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Microbial Production of Biosurfactants from Some El-Korma Governorate Microbial Isolates for Bioremediation of Crude Oil Spills in the Different Environments

Bayoumi,R.A.[1*],Atta,H.A.[1],El-Sehrawey[2],M.A.,Selim,S.M[1].

[1] Biotechnology Dept., Faculty of Science and Education , Taif Univ. (Khorma Branch), KSA.
[2]Biology Dept., Faculty of Science, Taif Univ., KSA.

ABSTRACT

Biosurfactants are surface –active amphipathic molecules produced by a plethora of microorganisms. The aim of the present study was production of biosurfactants from some bacterial isolates for bioremediation of crude oil spills. Twenty five bacterial isolates were isolated from twenty crude oil contaminated soils located in repairing cars stations in Khorma governorate,Taif, Kingdom of Saudi Arabia (KSA). Ten pure bacterial isolates were screened qualitatively and quantitatively for the ability to surface active compounds (SACs) biosynthesis in liquid culture media containing crude oil as sole carbon and energy source by blood agar lysis and oil spread techniques. The most potent bacterial isolate was selected and displayed highest reduction of surface tension by using surface tension measurement. The selected bacterial isolate was identified as ***Bacillus subtilis*-KG82-KSA** based on the morphological, physiological, biochemical characteristics and 16S rRNA gene sequencing analysis. Maximum SACs biosynthesis by ***B. subtilis*-KG82-KSA** strain respectively under study could be obtained using crude oil in concentrations of 0.5 (mg/100ml); initial pH values,7; sodium chloride concentrations, 1 (%);carbon sources, sucrose; best carbon source concentrations,2.5 (mg/100ml);nitrogen sources, ammonium ferrous sulphate ; best nitrogen concentrations, 0.133 (mg/100ml), vitamin sources, thiamine HCl; best vitamin concentrations, 200 (ppm) under shaking conditions of 100 rpm, incubation temperature and incubation period are at 40ºC for 7 days. The vegetable oils proved to increase SACs biosynthesis by ***B. subtilis*-KG82-KSA** bacterial strain. The bacterial strain showed high salt tolerance and their successful biosynthesis of SAC in a vast pH and temperature domain after only seven days and reduce surface tension. SAC could be biosynthesized from ***B. subtilis*-KG82-KSA** which could be extracted by chloroform: ethanol (2:1v/v) solvent and purified by using TLC and Silica gel chromatography. The SAC were identified as Iturin based on UV, IR and HPLC analysis techniques. Detection the qualitative and quantitative of amino acids were performed by amino acids analyzer, $^{-1}$H-Nuclear Magnetic Resonance (NMR) analysis, fatty acid analysis and sugar pattern. These results indicated that biosurfactant iturin has a strong potential to be applied as a remediation agent for the clean –up of oil spills in sea and soil positive effects on the bioremediation of crude oil spills.
KEY WORDS: Biosurfactants, Production, Purification, Identification, Iturin, *Bacillus subtilis*, Khorma governorate, Oil spill.

INTRODUCTION

Biosurfactants are surface active substances derived from living organisms, mainly from microbial origin with considerable potential in commercial applications in the food, agrochemical, cosmetic and pharmaceutical industries **(Morikawa *et al.*, 2000)**. They have a wide structural diversity, ranging from glycolipids, lipopeptides, and lipoproteins to fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants. This endows them with their unique properties, including better environmental compatibility, greater foaming properties, higher selectivity and biodegradability **(Das *et al.*, 2008; Saeki *et al.*, 2009; Nayak *et al.*, 2009)** in comparison to chemical surfactants. Microbial biosurfactants have advantages over chemical surfactants in biodegradability, ecological safety, low toxicity and structural diversity. Surface active compounds produced by microorganisms are of two main types, those that reduce surface tension at the air–water interface (biosurfactants) and those that reduce the interfacial tension

***Corresponding Author:** Prof.Dr. Reda Ahmed Bayoumi, Biotechnol. Dept., Faculty of Science (Khorma branch), Taif, Taif Univ. KSA. redaelbayoumi@yahoo.com

between immiscible liquids, or at the solid–liquid interface (bioemulsifiers). Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension **(Karanth *et al.,* 1999).**

Microorganisms capable of emulsifying and solubilizing hydrophobic contaminants *in situ* may have a distinct advantage over competitors in contaminated areas and therefore isolation from such areas and samples from such sites are often rich in microorganisms with desired characteristics for both in situ and ex situ bioremediation processes **(Cassidy and Hudak, 2001)**. An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase **(Desai and Banat, 1997).** Emulsions are commonly observed when liquid hydrocarbons and water are mixed during bioremediation or fermentation **(Atlas and Bartha, 1992).** These emulsions dramatically increase the area of the oil-water interface, thereby enhancing bioavailability. Formation of oil-water emulsions during bacterial growth on hydrocarbons is often attributed to biosurfactants **(Loredana *et al.,* 2004;Anyanwu *et al.*,2011; Panesar *et al.*, 2011; Parwa-Plociniczak *et al.*, 2011; Makkar *et al.*, 2011; Praveesh *et al.*, 2011; Vyas and Dave,2011 and Xu *et al.*, 2011)**

The bioremediation of soil contaminated with aromatic hydrocarbons and fossil fuel is limited by the poor availability of these hydrophobic contaminants to microorganisms. Surfactants can help to release hydrocarbons sorbed to soil organic matter by solubilization or emulsification and increase the aquatic concentrations of hydrophobic compounds, resulting in higher mass transfer rates **(Nereus *et al.*, 2005)**. Recent studies indicate that biosurfactants can enhance hydrocarbons biodegradation by increasing microbial accessibility to insoluble substrates. Biosurfactants have been tested in environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery and transfer of crude oil (**Das *et al.*, 2008; Saeki *et al.*, 2009; Nayak *et al.*, 2009)**.

Bacillus subtilis strains produce a broad spectrum of bioactive peptides with great potential for biotechnological and biopharmaceutical applications. A well-known class of such compounds includes the lipopeptide biosurfactants such as surfactin, fengycin and the iturin compounds (iturins, mycosubtilins, and bacillomycins), which are amphiphilic membrane active biosurfactants and peptide antibiotics with potent antimicrobial activities. All these agents occur as families of closely related isoforms which differ in the length and branching of the fatty acid side chains and in the amino acid substitutions in the peptide rings. The surfactin and iturin compounds are cyclic lipoheptapeptides which contain a β-hydroxy fatty acid and a β-amino fatty acid, respectively, as lipophilic components. Fengycin is a lipodecapeptide with a β-hydroxy fatty acid in its side chain. **(Joachim *et al.,* 2002)**.

