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Biosurfactant Production from Locally Isolated *Bacillus cereus*

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By

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بسمِ اللهِ الرّحَمنِ الرّحيمِ

وَاللهُ اَخرَجَكُم مِن بُطُونِ اُمَّهَاتِكُم لا تَعلَمُونَ شَيئاً وَجَعَلَ لَكُمُ السَمعَ وَالاَبصَارَ وَالاَفِئدَةَ لَعَلّكُم تَشكُرُونَ

صَدَقَ اللهُ العلي العَظيمِ
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قال رسول الله صلى الله عليه واله وسلم :

"جميع الناس هالِكون اِلا العالِمون وجميع العالِمون هالِكون اِلا العامِلون وجميع العامِلون هالِكون اِلا المُخلِصون"

صدق رسول الله صلى الله عليه واله وسلم

الاهداء

الى من كانت ام ابيها...

الى فلذة كبد الرسول وبضعته الطاهرة...

الى بنت رسول الله وزوجة ولي الله وام حجج الله...

الزهراء البتول صلوات الله عليها.

اقدم جهدي هذا

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We certify that this thesis was prepared under our supervision in Al-Nahrain University / College of Science as a partial requirement for the degree of Master of Science in Biotechnology.

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Summary

Sixty three isolates belonged to *Bacillus spp*. were isolated from 18 soil samples (uncontaminated and contaminated with hydrocarbons and fuels) collected from different locations in Baghdad and Basra governorates. All the isolates (63) were primarily screened for their ability in biosurfactant production depending on blood hemolysis activity at blood agar base, which referred that 34 isolates of the total (63 isolate) were biosurfactant producer. Biosurfactant production ability of the producer isolates was evaluated by surface tension (mN m^{-1}) measurement of cell-free broth after cultivation in mineral salt medium supplemented with sucrose as sole carbon source. Identification tests showed the best producer isolate was *Bacillus cereus* HI-2.

Optimum conditions for biosurfactant production from *B. cereus* HI-2 were determined. Results indicated that maximum biosurfactant production from this isolate was achieved by using soybean oil (4% v/v), ammonium nitrate (4 g/l), and potassium dihydrogen phosphate (0.5 g/l), at pH 7, 35°C, 180 rpm of shaking, for 72 hrs. Cell-free broth (crude biosurfactant) was collected and purified by acid precipitation method, and the biosurfactant yield was 0.453 gram per liter of culture medium under the optimum conditions.

Depending on the FTIR spectroscopy and some biochemical tests (Molisch's and Biuret tests) chemical nature of biosurfactant was characterized. Results elucidated that it was potentially a lipopeptide compound.

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List of Abbreviations

Abbreviations	
°C	Degree celsius
C:N	Carbon to nitrogen ratio
CMC	Critical micelle concentration
cm	Centimeter
DNA	Deoxyriboneuclic acid
D.W.	Distilled water
EA	Emulsification activity
FTIR	Fourier transformed infrared spectroscopy
g	gram
g/l	gram per liter
HPLC	High performance liquid chromatography
hrs	hours
Ib/in^2	Pound per square inches
Kg	Kilogram
mg / L	milligram per liter
min	minute
ml	milliliter
mNm^{-1}	millinuton per meter
µl	microliter
µm	micrometer
nm	nanometer
NMR	Neuclear megnatic resonance
O.D.	Optical density
%	Percent
rpm	round per minute
ST	surface tension
TLC	Thin layer chromatography
v/v	Volume by volume
w/v	weight by volume

Chapter One

Introduction

and

Literature Review

Introduction and Literature Review

1.1 Introduction

Surfactants are surface-active compounds, capable of reducing surface and interfacial tension at the interfaces between liquids, solids and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids. Biosurfactants are chemicals produced by microorganisms, which have both clearly defined hydrophilic and hydrophobic groups (Zouboulis *et al*., 2003).

When considering the natural roles of biosurfactants, in changing surface active phenomena, wetting and penetrating actions, spreading, hydrophylicity and hydrophobicity actions, microbial growth enhancement, metal sequestration and anti-microbial action (Kosaric, 2001), also their application concerning enhanced oil recovery , hydrocarbon bioremedation, agriculture, cosmetics, food processing (Desai and Banat, 1997), importance of surfactant sensors in environmental control of surfactant waste water, medical and health care applications, and antiviral activity of (surfactin) biosurfactant (Sak-Bosnar *et al*, 2004), it is important to emphasize that they are produced by a wide variety of diverse microorganisms and have very different chemical structures and surface properties (Ron and Rosenberg, 2001).

Most of the applications involve the use of chemically synthesized surfactants, so the production of surfactants in the United States and World wide was estimated at 3.4×10^3 Kg and 7×10^9 Kg in 1989, respectively, and the US surfactant industry shipments were 3.65billion dollar in 1989; but these compounds are usually toxic to the environment and most of them non-biodegradable, therefore, they may bio-accumulate, whereas their production

processes and by-products can be environmentally hazardous (Zouboulis *et al*., 2003).

Biosurfactants have several advantages as compared to their chemically synthesized counterparts, such as lower toxicity, higher biodegradability, availability of raw materials, acceptable production economics, specificity, and effectiveness (Kosaric, 2001). Due to all of these advantages, their importance in biotechnology is obvious.

But due to high production cost involved much interest and attention have been directed toward improving the efficiency of the current bioprocessing methodology and strain productivity and the use of cost-effective substrates (Internet 1).

A number of attempts have been made to increase biosurfactant productivity by manipulating physiological conditions and medium composition. Dvelopments in the area of optimization of fermentation conditions have resulted in a significant increase in production yields, making them more commercially attractive (Desai and Banat, 1997).

According to those mentioned above and because of the limited studies about biosurfactants production from Gram-positive bacteria, the aim of this study was to shed light on**:**

1. Isolation and identification of *Bacillus* spp. and screening the isolates for their ability to produce biosurfactants.
2. Determination of the optimum conditions for biosurfactants production.
3. Purification and characterization of some properties of the produced biosurfactants.

1.2 Genus *Bacillus*

cells belongs to this genus are rod-shaped occur singly or in chains which may be of considerable length; endospores very resistant to many adverse conditions, formed; not more than one per cell; sporulation not repressed by exposure to air. Gram-positive, aerobic or facultatively anaerobic. Colony morphology and size very variable; exhibit a wide diversity of physiological ability; catalase formed by most species (Claus and Berkeley, 1986).

