glycerol** | 0.251± 0.216 | 26.6 | 7 | 1.661± 0.064 |
| **Lactose** | 0.045± 0.053 | nil | 0.2 | 1.317± 0.096 |
| **Glucose** | 0.044± 0.072 | nil | 0.3 | 1.825± 0.083 |
| **Mannitol** | 0.043± 0.121 | nil | 0.3 | 1.971± 0.072 |

Different olive oil concentrations were assessed as source of carbon for producing the biosurfactant by *S. epidermidis*. The results were given in (Table 2) demonstrated the best concentration of olive oil. The concentration 2 % of olive oil was gave the optimum results for emulsification activity (1.240± 0.063), E24% (60 %), oil spreading fourteen cm and biomass 2.331± 0.084 gm with significant differences of other olive oil concentrations (P ≤ 0.05).

**Table 2.**  Effect of olive oil concentration on biosurfactant production by *S. epidermidis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Olive oil (%)** | **Emulsification activity/ 540 nm** | **Emulsification index (E24%)** | **Oil spreading/ cm** | **Biomass gm/l** |
| **1** | 0.902± 0.061\* | 53.3 | 14 | 2.425± 0.042 |
| **1.5** | 1.015± 0.034 | 53.3 | 14 | 2.667± 0.052 |
| **2** | 1.240± 0.063 | 60 | 14 | 2.331± 0.084 |
| **2.5** | 1.138± 0.620 | 53.3 | 14 | 2.173± 0.062 |
| **3** | 0.823± 0.083 | 50 | 14 | 2.215± 0.033 |

\* Mean ± SD, n=3, P ≤ 0.05

Various sources of nitrogen were assessed as sole nitrogen source for producing the biosurfactant by *S. epidermidis*. The results showed as in (Table 3) that Glutamic acid was the best source of nitrogen for producing the biosurfactant with values Emulsification activity (1.446± 0.038), E24 % (60%), oil spreading (14.5 cm) and biomass 2.651± 0.0752 gm with significant differences of nitrogen sources (P ≤ 0.05). Urea was found to be the weakest source of nitrogen for producing the biosurfactant with emulsification activity (1.020± 0.214), E24 % (53.3), oil spreading 14cm and biomass 2.342± 0.085 gm.

**Table 3.** Effect of various nitrogen sources on biosurfactant production by *S. epidermidis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Nitrogen sources** | **Emulsification activity/ 540 nm** | **Emulsification index (E24%)** | **Oil spreading/ cm** | **Biomass gm/l** |
| **Glutamic acid** | 1.446± 0.038\* | 60 | 14.5 | 2.651± 0.075 |
| **KNO3** | 1.323± 0.034 | 60 | 14 | 2.723± 0.042 |
| **NH4NO3** | 1.257± 0.051 | 60 | 14 | 2.425± 0.041 |
| **Urea** | 1.020± 0.214 | 53.3 | 14 | 2.342± 0.085 |

\* Mean ± SD, n=3, P ≤ 0.05

Several concentrations of Glutamic acid were assessed as a sole source of nitrogen for producing the biosurfactant by *S. epidermidis*. The results were given in (Table 4) demonstrated the best concentration of Glutamic acid. The concentration 0.4 % of Glutamic acid was gave the optimum results with emulsification activity (1.587± 0.036), E24% (60 %), oil spreading 14.5 cm and biomass 2.535± 0.092 gm with significant differences of other concentrations of Glutamic acid (P ≤ 0.05).

**Table 4.** Effect of Glutamic acid concentration on biosurfactant production by *S. epidermidis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Glutamicacid concentration (%)** | **Emulsification activity/ 540 nm** | **Emulsification index (E24%)** | **Oil spreading/ cm** | **Biomass gm/l** |
| **0.1** | 1.325± 0.061\* | 60 | 14.5 | 2.551± 0.072 |
| **0.2** | 1.423± 0.112 | 60 | 14.5 | 2.624± 0.038 |
| **0.4** | 1.587± 0.036 | 60 | 14.5 | 2.535± 0.092 |
| **0.6** | 1.302± 0.081 | 60 | 14.5 | 2.723± 0.022 |

\* Mean ± SD, n=3, P ≤ 0.05

Several sizes of inoculum were examined to assess the best inoculum size for the biosurfactant production by *S. epidermidis.* The results were provided in (Table 5) demonstrated the inoculum size above 3% had no significant effect on biosurfactant production. The concentration 3% of inoculum size was gave the best finding with emulsification activity (1.621± 0.033), E24% (60 %), oil spreading fifteen cm and biomass 2.923± 0.083 gm with significant differences of other concentrations of inoculum size (P ≤ 0.05).

**Table 5.** Effect of inoculum size on biosurfactant production by *S. epidermidis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Inoculum size (%)** | **Emulsification activity/ 540 nm** | **Emulsification index (E24%)** | **Oil spreading/ cm** | **Biomass gm/l** |
| **1** | 1.503± 0.061\* | 60 | 14.5 | 2.517± 0.034 |
| **2** | 1.578± 0.201 | 60 | 14.5 | 2.821± 0.022 |
| **3** | 1.621± 0.033 | 60 | 15 | 2.923± 0.083 |
| **4** | 1.523± 0.071 | 60 | 14.5 | 2.512± 0.502 |
| **5** | 1.417± 0.095 | 60 | 14.5 | 2.624± 0.0104 |

\* Mean ± SD, n=3, P ≤ 0.05

Several incubation periods were examined to determine the optimum incubation period for producing the biosurfactant by *S. epidermidis*. The results were provided in (Table 6) demonstrated the incubation period of 6 days was gave the best findings with emulsification activity (1.678 ± 0.052), E24% (60 %), oil spreading fifteen cm and biomass 2.622± 0.015 gm along with significant differences of other incubation period (P ≤ 0.05). The values of screening tests were raised with raising the incubation days until five, but the values screening tests decreased at 6 and 7 days of incubation.