The present investigation was carried out on the isolation and characterization of biosurfactant producing *B.subtilus*-KG82-KSA. Further, the isolation and characterization of biosurfactants and its applications in the bioremediation of crude oil were studied.

MATERIALS AND METHODS

1- Soil samples collection: Twenty crude oil polluted soil samples were collected from different crude oil polluted localities from repairing cars stations located in Khorma governorate, Taif, KSA. Soil samples were transported to the laboratory in ice tank to complete the crude oil utilizing heterotrophic microbial isolation and stored at 4°C immediately. Each soil sample (1 g) was added into 100 ml of minimal medium (MM) or minimal salts medium (MSM) media supplemented with crude oil (2g /L) and cultivated at 37 °C for five days on rotary shaker at 180 rpm. The cultures were then transferred to MM and MSM fresh medium containing crude oil and incubated under the same conditions. The purity of the culture was confirmed by plating of LB agar.

2- Culture medium and growth conditions: Two media were used to biosurfactant biosynthesizing. First medium was minimal medium (MM) was prepared according to **Abu-Ruwaida *et al.* (1991)**. The second medium minimal salts medium (MSM) was prepared according to **Kästenar *et al.* (1994)**. MM used for isolation and cultivation of biosurfactant biosynthesizing bacteria. The composition of this medium was (g/l): Na_2HPO_4, 2.2;KH_2PO_4, 1.4; $MgSO_4 \cdot 7H_2O$, 0.6;$(NH_4)_2SO_4$, 3; yeast extract,1; $CaCl_2 \cdot 7H_2O$, 0.02; NaCl,0.05; $FeSO_4 \cdot 7H_2O$, 0.01; 1ml of trace elements (solution contained (mg/100ml) of distilled water was: $CuSO_4.5H_2O$, 0.5;H_3BO_3, 1.0; $MnSO_4.5H_2O$, 1.0; $ZnSO_4$, 0.7 and MoO_3, 1.0) and 2% (v/v) crude oil was used as the sole carbon source (Gulf Petroleum Co.). pH was adjusted to 7.0 $\pm$ 0.2, distributed and sterilized at 121°C for 15 min. Ten grams of mixed soil samples were inoculated aerobically in 100ml of

medium at 30°C for 7 days without shaking. After incubation the growth was took for spried on nutrient agar (NA) and Tryptic soy agar (TSA) media.

Biosurfactant biosynthesizing bacterial isolates were isolated from soil samples by plate culture technique on minimal salts medium (MSM). MSM contained (g/l) of distilled water: Na_2HPO_4, 2.13; KH_2PO_4, 1.3; NH_4Cl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; & 0.5% (v/v) crude oil was used as the sole carbon source (Gulf Petroleum Co.). pH was adjusted to 7.0 $\pm$ 0.2, distributed and sterilized at 121°C for 15 min. MSM was inoculated with one gram of soil sample and incubated at 30°C for 7 days. After incubation the bacterial growth was took for spried on three plates of each nutrient agar (NA) and Tryptic soy agar (TSA) media (**Kästenar *et al.* 1994**).

3- Screening of biosurfactant biosynthesizing bacteria isolates: The first method, which was used to screen biosurfactant biosynthesizing isolates, was blood agar lysis (Hemolytic activity) according to **Palmisano *et al.* (2001). Carrillo *et al.* (1996)** were recommended to use the hemolytic activity as a primary screening of biosurfactant activity. The second method, which was used to screen biosurfactant biosynthesizing isolates, was oil spread technique (OST) according to **Youssef *et al.* (2004).**

a- **Hemolytic activity (HA) Palmisano *et al.* (2001):** The biosurfactant biosynthesis isolates were screened on blood agar plates containing 5% (v/v) blood and inoculated with bacterial isolates at room temperature for 24-48hr under aseptic conditions. Hemolytic activity was detected as the presence of a clear zone around a colony. The plates were visually inspected for zones of clearing (mm) around the colonies, indicative of biosurfactant biosynthesis. The diameter of the clear zones depends on the concentration of the biosurfactant **(Mulligan *et al.*, 1984)**. Five plates for each isolate were inoculated and clear zones in several different areas of each plate were analyzed.

b- **Oil spread technique (OST) Youssef *et al.* (2004):** Fifty ml of distilled water was added to a large Petri-dish (25cm diameter) followed by addition of 20µl of crude oil to the surface of the water. Ten microliters of culture filtrates was put on the crude oil surface. The diameter of the clear zone (mm) on the oil surface was measured and related to the concentration of biosurfactant.

4- Identification of the bacterial isolates: Biosurfactant producing bacterium was identified on the basis of morphological, physiological and the biochemical characteristics according to Bergey's Manual of Determinative Bacteriology **(Hensyl *et al.*, 1994)** and 16 S rRNA gene sequencing. Surface active biosynthesizing bacterial colonies were obtained from TSA and suspended in nuclease-free water. DNA was extracted from **82KG-KSA** isolate and purified and 16S rRNA gene amplified and sequenced by following a standard procedure according to **Yoon *et al.* (1998)**. A representative consensus sequence was compared to known sequences using the BLAST program **(Altschul *et al.*, 1990)**. Finally, the 16S rRNA gene nucleotide sequence of each unique isolate was submitted to the National Center for Biotechnology Information (NCBI) database and assigned an accession number construction of the phylogenetic tree. Sequences representing a broad range of biosurfactant-biosynthesizing microorganisms from this study and from the literature were retrieved from the NCBI 16S rRNA gene database and downloaded in FASTA format. Sequences were aligned using ClustalX version 1.81 **(Thompson *et al.*, 1994)**.

