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Pol. J. Environ. Stud. Vol. 30, No. 5 (2021), 1-9
DOI: *10.15244/pjoes/132807* ONLINE PUBLICATION DATE:

Original Research

A Study on Larvicidal Activity and Phylogenetic Analysis of *Staphylococcus epidermidis* as a Biosurfactant-Producing Bacterium

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Received: 4 December 2020
Accepted: 25 January 2021

Abstract

Biosurfactants, produced by microorganisms, are surface-active compounds with abundant applications in miscellaneous industries like food, cosmetics, bioremediation, agriculture, and so on. The primary objective of this study was to investigate the larvicidal activity of biosurfactants produced by *Staphylococcus epidermidis* (*S. epidermidis*) isolated from petrochemical wastes. As the secondary objective, this study was to perform a phylogenetic analysis of this bacterium. To this end, the type of the extracted biosurfactant was characterized using the thin-layer chromatography (TLC) and the gas chromatography/mass spectrometry (GC-MS). The isolated bacterium was then identified through molecular tests based on 16S ribosomal ribonucleic acid (16S rRNA) and serum response factor (*srf*) genes. Likewise, the phylogenetic tree of the bacterium was deduced via the Molecular Evolutionary Genetics Analysis (MEGA-X) software. Besides, the larvicidal activity of the biosurfactant on the larvae of flour beetles, *Tribolium castaneum* (*T. castaneum*), was examined. The mean mortality rate and the lethal dose (LD50) were further calculated using the Statistical Analysis System (SAS) and the Probit Analysis-MSChart software. Totally, nine diverse types of colonies were isolated and three of them were found with potential activity to produce biosurfactants. The best bacterium was accordingly selected for subsequent experiments recruiting the oil displacement process and the emulsification index (E24). The extracted biosurfactant was then characterized as a lipopeptide. Moreover, biochemical tests showed that the bacterium was Gram-positive cocci of gamma species. Hemolysis and molecular tests also confirmed that the bacterium was *S. epidermidis*. As well, the presence of the SRF gene in the isolation was established via the polymerase chain reaction (PCR). The mean mortality rate revealed that the mentioned bacterial biosurfactant at the concentration of 10000 µg/g and on the ninth day of the treatment had the highest larval mortality rate (63.33%). In addition, the LD50 equal to 25001.7 µg/g was calculated for *S. epidermidis* on the ninth day. The results demonstrated that the biosurfactant

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produced by this bacterium was endowed with an acceptable ability to control *T. castaneum* and it could be used in further research on the larvae of other insects.

Keywords: biosurfactant, lipopeptide, larvicidal, flour beetle, *Staphylococcus epidermidis*

Introduction

Biosurfactants are surface-active compounds with hydrophilic and hydrophobic parts that emulsify hydrocarbons and give rise to dissolution [1]. There are various types of biosurfactants, such as phospholipid, glycolipid, lipopolysaccharide, lipoprotein, lipopeptide, fatty acid, natural lipid, and flavolipid [2]. Biosurfactants have further demonstrated more advantages compared with chemical types such as their low toxicity, degradability, environmentally-friendly, extra- foam production, high selectivity, as well as specific activity with potential of hydrogen (pH), temperature, and extreme salinity [3]. Currently, they are being employed in diverse fields such as food industry, removal of oil and petroleum contaminations, bioremediation, and biopesticides. The use of biosurfactants as biopesticides has been also growing fast thanks to their advantages compared with chemical materials [4].