1.2.1 *Bacillus* Species and Related Genera

Established by Cohn in (1872) as cited in Fritze, (2004), the genus *Bacillus* has undergone considerable taxonomic changes. As of 1986 (Clause and Berkeley, 1986), the genus *Bacillus* has encompassed a variety of phenotypically heterogeneous species exhibiting a wide range of nutritional requirements, physiological and metabolic diversity and DNA base composition (Xu and Côte, 2003). Numerical classification based on a series of phenetic characters has been used for the classification of 368 *Bacillus* strains into 89 clusters (Priest *et al*., 1988). Further characterization at the genotypic and phenotypic levels of selected *Bacillus* species have led to the creation of several new genera: *Amphibacillus*, *Alicyclobacillus*, *Paenibacillus*, *Aneurinibacillus*, *Brevibacillus*, *Virgibacillus*, *Gracilibacillus*, *Salibacillus*, *Filobacillus*, *Geobacillus*, *Ureibacillus*, *Jeotgalibacillus* and *Marinibacillus* (Niimura *et al*., 1990; Wisotzkey *et al*., 1992; Ash *et al*., 1993; Shida *et al*., 1996b; Heyndrickx *et al*., 1998; Wainø *et al*., 1999; Fortina *et al*., 2001; Nazina *et al*., 2001; Schlesner *et al*., 2001; Yoon *et al*., 2001).

1.2.2 Biosurfactant Producer *Bacillus* spp.

According to the researches deals with biosurfactants production from genus *Bacillus*, the most producers were belonged to *B. cereus*, *B. subtilis*, *B. licheniformis*, *B. sphaericus*, *B. thuringiensis*, *B. stearothermophilus* and *B. pumilus* (Jacques *et al*., 1999; Jennings and Tanner, 2000; Noudeh *et al*., 2003; Tuleva *et al*., 2005).

1.3 Surface-Active Agents

Molecules and ions that are adsorbed at interfaces are termed "surface-active agents" or "surfactants". An alternative expression is "amphiphile" which suggest that the molecule or ion has a certain affinity for both polar and nonpolar solvents. Depending on the number and nature of the polar and nonpolar groups present, the amphiphile may be predominantly "hydrophilic" (water-loving), "lipophilic" (oil-loving), or reasonably well balanced between these two extremes. For example, straight-chain alcohols, amines, and acids are amphiphiles that change from being predominantly hydrophilic to lipophilic as the number of carbon atoms in the alkyl chain is increased. Thus, ethyl alcohol is miscible with water in all proportions (Martin *et al*., 1994).

The amphiphilic nature of surfactants leads to their arrangement to different complex forms as shown in (Figure 1-1). Almost all surfactants currently in use are chemically derived from petroleum (Fiechter, 1992; Klekner and Kosaric, 1993; Banat, 1995). It is the amphiphilic nature of surface-active agents that causes them to be absorbed at interfaces, whether these are liquid-gas or

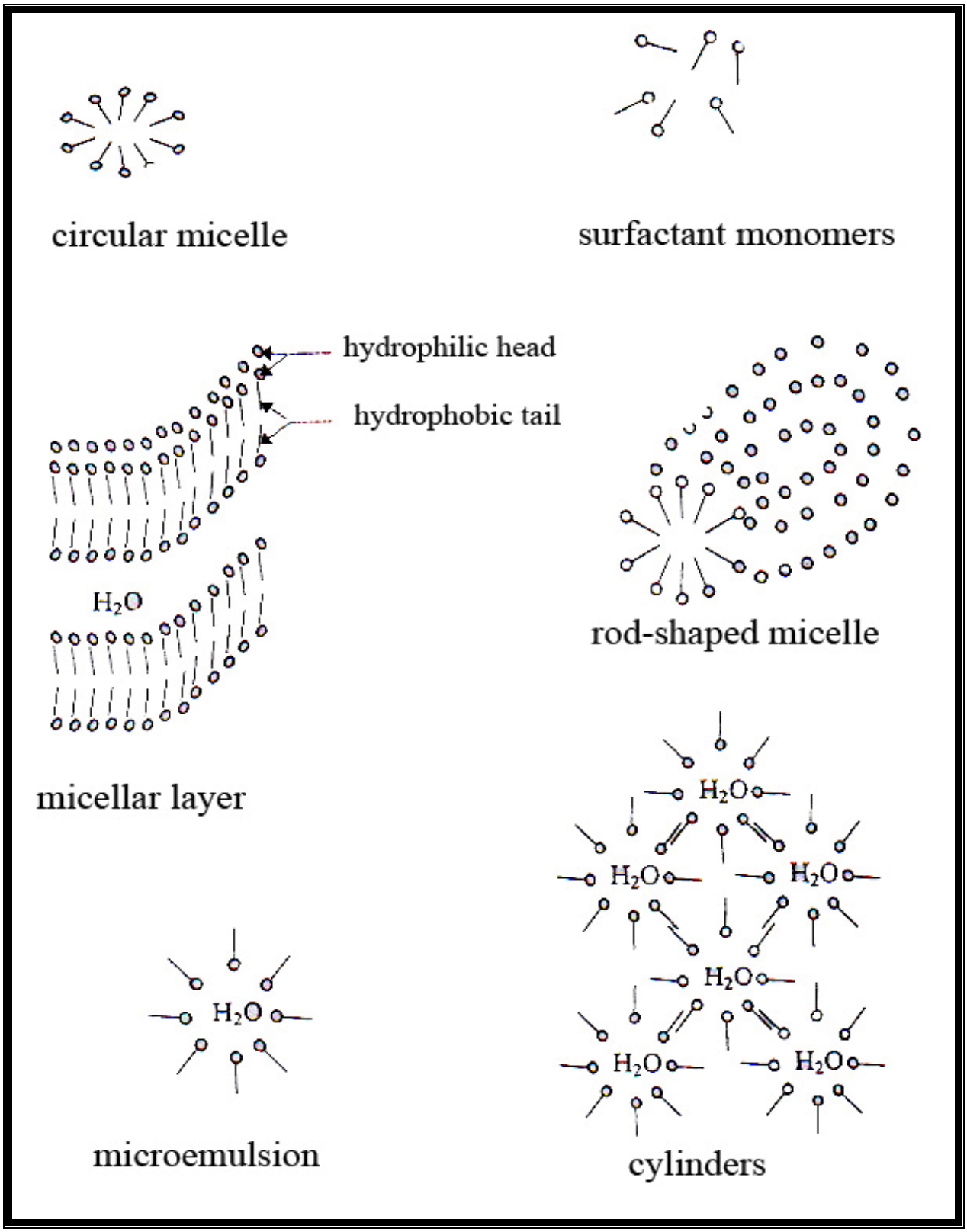


Figure (1-1) Amphipathic structure of surface containing hydrophobic and hydrophilic moiety in one molecule (Desai *et al.*, 1994)

liquid-liquid. Thus, in an aqueous dispersion of amyl alcohol ($C_5H_{11}OH$), the polar alcoholic group is able to associate with water molecules, the nonpolar portion is rejected, however, because the adhesive forces it can develop with water are small in comparison to the cohesive forces between adjacent water molecules. As a result, the amphiphile is adsorbed at the interface. The situation for a fatty acid at the air-water and oil-water interface is shown in (Figure 1-2).