5- Construction of a standard curve for assaying the biosurfactants activities by using the oil spreading technique and hemolytic activity:

a- Oil spreading technique: According to **Morikawa *et al.* (2000),** the activity of biosurfactants were estimated in term of mean diameters of clearing zones (mm) using a standard curve constructed for such a purpose and using the oil spreading technique (OST) mm to detect the biosurfactant biosynthesis by diverse bacterial isolates. Screening the biosurfactant was determined by drawing the relationship between the diameter of clearing zone obtained by the OST (mm) and the concentrations of surfactant standard (g/l). The diameter of clearing zone linearly was increased with the concentration of standard surfactant range of 0.2 to 2.4 (g/l) by using OST.

b- Hemolytic activity: According to **Yonebayashi *et al.* (2000),** the activity of biosurfactants were estimated in term of mean diameters of clearing zones (mm) using a standard curve constructed for such a purpose and using the HA to detect the biosurfactant biosynthesis by diverse bacterial isolates. Screening the biosurfactant was determined by drawing the relationship between the diameters of oil clearing zone (mm) and the standard surfactant concentrations (g/l). The diameter of clearing zone linearly was increased with the concentration of standard surfactant range of 0.2 to 2.4 (g/l) by using HA. The concentrations of surface active compounds can then be determined using the HA and OST techniques.

6- Surface tension measurements according to Willumsen and Karlson, (1997): A freshly prepared solution of cell-free supernatant at concentration of 0.01gm/10ml was prepared in distilled water and the surface tension value was measured. The average of three results from same culture was measured by using Kruss Digital-Tensiometer 10, Hamburg Germany with platinum ring at 25°C temperature overlaid, at Egyptian Petroleum Research Institute (EPRI).

7- Preliminary surface active compound (SAC) biosynthesis studies: The surface active compound (SACs) which biosynthesized from one selected bacterial **KG82-KSA** isolate were choice as best screened isolate. To obtaining the highest yield from grown on crude oil as carbon source, that required studied the optimum conditions for SAC stimulating biosynthesis.

A- Environmental characteristics: The most potent bacterial isolate was allowed to grow on minimal salts broth medium supplied with crude oil as only of carbon and energy sources and incubated at 40°C for 2, 4, 6, 7, 8, 10, 12, 14 and 16 days, difference incubation temperatures via 5, 10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55 and 60°C, different pH values ranging from 5.6 - 8.0 viz 5.6, 6.0, 6.5, 7.0, 7.5 and 8.0. The inoculated flasks were incubated under shaking and also at static conditions. At the end of incubation period the SACs were assayed by using HA or OST.

B- Nutritional characteristics: Effect of different substrate (crude oil) concentration; sodium chloride concentrations; carbon sources; carbon source concentrations; nitrogen sources; concentration nitrogen source concentrations; vitamin requirements; vitamin concentrations requirement; vegetable oil sources; protein sources; $FeSO_4 \cdot 7H_2O$ and $MnSO_4.5H_2O$ concentrations and cheap media substrate were studied for SAC biosynthesis:

8- Biosurfactant biosynthesis, extraction and purification:

After determination of the optimum conditions from the most potent bacterial isolate. The following step of SACs purification of **KG82-KSA** isolate was performed. The purification processes were started with isolation and removal the cells from the broth culture by filtration and centrifugation at 8000 rpm for 15 min. SACs were then isolated from the clear broth extracted by chloroform and methanol (2:1). The lower layer was concentrated by evaporation in a rotary evaporator at 35°C and collected in 5 ml of a mixture of chloroform and methanol (2:1) for preparation at another analysis. The biosurfactant purification was following this sequence: Filtration and centrifugation, Solvent separation according to **Kim *et al.,* (2000)**, Evaporation and TLC purification according to **Sung-chyr *et al.,*(1994)**, Silica gel separation according to **Joseph *et al.,*(2006)**.

9- Biosurfactant identification: After the purification processes, the biosurfactant biosynthesized from **KG82-KSA** isolate was identified. We used several analyses for determination of the biosurfactant structure and confirmed the identification processes. TLC identification test. UV detection test. IR analysis according to **Sung-chyr *et al.,* (1994)**. HPLC analysis. Amino acid analysis according to **Noha *et al.,*(2005)**. $^{-1}$HNMR analysis according to **Zengguo *et al.,(*2007)**. Fatty acid analysis_according to **Jorge *et al.,*(2003)**. Sugar pattern analysis according to **(Toujima *et al.,* 2000)**.

RESULTS

Twenty contaminated soil samples were collected from different crude oil polluted soil from repairing cars stations in Khorma governorate, Taif, KSA. Twenty five selected distinct morphological bacterial colonies were isolated by using the initial screening on minimal media supplemented with crude oil as carbon and energy source according to **Abu-Ruwaida *et al.* (1991)** and minimal salts media according to **Kästenar *et al.* (1994)** and then tested qualitatively and quantitatively for surface active compounds (SACs) biosynthesis with the hemolytic activity (HA) according to **Palmisano *et al.* (2001)** and quantitatively by oil spread technique (OST) according to **Youssef *et al.* (2004,2007)**. Ten pure bacterial isolates were selected as best SAC producers. Five bacterial isolates were isolated from crude oil polluted soil samples and three isolates from treatment cars stations polluted soils samples. This distribution may represent the ability of the microorganisms to survive in these soils or may be a response to the type of the contaminant point. Eight pure bacterial isolates were selected as SAC bacterial producers by using HA and OST. Only one isolate was best SACs biosynthesizing, the screened isolate was KG82-KSA which displayed the highest lysed red blood cells in blood agar with scores ranging between (+++) corresponding to complete haemolysis with a diameter of lysis >10mm but < 30mm.

Identification of the bacterial isolates: The most purified SAC bacterial isolate was identified on the basis of morphological, physiological and biochemical characteristics were carried out according to Bergey's Manual of Determinative Bacteriology (**Hensyl *et al.,* 1994**). The phylogenetic technique was used for complete identification of selected isolate as highest reduction of surface tension isolate **KG82-KSA**. Cluster analysis of the 16S rRNA gene sequences of **KG82-KSA** bacterial isolate revealed 79% similarity to ***B. subtilis*-KG82-KSA**, which belong to bacterial division: ***Bacillaceae*** family.

Parameters controlling the biosynthesis of SACs by *B. subtilis*-KG82-KSA strain:

The identified bacterial strain ***B. subtilis*-KG82-KSA** proved to be the most potent SAC biosynthesized from soil samples polluted with crude oil. They were selected for investigate some parameters affecting the biosynthesis of surface active compounds from crude oil as sole carbon and energy sources.