Today, due to human knowledge about the possibility of contamination and the health threats posed by many common synthetic chemical toxins, much attention has been driven to the production of biotechnology products to promote sustainable agriculture. Although synthetic pesticides can protect crops by suppressing arthropod pests, they have severe hazards for health and the environment because of their toxicological properties and harmful side effects [5, 6]. The main problems associated with chemical pesticides are their effects on non-target organisms like humans, pets, beneficial insects, and wildlife, as well as their persistence in nature, which has been often long [7]. Biopesticides can be developed by beneficial microorganisms such as fungi and bacteria. They are also environmentally-friendly alternatives to sustainable pest control due to their safety for human and non-target organisms [8]. Accordingly, biosurfactants are known for their antimicrobial and biopesticide activity against human and plant pathogens [9]. Among pests, flour beetles, *Tribolium castaneum* (*T. castaneum*), are noteworthy in the agricultural industry because of their resistance to various insecticides [10]. This species is a pest that attacks stored grains, flour, cereals, and other crops [11] and reduces annual statistical yield. Therefore, this insect is considered as a very destructive pest for storage grains [12]. Hence, the present study aimed to isolate a biosurfactant-producing bacterium, *Staphylococcus epidermidis* (*S. epidermidis*), from the petrochemical wastes in Shiraz Province, Iran, and to determine the extracted biosurfactant type. The given study was also to perform a phylogenetic analysis on the presence of the biosurfactant gene of this bacterium. Finally, the larvicidal activity of the extracted biosurfactant on the larvae of *T. castaneum* as a biological control was investigated.

Materials and Methods

Isolation of Biosurfactant-Producing Bacterium

A total number of 50 soil samples were randomly collected based on the distribution of the accessible area to the petrochemical wastes situated in Shiraz Province, Iran. The topsoil was then removed, and each sample was collected with a sterile spatula and transferred to the microbiological laboratory in sterile polyethylene bags. Afterwards, the samples were homogenized, and subsequently 1 gram of soil was added to 9 cc of sterile distilled water. The samples were serially diluted (10^{-1}-10^{-6}), and then 0.1 ml of each dilution was transferred to the blood agar (BA; Liofilchem, Italy). The plates were further incubated at 37ºC for 24 hrs and the colonies were purified for further steps.

Evaluation of Biosurfactant Production

The early tests for the isolation of the biosurfactant-producing bacteria included surface activity tests such as drop-collapse test, oil displacement process, and tensiometer measurements. Subsequently, hemolysis and the emulsification index (E24) and its stability were checked. These tests were completed with three replicates.

The purified bacterium was then inoculated in the brain heart infusion broth for 24-48 hrs at 37ºC under shaking conditions (300 rpm). After the incubation period, the cell-free supernatant was kept on ice and ethanol and centrifuged. Ultimately, the pellet solved in water, named the microsurf, was evaluated for surface activity and characterization of the biosurfactant.

Drop-Collapse Test

In this step, the cell-free broth (10 ml) was added to the center of one oil (namely, paraffin) drop in a clean glass Petri dish to achieve the drop collapse. The collapse was then detected in a shorter time with the higher activity of the surfactant [13].

Oil Displacement Process

For this purpose, 50 ml distilled water was added into a clean Petri dish, then 50 µL of different oils, including paraffin, olive, and coconut, was added to the water surface and 20 µL of the cell-free broth

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supernatant was added to the center of the oils, wherein the size of the resultant oil-displaced circle area reflected the activity of the surfactant. Distilled water and polysorbate 80 (Tween) 80 were also used as negative and positive controls [14].

Tensiometer Measurements

For tensiometer measurements, 10 ml of the cell-free broth was added into a clean glass beaker and placed on a tensiometer platform (Sigma 703D, KSV Finland). The surface tension measurement was then determined using the Du Noüy ring method via interfacial tensiometer at room temperature [15].

Hemolysis

The next step to confirm the biosurfactant-producing bacterium was hemolysis [16]. For this purpose, the isolated and purified colony was inoculated on the BA at 37ºC for 24 hrs.

Evaluation of E24 Index and its Stability

To this end, 1 ml of the cell-free broth was added to 1 ml of different types of hydrocarbons (viz. paraffin, olive, and coconut oils) and vortexed for 30 sec to access the E24. The tubes were also kept at room temperature for 24 hrs. Afterwards, the E24 was measured using the following equation.

$$E24 = \frac{\text{Height of the emulsion layer (mm)}}{\text{Overall height of the mixture (mm)}} \times 100$$

The percentage of the total height occupied by the emulsion was expressed as the emulsifying activity. For the stability of emulsification, the emulsified product was kept at room temperature for 0-100 days and phase separation was checked [17]. The bacterium with a displacement of all types of oils and higher E24 were ultimately selected for subsequent experiments.