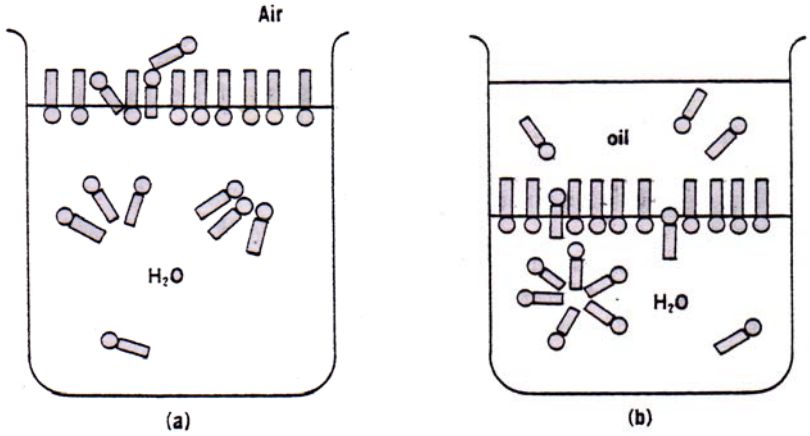


Figure (1-2) Schematic representation of the adsorption of fatty acid molecules at a water-air interface (a) and at a water-oil interface (b) (Martin *et al.*, 1994).

At the air-water interface, the lipophilic chains are directed upward into the air, oil-water interface, they are associated with the oil phase. In order for the amphiphile to be concentrated at the interface, it must be balanced with the proper amount of water- and oil-soluble groups. If the molecule is too hydrophilic, it remains within the body of the aqueous phase and exerts no effect at interface. Likewise, if it is too lipophilic, it dissolves completely in the oil phase and little appears at the interface (Martin *et al.*, 1994).

1.4 Nature of Surfactants

Chemically, surfactants are amphiphilic molecules consisting of a hydrophilic and hydrophobic domain. The former can be non-ionic, positively or negatively charged or amphoteric; but the latter is usually a hydrocarbon (Georgiou *et al*., 1992; Desai *et al.*, 1994). Since both the hydrophilic and hydrophobic groups reside within the same molecule, surfactants tend to partition preferentially at the interfaces between fluid phases with a different degree of polarity and hydrogen bonding (like that of oil/water or air/water interfaces). Unique properties of the surfactant molecules were driven by the reduction of interfacial energy (interfacial tension) and surface tension through the formation of an ordered molecular film at the interface. These properties allow them to be used extensively in industrial applications involving emulsification, foaming, detergence, wetting and phase dispersion or solubilization (Internet 1).

The three general characteristics of surfactants are enrichment at interfaces, lowering of interfacial tension, and micelle formation, so to compare surfactants, the surface or interfacial tension is used as a measure of effectivity. These characteristics are also applicable to biosurfactants; however, not all of them may be applicable to polymeric microbial surface-active agents.

The difference between lipids and surface-active agents is based on the ratio of the hydrophobic to the hydrophilic regions. This relation is expressed for synthetic surfactants as the hydrophilic/lipophilic balance value and can vary over a wide range (Neu, 1996).

1.5 Classes of Surfactants

Surfactants fall in the following classification according to the nature of the hydrophilic group:

- Anionic: Hydrophilic head is negatively charged;
- Cationic: Hydrophilic head is positively charged;
- Nonionic: Hydrophilic head is polar but not fully charged;
- Amphoteric: Molecule has both potential positive and negative groups: charge depends on pH of the medium (Salager, 2002).

1.6 Biosurfactants

Biosurfactants are a specialized class of surfactants that are produced by bacteria, yeast, and fungi as extracellular or membrane - associated surface active compounds called biosurfactants (Banat *et al*., 1991 and Banat, 1995a; Desai and Banat, 1997). The term biosurfactant refers to any type of compound produced by microorganisms with surface active or emulsifying properties (Rosenberg, 1986; Hommel, 1990; Fiechter, 1992). These can be divided into low-molecular-weight molecules that lower surface and interfacial tensions efficiently (Cooper and Zajic, 1980) and high-molecular-weight polymers that bind tightly to surfaces (Rosenberg and Ron, 1997 and 1999).

Industrially, these biosurfactants are becoming more attractive because of their possible exploitation as emulsifiers, wetting agents, foaming agents, food ingredients, and detergents to name a few. The term “Bioemulsifiers” has also been used to describe surface-active agents forming emulsion (when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase) (Fiechter, 1992). Some surfactants and biosurfactants are micelle-forming agents and some are not. Micelles are highly organized structures consisting of a grope of surfactant molecules aggregating in a specific orientation (Mittal, 1977).

Micelles may take on assorted shapes such as rods, bilayered sheets, vesicles, and worm-like structures (Champion, 1995; Jianhai, 2001; Al-Anber *et al*., 2003). The most typical example of a micelle is the spherical micelle in which the hydrophilic heads of multiple surfactant molecules align along the surface of a sphere with their hydrophobic tails directed inward excluding water from the interior of the sphere. In this manner, micelles are proficient at solubilizing organic substances in an aqueous solution in which they are typically immiscible. There are many advantages of biosurfactants if compared to their chemically synthesized counterparts. Some of these are: biodegradability, generally low toxicity, biocompatibility and digestability, availability of raw, acceptable production economics, use in environmental control, specificity i.e. depending on strain and concentration used, and effectiveness at extreme temperatures, pH and salinity (Kosaric, 2001).

