**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 produced 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 bacteriumwas isolated for first time in world from crude oil of 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 determine the optimal conditions for biosurfactant production. The results demonstrated that olive oil was the optimal carbon source with concentration 2 % and the glutamic acid was the best nitrogen source with concentration 0.2 %. 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 produced mainly by hydrocarbon-utilizing microorganisms which either released into the surrounding 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, agriculture, food industries, environmental remediation 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., *Bacillus* sp., *Acinetobacter sp., Rhodococcus* sp., *Lactobacillus* 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 16S rDNA gene sequence analysis. The bacterial DNA was isolated by using Presto™ Mini g DNA bacteria kit from the Geneaid company. The 16S rDNA gene was amplified by the polymerase chain reaction (PCR) using the bacterial universal primers 27F (5AGAGTTTGA TCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The PCR reactions were made in a total volume of 50 μl. An initial denaturation step of 96 °C for 3 min followed by 27 cycles of 96 °C for 30s, annealing temperature of 56°C for 25s and extension at 72°C for 15s and final extension at 72°C for 10 min (Miyoshi *et al.* 2005). The Amplified DNA was purified and sequenced by Macrogen Company (South Korea). The 16S rDNA gene sequence was corrected and compared with bacterial nucleotide sequences available at NCBI using BLAST tools “http://www.ncbi.nlm.nih.gov“ to find the sequence homology 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 (MSM) was adopted 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 sterilized by autoclaving at 121 °C for 15 min and inoculated with 5 ml (106 CFU/ml) of an activated inoculum. The culture 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 remove 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 based on the method described by Youssef  
*et al.* (2009). The bacterium was streaked on 5% human blood agar plate 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 performed by adding 40 ml of distilled water to a Petri dish (size of 15 cm diameter), 20 µl of crude oil was added onto the water surface forming a thin layer. Then, 10 µl of supernatant was added to the oil surface. The diameter of the clearing zone was measured and compared with 10 µl of distilled water as a negative control (Satpute *et al.* 2010).

**Determination of 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

**Determination of emulsification activity**

The supernatant (0.5 ml) was added to a screw-capped 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-capped 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**