A- Environmental characteristics:

a- Incubation period: The most potent SAC biosynthesizing ***B. subtilis*-KG82-KSA** strain was allowed to grow aerobically on minimal salts broth medium supplemented with crude oil. SAC was optimally biosynthesized within 7 days of incubation period.

b- Incubation temperature: The optimum incubation temperature for SAC biosynthesis by ***B. subtilis*-KG82-KSA** at 40°C, at this particular degree of temperature, the highest yield of SAC biosynthesis by HA was 1. 8 (g/l) and OST found to be 1.50 (g/l).

c- Initial pH value: The optimum pH value for SAC biosynthesis by ***B. subtilis*-KG82-KSA** was 7, at this pH value the highest yield of SAC reached up to 1.4 (g/l) by HA and 1.9 (g/l) by using OST.

d- Static and submerged condition: The incubation conditions were affected on SAC biosynthesis by ***B .subtilis*-KG82-KSA** bacterial stain. The maximum yield of SAC biosynthesis for ***B. subtilis*-KG82-KSA** strain was at shaking state. ***B. subtilis*-KG82-KSA** biosynthesized the maximum yield of SAC by HA was 1.8 (g/l) and by OST was 2.0 (g/l), which meaning that, the shaking was best condition than static state in all tested isolates.

B- Nutritional characteristics:

a- Different substrate (crude oil) concentrations: The optimum SAC biosynthesis by ***B. subtilis*-KG82-KSA** at 0.5 (mg/100ml) which yielded the maximum biosynthesis was detected by HA was 0.7 (g/l) and by OST was 0.8 (g/l).

b- Different sodium chloride concentrations: The optimum NaCl concentration 1.0% (w/v) for SAC biosynthesis by ***B. subtilis*-KG82-KSA** was 1.4 (g/l) by HA and 1.8 (g/l) by OST.

c-Different carbon sources and determination of best concentrations: ***B. subtilis*-KG82-KSA** could utilize sucrose, Glucose, Galactose, Lactose, Sorbitol, Cellulose, Maltose, Mannitol and Fructose. The best carbon source with was sucrose which reached to 1.0 (g/l) by HA and 1.2 (g/l) by OST. The maximum yield of SAC was appeared at 1.5 (mg/100ml) of sucrose concentration which reached to 1.5 (g/l) by HA and 1.8 (g/l) by OST.

d- Different nitrogen sources and determination of best concentrations: ***B. subtilis*-KG82-KSA** could utilize $NaNO_3$, $(NH_4)HSO_4$, NH_4Cl, $NaNO_2$, Amm.oxalate, $NH_4Fe(SO_4)_2.12H_2O$, NH_4NO_3, $(NH_4)_2HPO_4$ and peptone. The best nitrogen source of was $NH_4Fe(SO_4)_2.12H_2O$ which reached to 0.8 (g/l) by HA and 1.4 (g/l) by OST, while the best nitrogen concentration was 1.33 (mg/100ml) which reached to 1.8 (g/l) by HA and 2.0 (g/l) by OST.

e-Different vitamin requirements and determination of best concentrations: Effects of introducing seven different concentrations of different available five vitamins (folic acid, thiamin HCl, Pyrodxin HCl, Cyanocobalamine and riboflavin). ***B. subtilis*-KG82-KSA** synthesized 1.6 (g/l) SAC by HA and 2.0 (g/l) by OST with thiamin HCl at 200ppm it yields 2.9 (g/l) by HA and 2.0 (g/l) by OST.

f- Different vegetable oil sources: ***B. subtilis*-KG82-KSA** strain was gave the high yield of SAC by using the vegetable oil *Prunus amygdalus,* which gave 2.5 (g/l) by HA and 1.8 (g/l) by OST.

g- Different protein sources: ***B. subtilis*-KG82-KSA** strain was gave the high yield of SAC with gelatin which reached to 1.8 (g/l) by HA and 2.0 (g/l) by OST.

h- Different $FeSO_4 \cdot 7H_2O$ and $MnSO_4.5H_2O$ concentrations as growth factors on the SAC biosynthesis: The optimum $FeSO_4 \cdot 7H_2O$ concentration was 0.2 (g/l) in ***B. subtilis*-KG82-KSA** strain which reached to 1.8 (g/l) by HA and 1.9 (g/l) by OST. The optimum $MnSO_4.5H_2O$ concentration was 0.08 (g/l) for SAC synthesized by ***B. subtilis*-KG82-KSA** was 1.5 (g/l) by HA and 1.9 (g/l) by OST.

i- Different cheap media substrates: *B. subtilis*-KG82-KSA strain gave the high yield of SAC by using the cheap medium Rapeseed oil, which gave 1.25 (g/l) by HA and 1.3 (g/l) by OST.

C- Biosynthesis of surface active compound by *B. subtilis*-KG82-KSA from crude oil under all investigated optimal conditions:- The optimal conditions for biosynthesis the SAC by ***B. subtilis*-KG82-KSA** on crude oil as follows: - Incubation period (seven days), incubation temperature (40°C), initial pH value (7) and static and submerged condition (shaking state), while the different nutrient requirements included substrate concentration (0.5mg/100ml), NaCl concentration (1%). Concentration of different vitamins was thiamin HCl (200ppm), the carbon source requirement (2.5mg/100ml of sucrose) and the nitrogen source requirement 0.133 mg/100ml of $(NH_4)_2Fe(SO_4).12H_2O$. The protein source was gelatin, the concentration of $FeSO_4 \cdot 7H_2O$ was 0.01 (g/l) and $MnSO_4.5H_2O$ was 0.1 (g/l). The vegetable oil was *Prunus amygdalus*. The cheap medium for SAC was rapeseed oil. A summary of all data was recorded of the twelve parameters mentioned in table (1).

Purification and identification the surface active compound by using spectroscopical analysis:

The ***B. subtilis*-KG82-KSA** SAC was not detected after filtration and centrifugation by using HA and OST because the SAC has a low concentration. The supernatant of SACs was collected, separated and extracted by chloroform: ethanol solvent (2:1 v/v). The solvent was evaporated and concentrated, activities were measured by OST which gave 1.1 (g/l). The SAC activities was measured by HA to gave 1.0 (g/l). The separated SACs spots appeared on TLC plate were measured by using HA that gave 1.7 (g/l), while the OST was gave 1.8 (g/l). After separation of the SAC by using silica gel column chromatography, the high activity of SAC was synthetized by ***B. subtilis*-KG82-KSA** strain presented in fraction number 2 which gave 6.0 (g/l) by using the OST and 5.6(g/l) by using the HA.