Characterization of Biosurfactants

The extracted supernatants were analyzed using the thin-layer chromatography (TLC) and the gas chromatography/mass spectrometry (GC-MS) with three replicates. [18]. For the TLC, a spot of the crude biosurfactant was plated on the silica gel (F254) plate and separated on the plate with chloroform: methanol: water (20:10:0.5). Ninhydrin reagent was then sprayed to detect lipopeptide biosurfactants as red spots and anthrone reagent was used to find glycolipid biosurfactants as yellow spots [19]. GC (Agilent Technologies 7890B, USA) coupled with MS (Agilent Technologies 5977A, USA) was further practiced. The injector and detector temperatures were both 280ºC. The oven temperature was also programmed to start at 60ºC and the oven was heated to 210ºC (rate: 3ºC/min). Then, the temperature increased to 240ºC (rating: 20ºC/min) and kept constant for 8.5 min. As well, 1 µL of the sample was injected into the GC/MS in the split mode (split ratio: 20/1). The carrier gas was helium with a flow rate of 20 ml/min and the GC/MSD ChemStation (G1701EA, E.02.01.1177 USA) software was applied to analyze mass spectra and chromatograms.

Identification of Biosurfactant-Producing Bacterium

The identification of the isolates was evaluated using biochemical as well as molecular techniques with three replicates.

Biochemical Tests

The purified bacterium was characterized based on morphological, physiological, and biochemical properties [20]. The biochemical tests were fulfilled in three replicates with positive and negative controls. The applying tests included Gram staining, catalase test, oxidase test, citrate test, methyl red/voges-proskauer (MR/VP), sulfur, indole, motility (SIM) test; nitrate reduction reaction, gelatin hydrolysis, 6.5% salt tolerance test, rapid urease test (RUT), consumption of glucose and arabinose, malonate test, arginine dihydrolase test, principle of phenylalanine deaminase (PAD) test, bile esculin agar, oxidative/fermentative (O/F) test, ornithine decarboxylase test, and lysine decarboxylation test.

Molecular Test

For the phylogenetic analysis of gaining gene sequence based on 16S ribosomal ribonucleic acid (16S rRNA), the isolation was evaluated using GenBank and the Basic Local Alignment Search Tool (BLAST) [21]. For this purpose, a designed biosurfactant primer from the National Center for Biotechnology Information (NCBI) by the AllelID software and the 16S rRNA gene primer from the reference was used for the polymerase chain reaction (PCR) [22] (Table 1). The isolation of the bacterial deoxyribonucleic acid (DNA) from the sample was performed using a DNA extraction kit (Yekta Tajhiz Azma, Iran) through a columnar method. The total volume amounts for the PCR were 25 µL including PCR master mix (Taq polymerase, deoxynucleoside triphosphate [dNTP], magnesium chloride [$MgCl_2$], and buffer) in a total volume of 12.5 µL, 3 µL genomic DNA, 1 µL primer, and 7.5 µL distilled water. Then, the PCR was completed according to the following program: 95ºC for 5 min, 30 cycles of 95ºC for 20 sec, 58.4ºC for 15 sec, 72ºC for 15 sec, and a final extension at 72ºC for 2 min. The PCR was according to the following program for 16S rRNA: 95ºC for 5 min, 30 cycles of 95ºC for 30 sec, 58ºC for 20 sec, 72ºC for 30 sec, and a final extension at 72ºC for 4 min. Subsequently, the

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Table 1. Primers used for PCR in this study.