1.6.1 Types of Biosurfactants

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, the various surface-active compounds of microorganisms can be distinguished in terms of different criteria. The size of the molecules can span a wide range from low-molecular-weight surfactants through polymeric surfactants up to particulate surfactants (Rosenberg, 1986). Another criterion for categorizing microbial surface-active compounds is the biochemical nature of the molecules such as fatty acids, lipids, bacteriocines, peptides, and polysaccharides (Zajic and Panchal, 1976). A further way to classify microbial surface-active compounds is by the nature of the hydrophilic part of the surface-active compounds such as the carboxylate group of fatty acids, the glycerol of the glycerolipids, the carbohydrate of glycolipids, and the amino acids of peptidyl lipids (Cooper and Zajic, 1980). Other authors distinguish between different locations of surface-active compounds in terms of intracellular, cell surface, and extracellular pool (Kosaric *et al*., 1987).

Furthermore, microbial surface-active compounds can be grouped by the species of the producing organisms. The surface-active compounds of the microorganisms may also be divided according to the type of carbon source used to produce them, such as hydrocarbons, water-soluble molecules, or both (Haferburg *et al*., 1986).

1.6.2 Biosurfactant Classification

Biosurfactants are categorized mainly by their chemical composition and their microbial origin (Cui, 2004). They have definite structure: the lipophilic moiety is usually the hydrocarbon (alkyl) tail of one or more fatty acids, which may be saturated, unsaturated, hydroxylated or branched; and the hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di- or polysaccharides; the fatty acid is linked to the hydrophilic group by a glycosidic ester or amide bond. Most of them are either neutral or negatively charged, the anionic character being due to carboxylate groups. According to Desai and Desai (1993), the major classes of biosurfactant include:

- Glycolipids;
- Lipopeptides and Lipoproteins;
- Phospholipids and Fatty Acids;
- Polymeric surfactants; and
- Particulate surfactants.

The Microbial source of important types of microbial surfactants was shown in table (1-1).

Table (1-1) Major biosurfactants produced by microorganisms (Makkar and Cameotra, 2002).

Biosurfactant	Organisms
Rhamnolipids	*Pseudomonas. aeruginosa*
Trehalolipids	*Arthrobacter paraffineus, Rhodococcus erythropolis*, *Mycobacterium spp.*
Sophorolipids	*Candida lipolytica*, *Torulopsis bombicola*
Viscosin	*P. fluorescens*
Surfactin	*Bacillus subtilis*
Polymyxins	*B. polymyxa*
Gramicidin S	*B. brevis*
Phospholipids	*Acinetobacter* spp., *T. thiooxidans*
Lipopeptides	*B. pumilis*, *B. licheniformis*, *P.fluorescens*
Polyol lipids	*Rhodotorula glutinis*, *Rhodotorula graminis*
Serrawettin	*Serratia marcescens*
Fatty acids (corynomycolic acids, spiculisporic acids)	*Corynebacterium lepus*, *A. paraffineus*, *Penicillium spiculisporum*, *Talaromyces trachyspermus*
Sulfonylipids	*Capnocytophaga* spp.
Diglycosyl diglycerides	*Lactobacillus fermentii*
Alasan	*Acinetobacter radioresistens*
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*A. calcoaceticus*
Liposan	*C. lipolytica*
Mannan- lipoprotein	*Candia tropicalis*
Food emulsifier	*Candida utilis*
Insecticide emulsifier	*Pseudomonas tralucida*
Sulfated polysaccharide	*Halomonas eurihalina*
Acetyl heteropolysaccharide	*Sphingomonas paucimobilis*

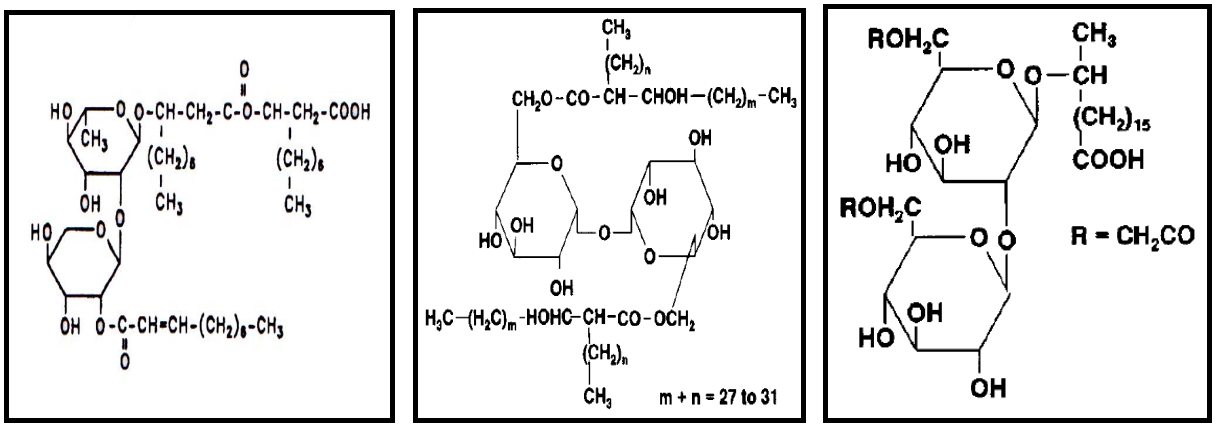
1.6.2.1 Glycolipids

Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids.

A- Rhamnolipids

A model micelle-forming biosurfactant molecule is the rhamnolipid molecule produced by the *Pseudomonas aeruginosa*. Production of rhamnose-containing glycolipids was first described in *P. aeruginosa* in 1940 by Jarvis and Johnson. Rhamnolipid is an anionic biosurfactant containing either one or two carboxylated sugar heads along with one or two lipid tails. For this reason, rhamnolipid is also classified as a glycolipid. Rhamnolipid may exist in its micellar form as a sphere, a vesicle, or a lamella (bilayered sheet) (Champion, 1995).

Rhamnolipid can be mono-headed (consisting of a six-carbon sugar) or multi-headed as well as mono-tailed (consisting of a seven-carbon alkyl) or multi-tailed. The chemical structures of two types of rhamnolipid, one dual-headed and dual-tailed, the other mono-headed and dual-tailed, are shown in (Figure 1-3A) Rhamnolipid's usual function is most probably to bring carbon sources into solution where they will be accessible by the bacterial cell. Rhamnolipids from *Pseudomonas* spp. have been demonstrated to lower the interfacial tension against *n*-hexadecane to 1 mN m^{-1} and the surface tension to 25 and to 30 mN m^{-1} (Lang and Wagner, 1987; Parra *et al*., 1989).



A **B** **C**

Figure (1-3) Structure of some common glycolipid biosurfactants (Desai and Banat, 1997).