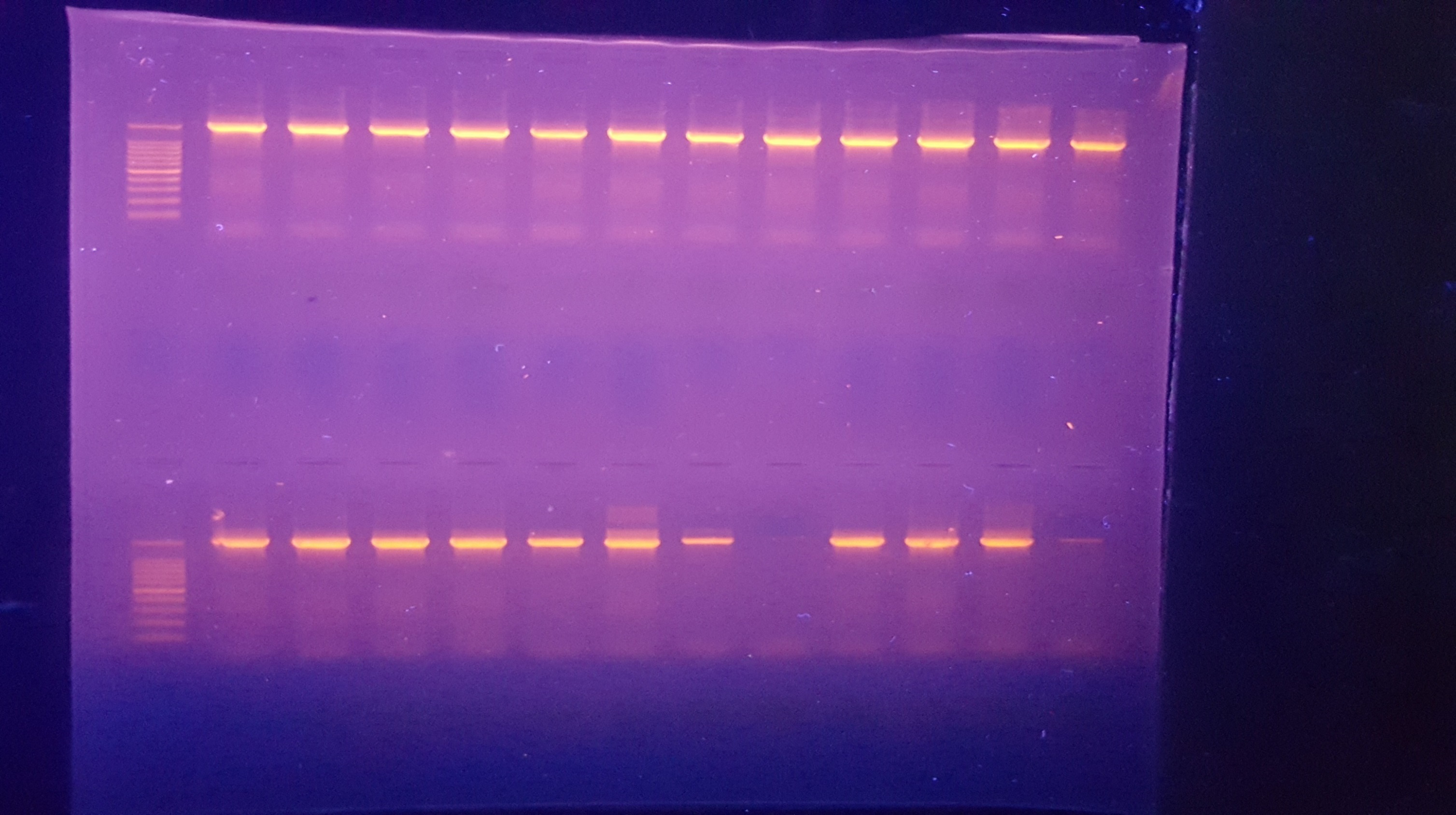
The culture media incubated at differentnutritional and environmental conditions to determine the optimum nutritional and environmental factors for producing the biosurfactant which included the following factors: (carbon source, carbon concentration, nitrogen source, nitrogen concentration, incubation period, and inoculum size), after that the production of biosurfactant was evaluated byOil spreading assay, emulsification activity and emulsification index. One hundred ml of production medium supplemented with 1% of one of the following carbon sources (glucose, corn oil, glycerol, lactose, mannitol, olive oil and sunflower oil) were assessed to select optimum carbon source in production medium and incubating at 30 °C for 7 days in a shaker incubator. Five concentrations of olive oil were examined (1 %, 1.5 %, 2 %, 2.5 % and 3 %) to choose optimum concentration keeping NH4NO3 as nitrogen source in production medium. 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 examined to find out the optimum nitrogen concentration using optimized olive oil source in production medium. The optimum inoculum size was evaluated by using different inoculum sizes (1 %, 2 %, 3 %, 4 % and 5 %) of the bacterium and incubated at 30 °C for 7 days in a shaker incubator. The optimum incubation period for producing the biosurfactant was determined by incubated at different incubation periods (1, 2, 3, 4, 5, 6 and 7 days).

**Statistical analysis**

One-way ANOVA was used to assess the differences among the different factors using SPSS software (version 20). P **<** 0.05 were considered as statistically significant. The average values presented for emulsification activity and biomass were estimated using 2 replications and expressed as mean ± standard deviation.

**Results**

**Molecular identification of bacterial isolate**

 The isolate was identified by amplified 16S rDNA gene, The PCR product was observed on agarose gel as shown in (Figure 1). The sequence analysis of 16S rDNA gene of the bacterial isolate was identified this isolate as *S. epidermidis.*