Genes	Primers	Sequence (5' to 3')	PCR product length
Biosurfactant	F	GTCGTTTCGCCCTCTCCAT	520bp
	R	CCAACCTTCCGCATCTCGTA	
16S rRNA	F	TACGGTTACCTTGTTACGACTT	1506bp
	R	AGAGTTTGATCCTGGCTCAG	

PCR products were analyzed by electrophoresis in 2% agarose gel for 45 min at 80 voltages. The data obtained from the PCR were utilized to draw the phylogenetic tree based on the unweighted pair group method with arithmetic mean (UPGMA).

Effect of Isolated Biosurfactant on *T. castaneum*

The flour beetles, *T. castaneum*, were obtained from the entomology laboratory of the Faculty of Agriculture, Islamic Azad University (Shiraz Branch), Shiraz, Iran. The samples were then placed in a dark room for a month at the temperature between 27-28ºC to feed the beetles with flour and to increase the number of the larvae. Different concentrations of the crude biosurfactants extracted from the selected bacterium (namely, 50, 100, 200, 400, 800, 1000, 1600, 2000, 5000, and 10000 µg/g) were also prepared and their effects on the third instar-larvae of *T. castaneum*, all in the same age and size, were investigated. Then, ten larvae were transferred to a sterile Petri dish containing 0.5 g of wheat flour impregnated with biosurfactant and kept at 28ºC. Besides, a sterile Petri dish containing 10 larvae with wheat flour and without biosurfactant was considered as a negative control. Three replicate experiments were further performed and mortality rate was recorded within nine days [9]. The mortality rate was calculated according to the Abbott's formula.

$$\text{Abbott's corrected mortality} = (\%\ \text{mortality in treatment} - \%\ \text{mortality in control}) / (100 - \%\ \text{mortality in control}) \times 100$$

Finally, the effect of the extracted biosurfactants on the larvae was determined by the Statistical Analysis System (SAS) and the LD50 was analyzed through Probit Analysis-MSChart software [23].

Results and Discussion

Isolation, Screening, and Evaluation of Biosurfactant Production

In this study, nine different types of colonies based on color, shape, and viscosity were isolated on the BA medium. Out of all, seven cases were Gram-positive and the other isolates were Gram-negative. Indeed, the petrochemical wastes and the petroleum-contaminated soils had led the bacteria to produce biosurfactants. In the presence of hydrocarbon pollution, the autochthonous bacteria in soil could adapt themselves in the same area using hydrocarbons as substrates for producing biosurfactants [24]. On the other hand, low solubility limits and their availability for decomposition by the bacteria induced them to produce biosurfactants [25, 26].

Drop-Collapse Test

The F3 isolate dispersed paraffin in a short time (3 sec), while the F2 isolated paraffin totally in a longer time (7 sec) and both were shorter than the F1 (11 sec). In this respect, Swaathy et al. had reported that four bacteria among five isolated cases from marine samples (namely, *Bacillus licheniformis* [*B.licheniformis*] ATCC 14580, *B. licheniformis* DSM13, *B.* spp. JS chromosome, and *B. subtilis* BEST 7613) had excellent oil displacement although time had not been stated [27].

Oil Displacement Process

Among all isolates, the F3 had a displacement of all oil types, while, the F1 and F2 only affected the paraffin. In this regard, Ekprasert et al. had found that *Enterobacter cloacae* isolated from petroleum-contaminated soil could produce biosurfactants with 42.2% E24 [28], lower than that for the isolate in the present study. In addition, Patel et al. had isolated *Staphylococcus epidermidis* KX781317 from oil-contaminated sites with different aspects [29].

Tensiometer Measurements

The surface tension for the isolates (viz. F1, F2, F3) and the distilled water as the control was 49.11, 52.96, 49.91, and 70 mN/m, respectively. The *Staphylococcus* sp. strain 1E isolated from crude oil-contaminated soil also had the surface tension equal to 25.9 mN/m [30].

Hemolysis

The hemolysis results showed ß hemolysis for the F1 and γ hemolysis for the F2 and F3 isolates.