B- Trehalolipids

Several structural types of microbial trehalolipid biosurfactants have been reported (Li *et al*., 1984). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium*, *Nocardia*, and *Corynebacterium*. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation (Asselineau and Asselineau, 1978; Cooper *et al*., 1989). Trehalose dimycolate produced by Rhodococcus erythropolis (Figure 1-3B) has been extensively studied (Desai and Banat, 1997). Trehalose lipids from *R. erythropolis* and *Arthrobacter* sp. lowered the surface and interfacial tensions in the culture broth to 25 to 40 and to 1 to 5 mN m^{-1}, respectively (Li *et al*., 1984; Rapp *et al*., 1979).

C- Sophorolipids

Sophorolipids, which are produced mainly by yeasts such as *Torulopsis bombicola*, consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid (Figure 1-3C). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides (Desai and Banat, 1997). It has been shown that *Candida bogoriensis* produce glycolipids in which sophorose is linked to docosanoic acid diacetate (Cutler and Light, 1979).

1.6.2.2 Lipopeptides and Lipoproteins

A large number of cyclic lipopetides including decapeptide antibiotics and lipopeptide antibiotics possess remarkable surface-active properties. The cyclic lipopeptide surfactin (Figure 1-4), produced by *Bacillus subtilis*, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN m^{-1} at concentrations as low as 0.5µg/ml (Arima *et al*., 1968). Viscosin, produced by the broccoli head rot pathogen *P. fluorescens*, is also a potent lipopeptide biosurfactant. It can reduce the water surface tension from 72 to 27 mN m^{-1} at a concentration of 4µg/ml (Laycock *et al*., 1991).

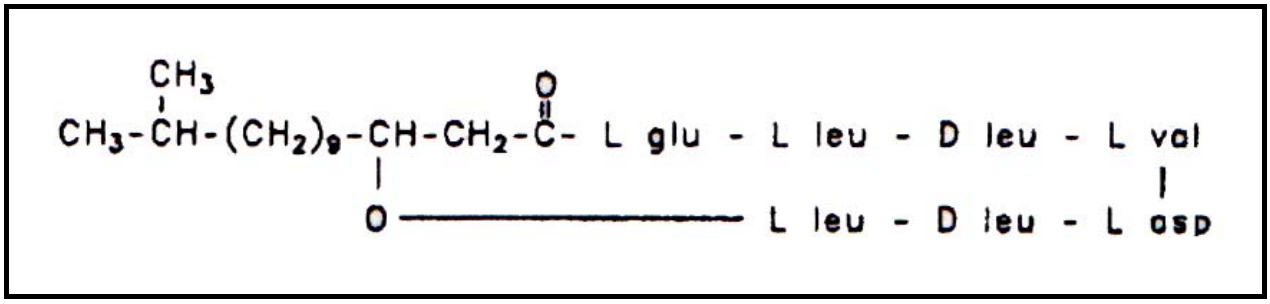


Figure (1-4) Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis* (Neu, 1996).

1.6.2.3 Phospholipids and Fatty Acids

Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on n-alkanes (Asselineau and Asselineau, 1978; Cirigliano and Carman, 1985). Phosphatidylethanol-amine produced by *Rhodococcus erythropolis* grown on n-alkane causes a lowering of interfacial tension between water and hexadecane to less than 1 mN m^{-1} and a CMC (critical micelle concentration) of 30 mg/l (Kretschmer *et al.*, 1982).

1.6.2.4 Polymeric Biosurfactants

Polymeric biosurfactants usually have a high molecular weight. The best studied polymeric biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. Emulsan, produced by *Acinetobacter calcoaceticus*, is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001% to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (Belsky *et al.*, 1979; Zosim *et al.*, 1982; Gutnick and Shabtai, 1987). Desai *et al.* (1988) demonstrated the production of bio-emulsifier by *P. fluorescens* during growth on gasoline. This bio-emulsifier is composed of 50% carbohydrate, 19.6% protein and 10% lipid

1.6.2.5 Particulate Biosurfactants

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion which plays an important role in alkan uptake by microbial cells (Kappeli and Finnerty, 1979). The membrane vesicles contain about 5 times as much phospholipid and about 350 times as much polysaccharide as does the outer membrane of the same organism (Desai and Banat, 1997). Surface activity in most hydrocarbon-degrading and pathogenic bacteria is attributed to several cell surface components, which include structures such as M protein and lipoteichoic acid in the case of group A streptococci, protein A in

Staphylococcus aureus, layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *Bacillus brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1 (Wilkinson and Galbraith, 1975; Fattom and Shilo, 1985).

1.7 Detection of Biosurfactants

In order to study microbial surfactant production, an accurate, rapid and sensitive detection method is required. A variety of different test methods have been described for the screening of potential biosurfactant-producing microorganisms.

(1) **Axisymmetric drop shape analysis (ADSA) method**: for the assessment of potential biosurfactant-producing bacteria. In this technique, drops of culture broth are placed on a fluoroethylene-propylene surface and the profile of the droplet is determined with a contour monitor. Surface tension is calculated from the droplet profiles by ADSA. Only biosurfactant-producing bacterial suspensions show reduction in surface tensions (Van der Vege *et al.,* 1991)

(2) **Colorimetric method**: biosurfactants estimation based on the fact that anionic surfactants are capable of reacting with the cationic indicator and forming a colored complex. This method is semi-quantitative as the diameter of the colored halo increases with surfactant concentration (Siegmund and Wagner, 1991)

(3) **A rapid drop-collapsing method**: in this method a water droplet will collapse while biosurfactant containing bacterial colonies (or bacterial suspensions) are added (Hildebrand, 1989). Jain *et al.* (1991) further developed this method by placing a drop of cell suspension on an oil-coated surface. In this method, drops containing biosurfactants collapse whereas non-surfactant containing drops remain stable.

(4) **A direct thin-layer chromatographic (TLC) technique:** The rapid characterization of biosurfactant-producing bacterial colonies was described by Matsuyama *et al.* (1987). They identified *Serratia macrescens* biosurfactants by applying a single bacterial colony directly to the TLC plate without any sample preparation procedure.

(5) **Surface tension method:** with a surface tension meter leads to an accurate quantification of the presence of a biosurfactant. Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic-lipophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. When a surfactant is added to air/water or oil/water systems at increasing concentrations, a reduction of surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supra molecular structures like micelles, bilayers, and vesicle. This value is known as the Critical Micelle Concentration (CMC) (Cui, 2004).