1- TLC purification: TLC standard and extracted ***B. subtilis*-KG82-KSA** SAC spots were recorded by using visual estimation (CS 9000 -Shimadzu). The retention factor (Rf) was 90.04 mm for standard, 80.03, 124.4 and 127.3 mm. The concentration of standard was gave 98.539 %, while the ***B. subtilis*-KG82-KSA** SACs were separated mainly to three peaks that means the present of some substrates which separated to give 8.292, 45.088 and 27.763 %.

2- Silica gel column chromatography: The elution pattern of SACs biosynthesised by ***B. subtilis*-KG82-KSA** on silica gel chromatography revealed that SACs were detected only in fractions numbers (2-3), proteins and fats were detected. The fractions number (4-9, 13-16) were included some fatts without activity. The active fractions of each SACs include the total protein, glycerol, glucose and SACs activity measured by HA and OST.

The high activity of purified SACs in ***B. subtilis*-KG82-KSA** was presented in fraction number 2 which gave 6.0 (g/l) by using the OST and 5.6 (g/l) by using the HA. Results of this step were presented graphically in figures (1).

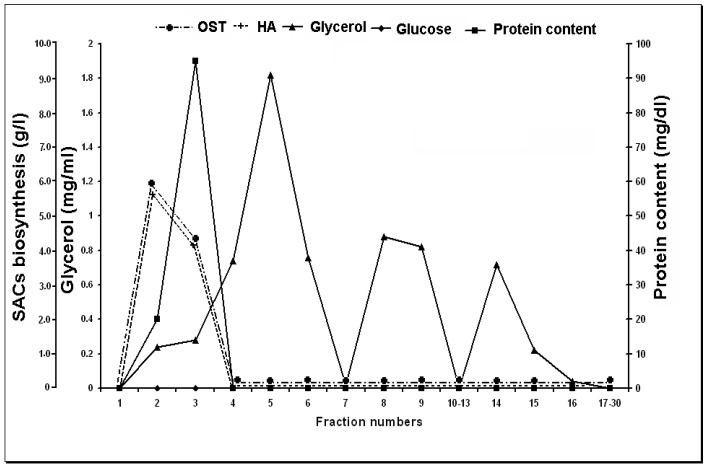

Figure (1): Fractionation pattern of the purified SAC biosynthesized by ***B. subtilis*-KG82-KSA** and extracted on silica gel column chromatography.

Table (1): Summary of the optimal conditions of SACs biosynthesis by *B. subtilis*-KG82-KSA allowed to growing on crude oil.

Culture conditions	Maximum yield of SACs
	Bacillus subtilis-KG82-KSA
Incubation period (day)	7
Incubation temperature (°C)	40
Initial pH value	7
Static and submerged condition	Shaking
Substrate concentration	0.5%
Different NaCl concentration	1%
Carbon source	Sucrose
Carbon source concentration (mg/100ml)	2..5
Nitrogen source	$NH_4Fe(SO_4)_2.12H_2O$
Nitrogen source concentration (mg/100ml)	0.133
Vitamin source (ppm)	Thiamin HCl
Vitamin concentration (ppm)	200
Protein source	Gelatin
Different of $FeSO_4 \cdot 7H_2O$ concentration (g/l)	0.01
Different of $MnSO_4.5H_2O$ concentration (g/l)	0..1
Different cheap media	Rapeseed oil
Vegetable oil source	*Prunus amygdalus*

II- Identification SAC sequence:

1- UV analysis: UV analysis spectroscopy was useful in interpretation the present synthesized SAC by ***B. subtilis*-KG82-KSA** strain purified by silica gel column. The reference solution (standard) gave a single peak and was determined at wave length 229.4 (nm), concentration equal 100% and absorption 1.1ג. The UV separated the purified SAC synthesized by ***B. subtilis*-KG82-KSA** to give a single peak at wave length 238.8 (nm) absorption read 1.049ג and the concentration 95.36% (Table (2).

Table (2): UV absorption spectra for the standard and purified SACs were biosynthesized by *B. subtilis*-KG82-KSA cultivated on crude oil.

UV test	Standard	*B. subtilis*-KG82-KSA
Wave length (nm)	229.4	238.8
Absorption (ג)	1.100	1.049
Concentration (%)	100	95.36
SACs activity (g/l)	1.0	0.95

2- Infrared (IR) spectrophotometers: The Infrared spectrums chart of the standard biosurfactant identifying their chemical groups: The 837.28 wave number was assignment and remarked to R-CH=C-R in tri-subst alkenes group. Also the 1458.61 wave number was assignment and remarked to CH_2 in aliphatic compounds. Also the secondary amides were observed at wave number 1127.05 and 1186.39 were assignment and remarked to –NHR. The 1510.49 wave number was benzene ring in aromatic compounds

assignment and remarked to ring stretch (shape band). The aliphatic chains were identified at 2926.47 wave number which assignment and remarked to CH stretch in C-CH_3 compounds and several bonds to indicate the methylene group. The wave number 1640.88 was assignment and remarked to C=O in tertiary group –$CONR_2$ of amides.

The Infrared spectrums chart of purified SAC biosynthesized by ***B. subtilis*-KG82-KSA** strain are characterized by the presence of peptides in amino acids that identifying the chemical groups: the 3449.17 wave number was assignment and remarked to: NH stretch; broad bond may have amines structure and 1650.72 wave number was assignment and remarked to CO stretch carbonyl group. The aliphatic chains were identified at 2932.41 wave number which assignment and remarked to CH to indicate the methylene group, that indicating that this compound is a lipopeptide. Also the 1434.73 wave number was assignment and remarked to H-C=O bend in aliphatic aldehydes. Also the ether was observed at wave number 1268.80 which assignment and remarked to C-O-C and wave number 1056.13 was assignment and remarked to R-(alkyl)-C-O stretch in alkyl aryl ethers. The wave number 3244.08 is an indication of NH_4 group presented (produced from medium). The wave number 2346.53 was assignment and remarked to P-H_2 stretch and phosphines in phosphotyrosine or phosphoserine. Standard biosurfactant and ***B. subtilis*-KG82-KSA** peaks were detected by using Infrared (IR) spectra analysis. All results were recorded in table (3).