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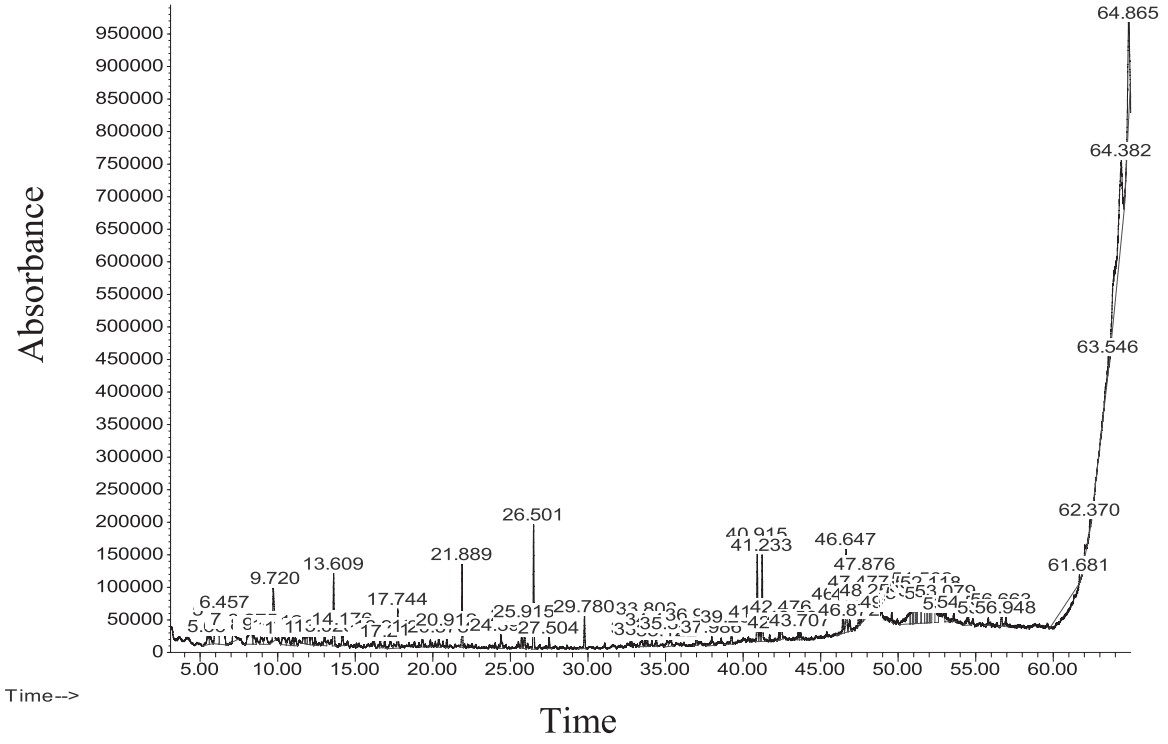

Fig. 1. GC mass analysis for F3 isolate.

As one of the main tests for the evaluation of biosurfactant production, hemolysis was reported for the first time for biosurfactants produced by *B. subtilis* [31]. Afterwards, scientists acknowledged that the given procedure could not be a significant test for screening biosurfactant-producing bacteria [32]. Therefore, surface activity test was utilized to confirm the isolates.

Evaluation of E24 Index and Its Stability

The E24 for the F1, F2 and F3 isolates was 10%, 21%, and 53%, respectively. To evaluate the emulsifying activity, the emulsification did not show any coagulation for the F3 isolate, while the F1 and F2 had different appearances. Hence, the F3 was selected and used for subsequent experiments because of its best oil displacement results and E24. The emulsifying activity of *Staphylococcus* sp. strain 1E biosurfactant against crude oil and olive oil had been also 98.9% and 42.48%, respectively [30].

Characterization of Biosurfactant

The presence of pink color in the TLC plate indicated lipopeptide biosurfactant. The GC-MS of

Table 2. Chemical compounds detected by GC-MS for F3.