(6) **Contact angle method**: in this method surface activity of bacteria can be obtained by measuring the contact angles obtained after adding the bacteria to a water drop. The contact angle is located at the interface between the droplet and the solid surface. In the absence of surfactant, water molecules adhere strongly to each other and so the water droplet retains a round appearance with a contact angle of more than 90°, while in the presence of biosurfactants, the adherence forces are reduced causing the droplet to spread out flat creating a contact angle of less than 90° (Bunster *et al.* 1989). This method may lack sensitivity towards low biosurfactant levels.

(7) **Fungal growth inhibition method:** which is used to detect biosurfactants with antibiotic activity (Itoh *et al.*, 1971).

1.8 Regulation of Biosurfactants Biosynthesis

In the biosurfactant amphipathic structure, the hydrophobic moiety is either long chain fatty acid or a hydroxy fatty acid or an α-alkyl-β-hydroxy fatty acid, and the hydrophilic moiety may be a carbohydrate, carboxylic acid, phosphate, amino acid, cyclic peptide, or an alcohol. According to Syldatk and Wagner (1987), there are four possible ways how the different moieties of biosurfactants and their linkage are synthesized: **(i)** the hydrophilic and hydrophobic moieties are synthesized *de novo* by two independent pathways; **(ii)** the hydrophilic moiety is synthesized *de novo* while the synthesis of the hydrophobic moiety utilizes a pre-existing substrate; **(iii)** the hydrophobic moiety is synthesized *de novo*, while the synthesis of the hydrophilic moiety is substrate dependent; and **(iv)** the synthesis of both the hydrophobic and hydrophilic moieties are formed from pre-existing substrates.

Generally, the regulation of biosurfactant production is governed by three main mechanisms, namely induction, repression and nitrogen and multivalent ions (Desai and Banat, 1997). Induction of biosurfactant synthesis can be achieved by addition of long chain fatty acids, hydrocarbons, or glycerides to the growth medium (Tulloch *et al*., 1962), like the induction of glycolipid in *P. aeruginosa* by addition of alkanes (Chakrabarty, 1985). Such mechanism appears to be the general regulatory mechanism used to control the onset synthesis of most lipopeptide biosurfactants (Besson and Michel, 1992; Desai and Banat, 1997). Repressive effect has been noted up on the addition of D-glucose, acetate, or tricarboxylic acids (Desai and Banat, 1997).

Nitrogen- or metal ion-dependent regulation also played a prominent role in biosurfactant synthesis. Lastly, the limitation of multivalent cations causes overproduction of biosurfactants (Itoh and Suzuki, 1974; Desai and Banat, 1997).

1.9 Function of Biosurfactants in Bacteria

Only limited information about the influence of biosurfactants on microorganisms themselves is available, however the common view attributes only one role for microbial surface active compounds, i.e. the growth of microorganisms on hydrocarbons (Neu, 1996), or enabling microorganisms to grow on poorly soluble hydrocarbons (Lang and Wagner, 1987; Hommel, 1990).

Strains that were know to degrade polycyclic aromatic hydrocarbons, also were biosurfactant producers (Deziel *et al*., 1996).

Hommel (1990), patented that biosurfactants may be active either in cell-bound or in extracellular form. For instance, emulsan is excreted into the medium, but is active only when cell bound. It has been proposed that the extracellular (anionic) biosurfactants function to promote uptake of pseudosolubilized substrate, while the cell-associated (nonionic) biosurfactants function to promote uptake of substrate by facilitating attachment of the cells to the separate-phase substrate.

The fact that many biosurfactant are produced even when the cells are not grown on hydrophobic substrates suggests that their function is not solely restricted to the stimulation of substrate availability (Koch *et al*., 1991). Another function might be to regulate cell adhesion to and cell detachment from surfaces, which controls motility of the cells (Rosenberg, 1986; Neu, 1996).

Besson and Chevanet (1985) were demonstrated that iturin A inhibited the growth of the producing organism, an inhibition which differs from its antifungal activities.

1.10 Factors Affecting Biosurfactant Production

1.10.1 Carbon Source

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol which were used for rhamnolipid production by *Pseudomonas spp*., give a product less than that obtained with water-immiscible substrates such as n-alkanes, olive oil, and sunflower oil (Syldatk *et al*., 1985; Patel and Desai, 1997). Hydrocarbons with less than 10 carbon atoms tend to be relatively easy to degrade as long as the concentration is not too high to be toxic to the organisms. Benzene, xylene, and toluene are examples of gasoline components that are easily degraded. Complex molecular structures, such as branched paraffins, olefins, or cyclic alkanes, are much more resistant to biodegradation (Kosaric, 2001). Glycolipid production by *T. bombicola* is stimulated by the addition of vegetable oils during growth on 10% D-glucose medium giving a yield of 80 g / l (Asmer *et al*., 1988). Carbon source plays an important role in yield and structure of microbial surfactants (Cui, 2004). Healy *et al*., (1996) found that *P. fluorescens* produce a rhamnolipid instead of viscosin when grown on virgin olive oil.

1.10.2 Nitrogen Source

Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production, whereas nitrate ions supported maximum amounts of biosurfactant in *B. subtilis* (Makkar and Cameotra, 1998), and in *P. aeruginosa* (MacElwee *et al*., 1990). Guerra-Santos *et al*., (1986) showed maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 18:1 and no surfactant production below a C:N ratio of 11:1, also Syldatk *et al*., (1985) showed that nitrogen limitation not only causes overproduction of biosurfactant, but also changes the composition of the biosurfactant produced.

Similarly, lichenysin-A production is enhanced 2 and 4 fold in *B. licheniformis* BAS 50 by addition of L-glutamic acid and L-asparagine, respectively to the medium (Desai and Banat, 1997). It has been also documented the production of surface active AP-6 by *P. fluorescens* 378 with no dependence on a C:N ratio (Persson *et al*., 1990a).

1.10.3 Multivalent Cations

The limitation of multivalent cations also causes an overproduction of biosurfactant (Cui, 2004). Iron limitation stimulates biosurfactant production in *P. fluorescens* (Persson *et al*., 1990a, Persson *et* al., 1990b). Guerra-Santos *et al*., (1986) demonstrated that by limiting the concentrations of salts of magnesium, calcium, potassium, sodium and trace elements, a higher yield of rhamnolipid can be achieved in *P. aeruginosa* DSM 2695. Iron limitation stimulates biosurfactant production in *P. aeruginosa* (Guerra-Santos *et al*., 1984; 1986), whereas addition of iron and manganese salts stimulates biosurfactant production in both *B. subtilis* (Cooper *et al*., 1981) and *Rhodococcus sp.* (Abu-Ruwaida *et al*., 1991).