3- High Performance Liquid Chromatography (HPLC): Analytical reverse-phase HPLC spectroscopy analysis showed the presence of three major peaks in both standard biosurfactant and SAC biosynthesized by ***B. subtilis*-KG82-KSA**. The standard biosurfactant detected the SAC at peaks A, B and C, the highest peak of standard was presented at peak B. Three peaks of area 54062, 95898 and 12658 (mm) with retention time 3.915, 4.533 and 5.771 (min). The height of peak A 4850 (mm) and width 0.39 (mm), while the height of peak B was 7156 (mm) and width 0.74 (mm), also the height of peak C was 1084 (mm) and width area 0.38 (mm). Peak A area concentration was 33.245, peak B 58.971 and peak C 7.784 (%).

Table (3): Infra red spectra analyses of standard SAC and purified SAC biosynthesized by *B. subtilis*-KG82-KSA cultivated on crude oil.

Peak No.	Standard	*Bacillus subtilis*-KG82-KSA	Peak No.	Standard	*Bacillus subtilis*-KG82-KSA
1	ND	ND	11	1510.49	ND
2	837.28	ND	12	1640.88	ND
3	ND	ND	13	ND	1650.72
4	ND	1056.13	14	ND	ND
5	1127.05	ND	15	ND	2346.53
6	ND	ND	16	ND	ND
7	1186.39	ND	17	2926.47	2932.41
8	ND	1268.80	18	ND	3244.08
9	ND	ND	19	ND	3449.17
10	1458.61	1434.73	/	/	/

ND: Not detected

The purified ***B. subtilis*-KG82-KSA** SAC was detected three peaks A, B and C, the highest SAC was presented at peak C. The three peaks detected at different areas 127884, 168095 and 1398334 (mm), retention time 3.925, 4.533 and 5.664 (min). The height of peak A was 11070 (mm) and width 0.44 (mm), while the height of peak B was 9939 (mm) and width (1.14), also the height of peak C was (92784) and width area (0.90). Peak A area concentration was (7.548%), peak B (9.921%) and peak C (82.531%). All results were presented in table (4).

4- Determination the quantity of amino acids of the purified SACs biosynthesized by *Bacillus subtilis*-KG82-KSA strain*:* The amino acid composition of the purified SAC synthesized by ***Bacillus subtilis*-KG82-KSA** can be detected by using amino acids analyzer. After purification processes we started in the SACs amino acids identification. The amino acids detected in the purified SAC ***B. subtilis*-KG82-KSA** strain contained aliphatic side chains amino acids (Glycine 1.191%, Valine 2.907% and Leucine 12.407%); amino acid with side chains containing hydroxylic group (Serine 0.758% and Tyrosine 1.622%); amino acids with side chains containing sulfur atom (Cystin 3.119); amino acids with side chains containing acidic group (Asparagine, 1.556% and glutamine, 1.086%); amino acid with side chains containing basic group (lysine, 0.706) and amino acids containing amino acid group (proline 3.827%). All data were listed in table (5).

Table (4): High Performance Liquid Chromatography (HPLC) analyses of the purified standard SAC and SAC biosynthesized by *Bacillus subtilis*-KG82-KSA cultivated on crude oil.

SACs	Peak symbol	Width (mm)	Height (mm)	Area concentration (%)	Area (mm)	Retention time (min)
Standard biosurfactant	A	0.39	4850	33.245	54062	3.915
	B	0.74	7156	58.971	95898	4.533
	C	0.38	1084	7.784	12658	5.771
Bacillus subtilis-KG82-KSA	A	0.44	11070	7.548	127884	3.925
	B	1.14	9939	9.921	168095	4.533
	C	0.9	92784	82.531	1398334	5.664

Table (5): Amino acid analysis of the purified SAC biosynthesized by *B. subtilis*-KG82-KSA cultivated on crude oil

Amino acid	*Bacillus subtilis*-KG82-KSA		
	Retention time (min)	Area (mm)	Con. (%/ µg/l)
Asparagin	12.55	1100968	1.556
Serine	16.05	536178	0.758
Glutamine	18.37	768804	1.086
Cystin	24.98	842876	1.191
Glycine	27.65	2206760	3.119
Valine	29.43	2056292	2.907
Leucine	33.30	8775856	12.407
Tyrosine	40.62	1147865	1.622
Lysine	54.40	499604	0.706
Proline	22.50	2706996	3.827
NH4	58.48	50088896	70.815

ND: Not detected, Con.: Concentration (%/ µg/l)

5- Fatty acids: After preparation the purified SAC synthesized by ***Bacillus subtilis*-KG82-KSA** the fatty acids were detected by using GC/MS technique. Fatty acids analysis were reversed that twenty nine peaks were detected, which including clearly four peaks represented to Myristic acid is detected at retention time 14.308 (min); area 202142503 (mm); height 9872067 (mm) and concentration 20.476 (%), Palmitic acid

was detected at retention time 16.579 (min); area 150939595 (mm); height 9554311 (mm) and concentration 15.798 (%). The Oleic acid was produced at retention time 19.634 (min); area 9279656 (mm); height 650669 (mm) and concentration 14.262 (%). The Stearic acid was produced at retention time 20.168 (min); area 169343586 (mm); height 11851819 (mm) and concentration 14.288 (%).Results of fatty acids using gas liquid chromatography were recorded in table (6).

Table (6): Fatty acid analyses of the purified SACs biosynthesized by *Bacillus subtilis*-KG82-KSA strain.

Fatty Acids	*Bacillus subtilis*-KG82-KSA			
	Retention time (min)	Height (mm)	Area (mm)	Concentration (%)
Myristic acid	**14.308**	**9872067**	**202142503**	**20.476**
Palmitic acid	**16.579**	**9554311**	**150939595**	**15.798**
Oleic acid	**19.634**	**650669**	**9279656**	**14.262**
Stearic acid	**20.168**	**1185181**	**169343586**	**14.288**

ND: Not detected

DISCUSSION

Petroleum hydrocarbons are important energy resources used by industry and in our daily life. At the same time petroleum is a major pollutant of the environment **(Chaillan *et al.,* 2006).** Oil pollution accidents are nowadays become a common phenomenon and have caused ecological and social catastrophes **(Burger, 1993).** Oil spill accidents results in significant contamination of the ocean and shoreline environments. One of the oil spill remediation techniques is the application of dispersants to oil slicks. The dispersants used for this purpose are composed of complex mixture of surfactants, solvents and additives.