Compound no.	RT (min)	Hit name	Mol weight (amu)	% Area
1	9.719	Undecane	156.188	2.884
2	46.646	11-octadecenoic acid, methyl ester	296.272	2.971
3	46.646	9-octadecenoic acid, methyl ester	296.272	2.971
4	47.475	Octadecanoic acid, methyl ester	298.287	1.086
5	47.876	9,12-octadecadienoic acid	280.24	4.357
6	41.233	Hexadecanoic acid, methyl ester	270.256	2.969
7	40.918	7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	276.173	3.139
8	36.992	Octadecane	254.297	0.646
9	34.403	Tetradecanoic acid, methyl ester	242.225	0.581
10	29.782	Hexadecane	226.266	1.156

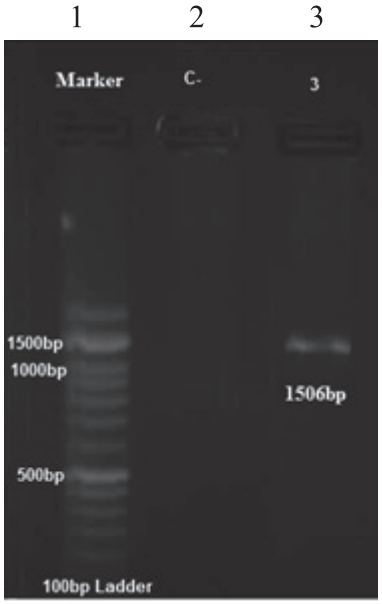

Fig. 2. PCR products by 2% agarose gel electrophoresis Lane 1: 100 bp DNA ladder marker, Lane 2: control, and Lane 3: F3 isolate.

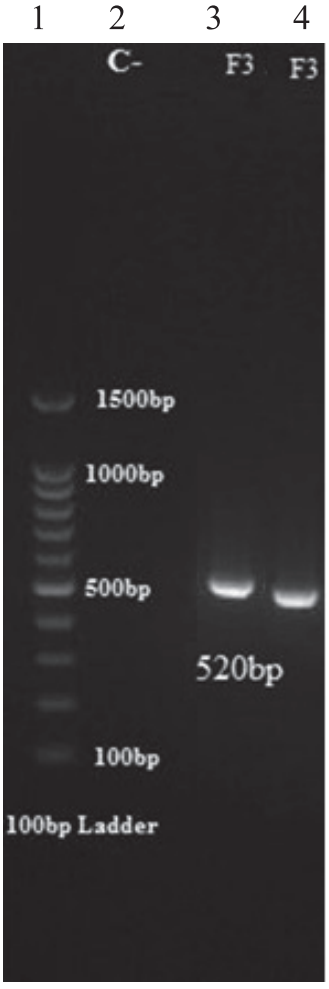

Fig. 3. PCR amplification of *srf* gene Lane 1: 100 bp DNA ladder marker, Lane 2: control, Lane 3: bacterial sample for F3 isolate, and Lane 4: PCR repeat for F3 isolate (F3 is *S.epidermidis*).

Table 3. Analysis of variance for mortality rate of *T. castaneum* larvae by biosurfactant extracted from *S. epidermidis* on different days after treatment

S.O.V	Degree of freedom	Mean square	F-statistic	Pr>F
Day (A)	4	10446.67	101.75	<0.0001**
Concentration (B)	9	422.22	4.11	0.0002**
A × B	36	96.67	0.94	0.5690ns
Error	100	102.67	-	-
C.V (%)	-	19.99		

the biosurfactant-producing bacterium also showed different peaks at various retention times (Fig. 1). The main one was thus identified from the standard library compounds and recorded (Table 2). In this line, Fazaeli et al. had further isolated two Gram-positive bacteria from Meighan wetland, Iran, with the capability to produce lipopeptide biosurfactant, using the TLC to recognize the type of the biosurfactant [14].