1500bp

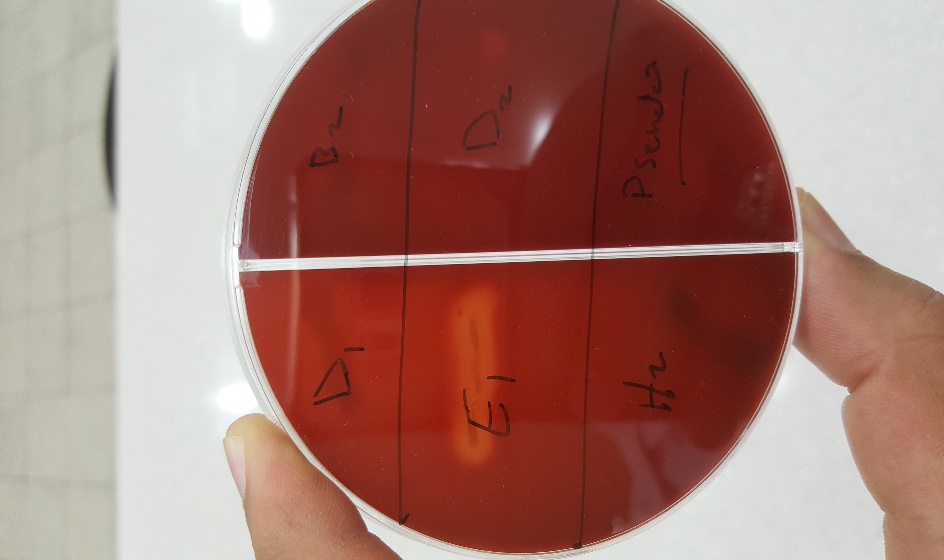
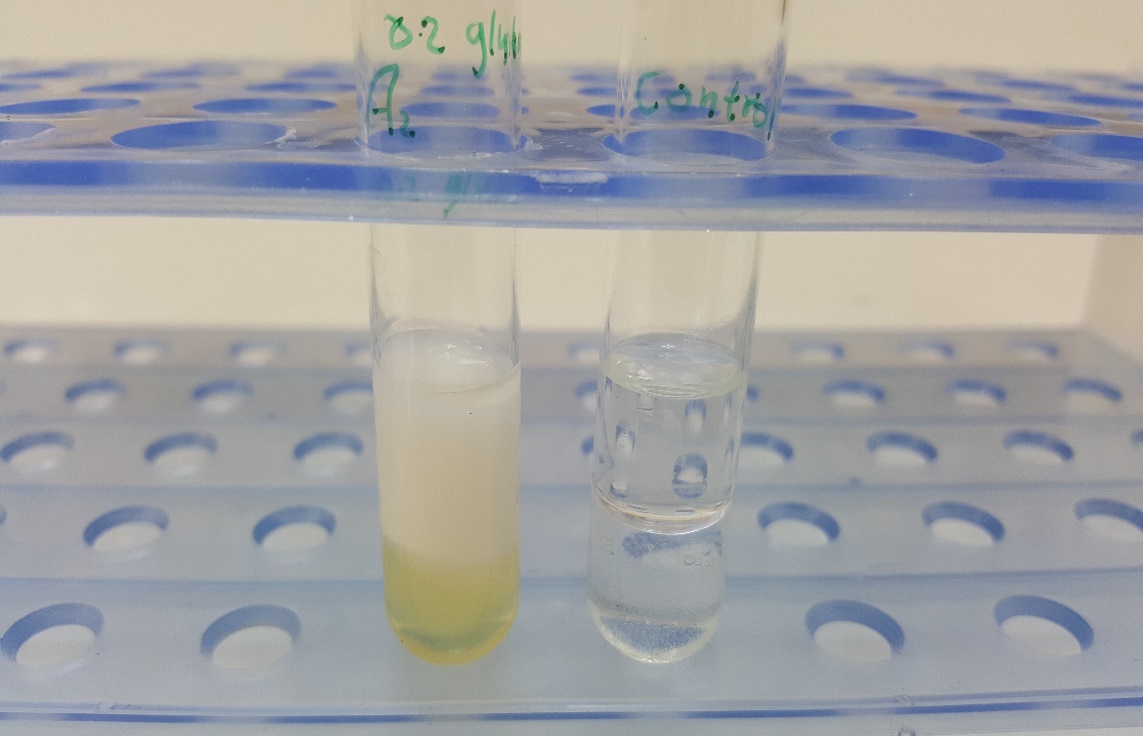
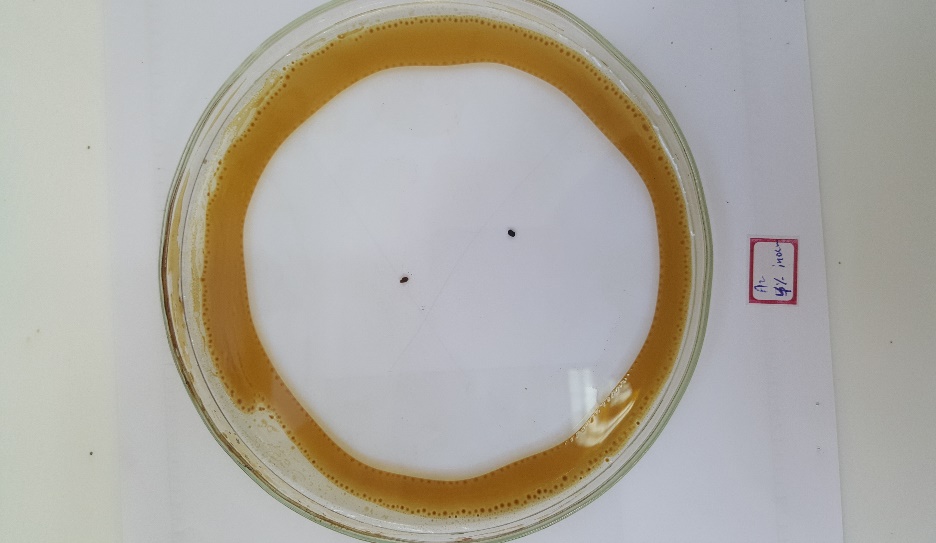
1000bp