Biosurfactants are a leading group of valuable microbial natural products with unique biochemical properties. From a biotechnology prospective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, agricultural and the petrochemical industries **(Nguyen *et al.,* 2008).**

Biosurfactants can be as effective as the synthetic chemical surfactants and for certain applications they have advantages such as high specificity. Most of the biosurfactants and many chemical surfactants employed for bioremediation purposes are biodegradable **(Pekdemir and Copur 2005)**. These molecules reaction their activity at extreme of temperatures, pH and salinity conditions **(Das *et al.*, 2008).** Various types of biosurfactants such as glycolipids, lipopolysaccharides, oligosaccharides and lipopeptides have been reported to be produced by diverse bacterial genera (**Mukherjee *et al.,* 2007**).

Biosurfactant production are sometimes detected by hemolytic activity (HA) **(Yonebayash *et al.,* 2000)** according to **Palmisano *et al.* (2001)** or oil spread technique (OST) according to **Youssef *et al.* (2004)** is reliable in detecting biosurfactant biosynthesis as determined by surface tension measurements. The oil spread technique (OST) has a larger dynamic range than surface tension. It also easy to perform and to standardize and less time-consuming than surface tension measurements, makes it applicable for large scanning studies. The bacterial lipopeptide iturin A is able to cause hemolysis of human erythrocytes in a dose dependent manner **(Francisco *et al.,* 2005;Anyanwu *et al.*,2011; Panesar *et al.*, 2011; Parwa-Plociniczak *et al.*, 2011; Makkar *et al.*, 2011; Praveesh *et al.*, 2011; Vyas and Dave,2011 and Xu *et al.*, 2011)**

Ten pure bacterial isolates were selected as best SAC producers by using hemolytic activity (HA) and oil spread technique (OST). Oil spread technique (OST) was used as indicator for production of biosurfactant by several studies **(Bicaa *et al.,* 1999; Bodour *et al.,* 2004)**.

The best three isolates were selected 25, 53 and 87 which displayed the highest lysed red blood cells in blood agar with scores ranging between (+++) corresponding to complete haemolysis with a diameter of lysis >10mm but < 30mm. The SAC were detected by HA to giving 0.8, 0.78 and 0.75 (g/l) respectively while the OST were gave 1.37, 1.25 and 1.18 (g/l) respectively.

The best highest SAC producer bacterial isolate was purified and identified on the basis of morphological, physiological and biochemical characteristics were carried out according to Bergey's Manual of Determinative Bacteriology **(Hensyl *et al.,* 1994)**. Finally we are using the phylogenetic technique by 16S rRNA for complete identification of the isolates KG82-KSA to give ***Bacillus subtilis*-KG82-KSA**.

Bacillus species are one of the major producers of microbial surfactants. These include bioactive lipopeptide molecules such as surfactin, fengycin, lichenysin, iturin, pumilacidin and bacillomycin **(Vater *et al.*, 2002)**. They facilitate the process of emulsification of hydrocarbons in aqueous phase by forming micelles thereby enhancing their availability for microbial uptake and degradation. Hence they have potential application in the field of bioremediation of persistent and recalcitrant organic pollutants.

Bioremediation is defined as the complete elimination or conversion of toxic recalcitrant compounds into non-toxic forms by a bacterium or microbial consortium. It is a cost effective technology because a microbial culture is used in this process and also a performance effective technique, as the end products of this process are mostly not harmful to environment.

Under certain conditions many microbes can be induced to produce extracellular biosurfactants. In this connection, the effect of different operating factors on biosurfactant production is of paramount importance. Many environmental factors, such as pH, temperature, salinity, type of nutrients, etc., can influence the physicochemical properties including carbon–nitrogen ratio, divalent cations, and specific substrate availability **(Adamczak and Bednarski, 2000)**.

The carbon substrate and type of carbon is an important factor affecting the quality production of microbial surfactants. The amount of biosurfactant synthesis depends greatly on the availability of carbon sources and on the balance between carbon and other limiting nutrients **(Abouseoud *et al.,* 2008)**.

Three strains of the best bacterial SACs producer was selected as ***Bacillus subtilis*-KG82-KSA** the optimum conditions were studied for reaching to the highest yield of SACs.

***Bacillus subtilis*-KG82-KSA** strain isolated, biosynthesized a high quantity of SACs which obtained with the following optimum conditions: Incubation period (seven days). The best incubation temperature 40°C. The optimum temperature for iturin A and surfactin production was observed at 25 and 37°C respectively. On the other hand, iturin A production was gradually decreased with the increase of the incubation temperature **(Rahman *et al.,* 2009).**

The initial pH value with ***Bacillus subtilis*-KG82-KSA** was 7. **Tababaee *et al.,* (2005)** reported in contrast that pH variation has no appreciable effect on biosurfactant production, but maximum biosurfactant production was at pH range from 6.2-7.2. Also **Abu-Ruwida *et al.* (1991)** observed biosurfactant production of *Rhodococcus* sp. at pH 6.5-7.2 that determined by surface tension. Also pH range for *Bacillus subtilis* B20 was 6.0–10.0 and salt concentration up to 5% (w/v) **(Sanket *et al.,* 2008)**.

The incubation condition (shaking state) for ***Bacillus subtilis*-KG82-KSA**, agitation rate affects the mass transfer efficiency of both oxygen and medium components and is considered crucial to the cell growth and biosurfactants formation of the strictly aerobic bacteria, especially when it was grown in a submerged under shake flask conditions.

The best crude oil concentration in ***B. subtilis*-KG82-KSA** (0.5mg/100ml). The NaCl concentration was 1.0%. Salt concentration also affected biosurfactants production depending on its effect of cellular activity which is very clear to the results obtained by **Yakimov *et al.* (1995)**. They isolated *Bacillus licheniformis* BAS50 which grew and produced a lipopeptide surfactant when grown on a variety of substrate at salinities of 13 % NaCl **(Jenny *et al.,* 1991)**. Thiamin HCl (200ppm) was the best concentration. Sucrose (2.5mg/100ml)) was the best carbon source requirement. **Batista *et al.* (2006)** reported that, glucose is a better carbon source than fructose and sucrose for biosurfactant production by Gram-positive and Gram-negative bacteria.