Bacterium Identification via Biochemical and Molecular Techniques

The selected bacterium (i.e. the F3 isolate) was Gram-positive cocci, catalase positive, and oxidase negative. It was also negative for MR, SIM, citrate, mannitol salt agar (MSA), malonate, arginine dihydrolase, PAD, bile esculin, and gelatin hydrolysis; in contrast, positive for VP, LD, OD, O/F, arabinose, urease, growth on salt 6.5%, and nitrate reduction.

The results obtained from the biochemical tests illustrated that the isolation belonged to the genus *Staphylococcus*, confirmed by the molecular techniques using 16S rRNA (Fig. 2). The presence of the *srf* gene in the isolation was also established through the PCR (Fig. 3). Besides, the analysis of the sequences based on the *srf* gene via the NCBI demonstrated the F3 isolate as *S. epidermidis* (Accession No: KP728026.1, homology: 94%). The phylogenetic analysis for the bacterium was inferred using the MEGA X software (Fig. 4).

Effect of Isolated Biosurfactant on *T. castaneum*

The results were evaluated on the first, third, fifth, seventh, and ninth days. The variance and mean larval mortality calculated using the SAS software is shown in Tables 3 and 4, respectively. A comparison between the effects of the biosurfactant on larval mortality demonstrated that the concentration of 10000 µg/g on a ninth day had the highest mortality (63.33%) on the larvae and the LD50 equal to 25001.7 µg/g was calculated for *S. epidermidis* on a ninth day by the Probit

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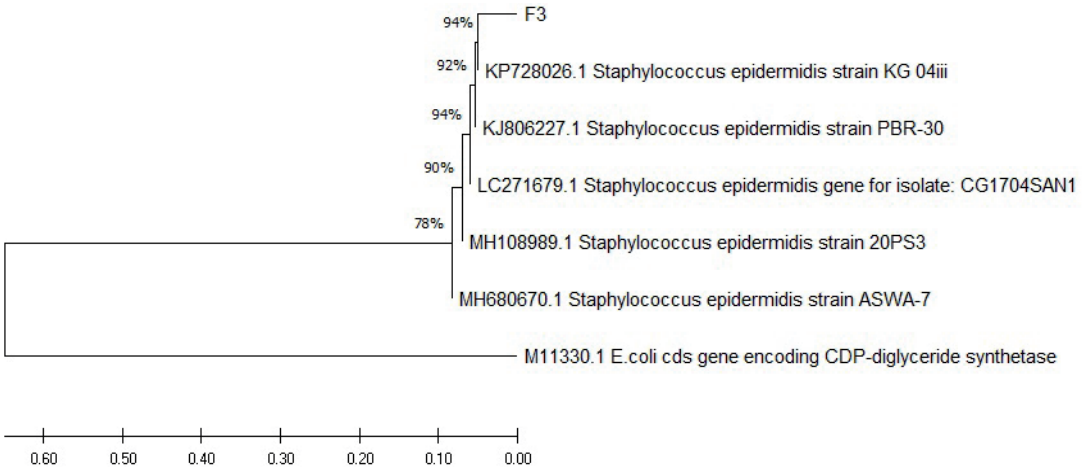

Fig. 4. Phylogenetic analysis for F3 isolate (*S. epidermidis* (Accession No: KP728026.1, homology: 94%)). The evolutionary history was inferred using the UPGMA. The optimal tree with the sum of branch length = 0.02545270 is also shown. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is illustrated next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. Evolutionary analyses were also conducted on the MEGA X software.