1.10.4 Environmental Factors

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity. The pH of the medium plays an important role in sophorolipid production by *T. bombicola*, and rhamnolipid production in *Pseudomonas spp*. was at its maximum at a pH range from 6-6.5 and decrease sharply above pH 7 (Guerra-Santos *et al*., 1984). Kim *et al*., (1997) was defined an optimum medium for the production of biosurfactant form *B. subtilis* C٩ at pH (8.0). Optimal surfactant production of *B. lecheniformis* BAS 50 was occurred at temperatures between 35-45ºC (Yakimov *et al*., 1995). Jacques *et al*., (1999) has shown that oxygen is an important parameter to

consider for the biosurfactant production in *B. subtilis* S499. Salt concentration also affected biosurfactant production depending on its effect on cellular activity (Desai and Banat, 1997).

1.11 Natural Roles of Biosurfactants

With increasing numbers of identified microbial emulsifiers, it becomes clear that microbial surfactants have very different structures, are produced by a wide variety of microorganisms and have very different surface properties. Thus, it is expected that bioemulsifiers have various roles, but it is impossible to draw any generalization or to identify one or more functions that are clearly common to all microbial surfactants (Ron and Rosenberg, 2001). Several hypothetical natural roles for biosurfactants have been suggested or demonstrated.

1.11.1 Increasing the Surface Area of Hydrophobic Water-Insoluble Substrates

For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface are between water and oil (Shreve *et al*., 1995). When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. Stated briefly, emulsification is a cell density-dependent phenomenon; that is, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to emulsify oil effectively. One way to reconcile the existing data with these theoretical considerations is to suggest that the emulsifying agents do play a natural role in oil degradation, but not in producing macroscopic emulsions in the bulk liquid (Ron and Rosenberg, 2001).

1.11.2 Increasing the Bioavailability of Hydrophobic Water-Insoluble Substrates

One of the major reasons for the prolonged persistence of high-molecular-weight hydrophobic compounds is their low water solubility, which increases their sorption to surfaces and limits their availability to biodegrading microorganisms (Ron and Rosenberg, 2001). When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited (Van Delden *et al*., 1998). Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or increasing their apparent water solubility (Deziel *et al*., 1996). Recently, it has been demonstrated that alasan increases the apparent solubility of polycyclic aromatic hydrobarbons (PAHs) five-to-20-fold and significantly increase their rate of biodegradation (Barkay *et al*., 1999; Rosenberg *et al*., 1999).

1.11.3 Binding of Heavy Metals

A rhamnolipid biosurfactant has shown to remove cadmium, lead and zinc from soil (Herman *et al*., 1995). The mechanism by which rhamnolipid reduces metal toxicity may involve a combination of rhamnolipid complexation of cadmium and rhamnolipid interaction with the cell surface to alter cadmium uptake. Polysaccharide high-molecular-weight emulsifiers interact with metals by binding them, as has been shown for the binding of uranium by emulsan of *A. calcoceticus* (Zosim *et al*., 1983).

1.11.4 Pathogenesis

Rhamnolipid is considered to be one of the virulence-associated exoproducts of *P. aeruginosa*. Its production is also carried out and regulated in correlation with that of other virulence factors. Thus, an essential protein in the synthesis of rhamnolipids is AlgC, which is also involved in the production of alginate and lipopolysaccharide (LPS). The production of rhamnolipids in *P.*

aeruginosa is under the cell density-dependent control system comprising RhlR-Rhll, which controls the synthesis of two other virulence factors, elastase and the LasA protease (Ron and Rosenberg, 2001).

1.11.5 Antimicrobial Activity

Several lipopeptide surfactants are potent antibiotics (Marahiel *et al*., 1993; Yakimov *et al*., 1995). These include the cyclic lipopeptide of *B. subtilis* surfactin or subtilysin (Peypoux *et al*., 1999) which is known as antibacterial, antitumor compound against Ehrilich ascites carcinoma cells and has anti HIV properties (Noudeh *et al*., 2003), the extracellular hydrophobic peptide, streptofactin is produced by *Stryptomyces tendae* (Richter *et al*., 1998) and the antifungal lipopeptide antibiotic plipastatin of *B. cereus* BMG302-fF67 (Tsuge *et al*., 1996) and the polymyxins produced by *B. polymyxa* and related bacilli (Suzuki *et al*., 1969).

1.11.6 Regulation the Attachment-Detachment of Microorganism to and from Surfaces

It was reported by Neu, (1996), if a biosurfactant is excreted, it can form a conditioning film on an interface, thereby stimulating certain microorganism to attach to the interface while inhibiting the attachment of others. For example, the cell surface hydrophobicity of *P. aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid (Zhang and Miller, 1994), whereas the cell surface hydrophobicity of *Acinetobacter* strains was reduced by the presence of its cell-bound emulsifier (Rosenberg and Rosenberg, 1983). These data suggest that microorganisms can use their biosrufactants to regulate their cell surface properties in order to attach or detach from surface according to need (Ron and Rosenberg, 2001).

1.11.7 Emulsifier Production of Quorum Sensing

Depending on the suggestion that the production of bioemulsifiers by bacteria is correlated with high bacterial cell density. This finding may reflect an indirect correlation with one or more physiological factors, such as the availability of energy, nitrogen or oxygen. It is easy to explain the need for bioemulsifier in bacteria growing on hydrocarbons. As these bacteria are growing at the oil-water interface, the production of emulsifiers when the density is high will increase the surface area of the drops, allowing more bacteria to feed. Alternatively, consumed, as in the case of oil that consists of many types of hydrocarbons, the production of the emylsifier of many types of hydrocarbons, the production of the emulsifier allows the bacteria to detach form the "used" droplet and find a new one (Ron and Rosenberg, 2001).

1.11.8 Role of Bioemulsifier in Biofilms

A recent finding indicates the existence of horizontal transfer of high-molecular-weight emulsifiers from the producing bacteria to heterologous bacteria. When the alasan-producing *A. radioresistens* KA53 was grown together with *A. calcoaceticus* RAG-1, alasan was released from the producing strain and became bound to the recipient RAG-1 cells (Osterreicher-Ravid *et al*., 2000). This horizontal transfer of bioemulsifiers from one bacterial species to another has significant implications in natural microbial communities, co-aggregation and biofilms (Ron and Rosenberg, 2001).