**Table 6.** Effect of incubation period on biosurfactant production by *S. epidermidis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Incubation period** | **Emulsification activity/ 540 nm** | **Emulsification index (E24%)** | **Oil spreading/ cm** | **Biomass gm/l** |
| **1** | 0.186 ± 0.016\* | 3.3 | 4 | 0.374 ± 0.071 |
| **2** | 0.491 ± 0.063 | 26.6 | 8 | 0.873± 0.082 |
| **3** | 0.885 ± 0.221 | 53 | 10 | 1.943± 0.063 |
| **4** | 1.286 ± 0.036 | 60 | 14 | 2.412± 0.502 |
| **5** | 1.678 ± 0.052 | 60 | 15 | 2.622± 0.015 |
| **6** | 1.435 ± 0.027 | 60 | 14.5 | 2.853± 0.720 |
| **7** | 1.387 ± 0.241 | 60 | 14 | 2.752± 0.045 |

\* Mean ± SD, n=3, P ≤ 0.05

**Discussion**

The current5 study is aimed to identify biosurfactant producing bacteria from crude oil of oilfield and evaluate the nutritional and environmental factors on biosurfactant production. The molecular identification of bacterial isolate shows that isolate is *Staphylococcus epidermidis*. The majority of *Staphylococcus* species is known as the pathogens for human and animals. Several *Staphylococcus* strains isolated from many natural environments could be used for producing substances that high values for biotechnological application (Popowicz *et al.* 2006; Eddouaouda *et al.* 2011).

The screening tests that used to evaluate biosurfactant production were hemolytic assay, emulsification index test, oil spreading test and emulsification activity, these tests are simple and quick for screening and predicting biosurfactant production by bacteria. The hemolytic assay is used as primary screening method for detecting the ability of bacteria to produce biosurfactant. Walter *et al.* (2010) found that some biosurfactants can cause hemolysis but, that the hemolysis is not limited to biosurfactants and hemolysis could also be by bacterial lytic enzymes**.**

Emulsification index is an important test to select the potential producers of biosurfactants. The principle of emulsification index test as well as oil spreading test and emulsification activity is based on lowering of interfacial tension in liquids and the ability of biosurfactants to emulsify, regardless of their structure. (Alyousif *et al.* 2020b). The value of emulsification index and oil spreading test refers to the amount of biosurfactant that produce by bacterial isolate.

The results of screening tests demonstrated that the olive oil was the best source of carbon with 2 % concentration for producing biosurfactant. *S. epidermidis* strain was isolated from crude oil and thus substrate formed a natural carbon source for its natural environment. The ability of *S. epidermidis* for utilizing olive oil as source of carbon for biosurfactant production instead of other sources of carbon due to strain's ability to synthesize lipase enzyme which aids absorption of fatty acids existed in olive oil, and the growth of bacteria on carbohydrate substrates which causes a decrease in pH of the medium and thus inhibits production of biosurfactant (Abouseoud *et al.* 2008; Mnif *et al.* 2009). The same results demonstrated by Eddouaouda *et al.* (2011) the best carbon source for producing biosurfactant was olive oil by *Staphylococcus* sp. strain 1E among the other tested sources.

The current study revealed the glutamic acid was the optimal source of nitrogen with 0.2 % concentration for biosurfactant production. The bacteria are need nitrogen source for synthesizing enzymes that essential for their survival and metabolic processes (Okoliegbe and Agarry 2012). Rufino *et al.* (2008) demonstrated that the biosurfactant was optimally produced by *Candida lipolytica* by using glutamic acid as nitrogen source. While Subasioglu and Cansunar (2008) have shown rhamnolipid production by *P. aeruginosa* when it was used 2.0 g/l NaNO3 as the nitrogen source.

The results of current study were revealed maximum biosurfactant production was recorded at 3% of inoculum size. Alyousif *et al.* (2020b) reported the optimal size of inoculum for highest production of rhamnolipid biosurfactant from *P. aeruginosa* was 3%. While, Keskin *et al.* (2015) reported the optimal size of inoculum for highest production of biosurfactant from *S. xylosus* was 5%. The current study demonstrated the highest production of biosurfactant was recorded at 5 days of incubation period. The increasing the period of incubation may be led to the interference between bacterial metabolites and biosurfactant may led to decrease the activity of biosurfactant. Mani *et al.* (2016) reported that biosurfactant yielding produced by *S. saprophyticus* SBPS 15 increased with incubation period and reached maximum production after 66 h of incubation period. The obtained results during the current study constitute a stimulus for future studies to determine the chemical structure of produced biosurfactant, evaluate the potential antibacterial activity of biosurfactant and determine the possibility of using it in the bioremediation of pollutants.

**Conclusions**

The present study revealed that *S. epidermidis* isolated from crude oil of oilfield reservoir for first time was efficient biosurfactant producing bacterium. The bacterium was evaluated for Several factors to determine the optimal conditions for producing of biosurfactant. The finding revealed that optimum conditions for producing of biosurfactant were olive oil (2%), glutamic acid (0.2%), 5 days incubation period and 3% inoculum size.

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**Conflicts of Interest**

The authors declare no conflict of interest.

**Author Contributions**

All authors equally contribution to this work.

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