Analysis-MSChart software. In 2012, Kamal et al. had worked on filter papers impregnated with rhamnolipids extracted from *Pseudomonas* sp. ICTB-745 strains, reporting that it had no insecticidal effects on *T. castaneum*, probably due to the presence of waxes on the surface of the beetle cuticle, inhibiting the penetration of the biosurfactant [33]. While, other scientists had shown that, *B. subtilis* SPB1 had insecticidal activity against *Ectomyelois ceratoniae* and *Ephestia kuenella* larvae by lipopeptide biosurfactant [34, 35]. Furthermore, ochrosin extracted from *Ochrobactrum* sp. strain BS-206 had exhibited insecticidal activity against the adults of three major stored-product grain pests like *T. castaneum*, *Callosobruchus chinensis*, and *Sitophilus oryzae* with LC50 values of 7.3, 8.9, and 7.6 µg/cc, respectively [36]. Besides, other researchers had established that *B. amyloliquefaciens* strain AG1 could produce lipopeptide and polyketide biosurfactants, showing the LC50 of about 180 ng/cm towards *Tuta absoluta* larvae. They had also found severe injuries of the midgut tissues including the separation and disintegration of the epithelial layer and the cellular vacuolization, which could be due to the biosurfactant [37]. Indeed, lipopeptide biosurfactants are well known for its ability to disrupt the physical characteristics of cell membranes and to cause metabolism disruption.

Table 4. Mean mortality rate (M±SE) of *T. castaneum* larvae by biosurfactant extracted from *S. epidermidis*.

Day / Concentration (µg/g)	Mortality rate				
	1 day	3 days	5 days	7 days	9 days
50	6.67±3.33 i-k	6.67±3.33 i-k	16.67±6.67 f-k	26.67±3.33 d-i	33.33±3.33 c-g
100	6.67±3.33 i-k	10.00±5.77 h-k	30.00±5.77 d-h	33.33±3.33 c-g	40.00±10.0 b-e
200	3.33±3.33 jk	10.00±5.77 h-k	23.33±3.33 e-j	36.67±3.33 c-f	50.00±10.0 a-c
400	3.33±3.33 jk	13.33±3.33 g-k	26.67±3.33 d-i	36.67±3.33 c-f	46.67±8.81 a-d
800	13.33±3.33 g-k	13.33±6.67 g-k	33.33±6.67 c-g	46.67±3.33 a-d	56.67±3.33 ab
1000	3.33±3.33 jk	13.33±3.33 g-k	20.00±10.0 e-k	33.33±6.67 c-g	56.67±8.81 ab
1600	0.0±0.0 k	3.33±3.33 jk	10.00±5.77 h-k	26.67±3.33 d-i	56.67±6.66 ab
2000	3.33±3.33 jk	6.67±3.33 i-k	20.00±5.77 e-k	36.67±3.33 c-f	61.33±6.66 a
5000	6.67±6.67 i-k	6.67±6.67 i-k	16.67±6.67 f-k	23.33±3.33 e-j	60.00±5.77 a
10000	20.00±10.0 e-k	23.33±8.81 e-j	30.00±10.0 d-h	40.00±5.77 b-e	63.33±6.66 a

The means with the same letters in each column and row do not show a significant difference in the Duncan's new multiple range test at 1% probability level.

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They can also modify membrane permeability by channel formation or membrane solubilization through their insertion into lipid bilayers due to hydrophobic interactions [35].

Conclusion

In this study, Gram-positive cocci were isolated from petrochemical wastes that are best for the E24 and the oil displacement process. This bacterium could produce lipopeptide biosurfactant and then the presence of the *srf* gene was illustrated via the PCR. Based on chemical tests and phylogenetic analysis, it belonged to *S. epidermidis*. According to the results obtained from the effects of the biosurfactant on *T. castaneum*, it was confirmed that the concentration of 10000 µg/g on the ninth day of treatment had the highest mortality of 47.56% with the LD50 concentration 25001.7 µg/g on the ninth day.

The results of this experiment also showed that the lipopeptide biosurfactant produced by the *S. epidermidis* was endowed with an acceptable ability to control this resistant pest and could be used in further research on the larvae of other insects. Accordingly, it is suggested to reflect on their effects on pests in more detail in other studies. Furthermore, their gastrointestinal and contact effects on other pests should be delineated.

Acknowledgments

The authors hereby would like to extend their gratitude to the Research Council of Islamic Azad University (Shiraz Branch), Shiraz, Iran, for the confirmation of this project as a Doctoral Thesis in Microbiology (Thesis Code No: 16330507952022).

Conflict of Interest

The authors declare no conflict of interest.

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