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 improved biosurfactant production**

Several factors were evaluated in the current study to determine the optimal conditions for biosurfactant production by *S. epidermidis*. Various carbon sources were evaluated for producing the biosurfactant. The results provided in (Table 1) demonstrated that the olive oil was the best carbon source for producing the biosurfactant with emulsification activity (0.896± 0.055), E24% (53.3 %), oil spreading 14 cm and biomass 2.237± 0.035 gm. Mannitol was found to be the poorest carbon source 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 |

several concentrations of olive oil were assessed as a sole source of carbon for producing the biosurfactant by *S. epidermidis*. The results were provided in (Table 2) demonstrated the optimum olive oil concentration. The concentration 2 % of olive oil was gave the best results with Emulsification activity (1.240± 0.063), E24% (60 %), oil spreading 14 cm and biomass 2.331± 0.084 gm with significant differences with other concentrations of olive oil (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 nitrogen sources 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 with nitrogen sources (P ≤ 0.05). Urea was found to be the poorest nitrogen source 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 provided in (Table 4) demonstrated the optimum Glutamic acid concentration. The concentration 0.4 % of Glutamic acid was gave the best 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 with 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 determine the optimum inoculum size for producing the biosurfactant 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 15 cm and biomass 2.923± 0.083 gm with significant differences with 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 values of emulsification activity (1.678 ± 0.052), E24% (60 %), oil spreading 15 cm and biomass 2.622± 0.015 gm along with significant differences with other incubation period (P ≤ 0.05). The values of screening tests were increased with increasing the number of incubation days until 5, while biomass kept on increasing along with increasing of incubation period, but the values of emulsification activity, E24%, and oil spreading 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 present study is aimed to isolate and identify biosurfactant producing bacteria from crude oil of oilfield and evaluate the effect of nutritional factors 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 one of the important tests to select the potential producers of biosurfactants. The principle of emulsification index as well as oil spreading test and emulsification activity based on interfacial tension reduction of the liquids and emulsification ability of biosurfactants regardless the structure of biosurfactant (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 optimal carbon source with concentration 2 % 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 carbon source for producing biosurfactant rather than other carbon sources may be due to strain's ability to produce lipase enzyme which aids assimilation of fatty acids found in olive oil, and the growth of bacteria on carbohydrate substrates which causes a decrease in pH of the medium and thus inhibits biosurfactant production (Abouseoud *et al.* 2008; Mnif *et al.* 2009). The same results reported by Eddouaouda *et al.* (2011) that olive oil was the optimal carbon source for producing biosurfactant by *Staphylococcus* sp. strain 1E among the other tested sources.

The current study revealed the glutamic acid was the best source of nitrogen with concentration 0.2 % for biosurfactant production. The bacteria are need nitrogen source for synthesizing enzymes that essential for growth and metabolic pathways (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 highest biosurfactant production was recorded at 3% inoculum size. Alyousif *et al.* (2020b) reported the optimum inoculum size for maximum production of rhamnolipid biosurfactant from *P. aeruginosa* was 3%. While, Keskin *et al.* (2015) found the optimum inoculum size for maximum production of biosurfactant from *S. xylosus* was 5%. The current study demonstrated highest biosurfactant production was recorded at incubation period of 5 days. The increasing incubation period may be led to the interference between bacterial secondary metabolites and biosurfactant which led to decrease biosurfactant activity. 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 current study demonstrated that bacterium *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 biosurfactant production. The results demonstrated that optimum conditions for biosurfactant production were olive oil (2%) as carbon source, glutamic acid (0.2%) as a nitrogen source, 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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