The best nitrogen source requirement (0.133mg/100ml of $(NH_4)_2Fe(SO_4).12H_2O$). The best protein source was pepsin. The best concentration of $FeSO_4 \cdot 7H_2O$ was (0.01 (g/l), while the best $MnSO_4.5H_2O$ concentration was (0.04 (g/l).

Bento *et al.* (2005) reported that, fore *Bacillus* sp displayed a substantial capacity to decrease surface tension and increase the emulsification activity. Some strains of *Bacillus licheniformis* biosynthesised the production quite similar biosufactant to surfactin **(Jenny *et al.,* 1991)**.

The purification procedure included several steps that following:

First step: Filtration and centrifugation: Cell free filtrate was obtained by filter paper and filtrated again through 0.45µ filter and centrifuged at 8000prm for 15 min to separate small particles and obtained of the clear biosurfactant filtrate.
Second step: solvent separation: Separated and extracted by chloroform: ethanol solvent (2:1 v/v).
Third step: Concentration and separation of surface active compound(s) was carried out by using by evaporation and TLC purification to obtain the active separated spot.
Fourth step: Separating the SACs by silica gel column chromatography to obtained the active fractions.

UV, TLC, IR and HPLC analysis techniques were used for indicating and identification the biosurfactant products. The SAC biosynthesized by ***Bacillus subtilis*-KG82-KSA** can be detected by using UV (98%) analysis technique and confirmed the identification step by using TLC, IR and HPLC analysis techniques.

Microbial surfactants, which are secreted by different groups of bacteria, composed of lipid, phospholipids, polysaccharide, protein and other biological macromolecules and contain various functional groups including carboxyl, amino and phosphate groups **(Christofi and Ivehina, 2002)**.

There are many complex molecules included in biosurfactants, e.g. glycolipids, lipopeptides, fatty acids, polysaccharide protein complexes, peptides, phospholipids and neutral lipids. For instance, the biosurfactant obtained from *Streptococcus thermophilus* A was a multicomponent biosurfactant, consisting of protein and polysaccharides **(Rodrigues *et al.,* 2006)**.

The Infrared spectrums chart of purified SAC biosynthesized by ***Bacillus subtilis*-KG82-KSA** strain are characterized by the presence of peptides in amino acids that identifying the chemical groups: the 3449.17 wave number was assignment and remarked to: NH stretch; broad bond may have amines structure and 1650.72 wave number was assignment and remarked to CO stretch carbonyl group. The aliphatic chains were identified at 2932.41 wave number which assignment and remarked to CH to indicate the methylene group, that indicating that this compound is a lipopeptide. Also the 1434.73 wave number was assignment and remarked to H-C=O bend in aliphatic aldehydes. Also the ether was observed at wave number 1268.80 which assignment and remarked to C-O-C and wave number 1056.13 was assignment and remarked to R-(alkyl)-C-O stretch in alkyl aryl ethers. The wave number 3244.08 is an indication of NH_4 group presented (produced from medium). The wave number 2346.53 was assignment and remarked to P-H_2 stretch and phosphines in phosphotyrosine or phosphoserine. The IR spectrum characteristic showed similarity structure as iturin. The bands at 2956–2924 cm^{-1}, 2869 cm^{-1} and at 1463 cm^{-1}, 1377 cm^{-1} reflect aliphatic chains ($–CH_3$, $–CH_2–$) of the fraction, whilst a strong band observed at 1734 cm^{-1} is due to a carbonyl group. These results infer that biosurfactant produced contains aliphatic hydrocarbons as well as a peptide like moiety **(Abouseoud *et al.,* 2008)**.

Also the result of FTIR spectrum of *B. subtilis* 20B indicates that the product contains aliphatic hydrocarbons as well as a peptide like moiety. This spectrum shows similarity with lipopeptide biosurfactant surfactin **(Carrera *et al.,* 1993)**.

The purified SAC of ***Bacillus subtilis*-KG82-KSA** was detected three peaks A, B and C by using HPLC analysis technique, the highest SAC was presented at peak C. The three peaks appeared at retention time 3.925, 4.533 and 5.664 (min). The heighest activity presented at peak C concentration (82.531%). In HPLC, three major peaks were observed between 5.6 and 10.9 min which showed the activity in *B. subtilis* B20 **(Sanket *et al.,* 2008)**.

Biosurfactants are compounds produced by a variety of microorganisms that are capable of lowering surface and/or interfacial tension by partitioning at the water-air and water-oil interfaces. They can have a variety of structures, including fatty acids, neutral lipids, phospholipids, glycolipids, and lipopeptides **(Noha *et al.,* 2005)**.

Many strains of *Bacillus* are known to produce lipopeptides with remarkable surface-active properties. The most prominent of these powerful lipopeptides is surfactin from *Bacillus subtilis*. Some *B. subtilis* strains are also known to produce other, structurally related lipoheptapeptides like iturin and bacillomycin, or the lipodecapeptides fengycin and plipastatin **(Dirk *et al.,* 1999)**.

Complete the identification steps for biosurfactant by detected the amino acid groups and detected the fatty acid side chain of chemical structure. For ensure of the chemical groups we are used ^{1}H-nuclear magnetic resonance (NMR) as confirmatory analysis.

The amino acid composition of the purified SAC biosynthesized by ***B. subtilis*-KG82-KSA** was detected. The amino acids was detected in the purified SAC biosynthesized ***B. subtilis*-KG82-KSA** in the eluted fraction number 2 were included seven amino acids Glycine, Valine, Leucine, Serine, Tyrosine, Cystin, Asparagine, glutamine, lysine, and proline.

In this study, we purified the surface active compound from ***B. subtilis*-KG82-KSA** to determine the structure of this compound by amino acid analysis and various spectroscopic techniques. This result together with amino acid analysis and ^{1}H-NMR data indicated that the peptide sequence of the ***B. subtilis*-KG82-KSA** surfactant is identical to that of iturin.

At the end of identification the SACs biosynthesized by ***B. subtilis*-KG82-KSA** was identified as iturin. The lipopeptides iturin compounds (iturins, mycosubtilins and bacillomycins) which are amphiphilic membrane active biosurfactants and peptide antibiotics with potent antimicrobial activities **(Sanket *et al.*, 2008).** Bacteria of the genus *Bacillus* are known as producers of a number of peptides with antibiotic properties against bacteria, fungi and yeasts **(Asaka and Shoda, 1996).**

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