Chapter Two

Materials

and

Methods

Materials and Methods

- **Materials**

2.1 Equipments and Apparatus

The following equipments and apparatus were used throughout the study:-

Equipment	Company (Origin)
Autoclave	Tomy (Japan)
Balance	Ohans (France)
Compound light microscope	Olympus (Japan)
Cooled centrifuge	MSE (U.K)
Cooled shaker incubator	Gallenkamp (England)
Distillator	GFL (Germany)
Oven	Sanyo (Japan)
Freeze-Dryer (Lyophilizer)	Virtis (USA)
Hot plate magnetic stirrer	Gallenkamp
Incubator	Gallenkamp
Millipore filter unit	Millipore Corp (USA)
pH-meter	Mettler-Toledo (U. K.)
Sensitive balance	Delta Range (Switzerland)
Spectrophotometer	Aurora instrument Ltd. UV201 (U. K.)
Tensiometer	Karl kolb (Germany)
Vortex mixer	Sturat Scientific (U. K.)
Water bath	Atom (England)

2.2 Chemicals and Biological Materials

The following chemicals and biological materials were used throughout the study:

Material	Company (Origin)
Ethanol, Glycerol	Analar (England)
α-naphthol, Dipotassium hydrogen phosphate, Dodecane, Ethyl alcohol, HCl, H_2O_2, Mannitol, Peptone, NaOH, urea, Dichloromethane	BDH (England)
Ferric sulphate hydrate, Nickle dichloride hydrate, Sodium nitrate	Biolife (Italy)
Ammonium nitrate, ammonium oxalate, H_3BO_3, chloroform, cupperic sulphate, Ethylendiamine-tetraacetic acid (EDTA), Iodine, Methyl red, KI, KOH, Yeast extract, Tryptone, Zinc sulphate hydrate	Fluka (Switzerland)
Ammonium chloride	May and Baker (England)
Magnesium sulphate hydrate	Mereck (Germany)
Manganes sulphate hydrate	Riedel-DeHaeny (Germany)

2.3 Media

2.3.1 Biochemical Test Media

A. Ready Made Culture Media

These media were prepared as recommended by Manufacturer Company. pH was adjusted to 7.2 and sterilized by autoclaving.

- Methyl red-Voges Proskauer (MR-VP) broth.

- Nutrient agar (Fluka)
- Nutrient broth (Biolife)
- Simmon's citrate agar (Analar)

B. Prepared Culture Media:

- **Blood agar (Harley and Prescotte, 1996)**

Blood agar was used for the screening of biosurafactant producing microorganisms. It was prepared by dissolving 37g of blood agar base in D.W. pH was adjusted to 7.3 and complete volume to 950 ml with D.W. Sterilized by autoclaving. After cooling (45◦C), 50 ml of sterile, defibrinated sheep blood was added and mixed well.

- **Lecithinase Medium (Collee *et al*., 1996).**

It consists of the followings:

Nutrient agar	85 ml
Egg-yolk	15 ml

pH of previously prepared nutrient agar was adjusted to 7.0, sterilized by autoclaving, after cooling to 45^{o}C, 15ml of egg –yolk was aseptically added, mixed well and distributed into Petri dishes.

- **Mannitol Fermentation Medium (Collins and Lyne, 1987)**

This medium consists of the followings:

Component	g / l
$(NH_4)\ H_2PO_4$	1.0
KCl	0.2
$MgSO_4$	0.2
Agar	10.0

To the above basal medium, 4ml of 0.2% bromothymol blue was added, dissolving in D.W. and sterilized by autoclaving, and a final 1% of sterile (filtered) mannitol was added.

2.3.2 Biosurfactant Production Media

A- Jacques *et al*., Medium (1999)

This medium consists of the followings:

Component	g / l
KH_2PO_4	1.9
Sucrose	20
Peptone	30
$MnSO_4$ - $MgSO_4$ solution (2.4)	9 ml
Yeast extract	7
Trace elements solution (2.4)	1 ml

pH was adjusted to 7.0 and sterilized by autoclaving.

B- Mineral Salt (Bushnell – Hass) Medium (Patel and Desai, 1997)

This medium consists of the followings:

Component	g / l
KH_2PO_4	1
K_2HPO_4	1
NH_4NO_3	1
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
$FeSO_4.7H_2O$	0.05
Yeast extract	0.5
Trace element solution (2.4)	1 ml

pH was adjusted to 7.0 and sterilized by autoclaving.

2.4 Reagents and Solutions

- **Catalase Test Reagent (Atlas *et al*., 1995)**

This reagent composed of 3% H_2O_2.

- **Oxidase Test Reagent (Harley and Prescott, 1996)**

To prepare this reagent one gram of dimethyl-p-phenylenediamine dihydrochloride was dissolved in 100 ml distilled water and kept in dark bottle at 4°C.

- **Methyl Red Reagent (Harley and Prescott, 1996)**

It was prepared by dissolving 0.1 g of methyl red in 300 ml (95%) ethyl alcohol then the volume was completed to 500 ml with distilled water in a volumetric flask.

- **Barritt's Reagent (Harley and Prescott, 1996)**

This reagent was consisting of two solutions and as follows:

Solution A: 40% potassium hydroxide in distilled water.

Solution B: 5% α-naphthol in (95%) ethyl alcohol.

- **Molisch's Reagent (Sadasivam and Manickam, 1996)**

It was prepared by dissolving 5% 1-naphthol in alcohol (95%).

- **Gram's Stain (Harley and Prescott, 1996)**

It was prepared as follows:

Crystal Violet Solution:

This solution was prepared by mixing the following two solutions:

Solution A: Two grams of crystal violet was dissolved in 20 ml of 95% ethanol.

Solution B: 0.8 g of ammonium oxalate was dissolved in 80 ml distilled water.

The two solutions were mixed, filtered, and kept in a bottle.

Iodine Solution:

It was prepared by dissolving iodine (1g) and KI (2g), in 300 ml of distilled water.

Safranin Solution:

It was prepared by dissolving 0.25 g of safranin O in 10 ml of 95% ethanol, then it was completed to 100 ml with distilled water in a volumetric flask.

- **Trace Elements Solution (Vater *et al*., 2002)**

It was prepared by dissolving 2.32 g of $ZnSO_4.7H_2O$, 1.78g of $MnSO_4.4H_2O$, 1g of $CuSO_4.5H_2O$, 0.56 g of H_3BO_3, 1 g of EDTA, 0.004 g of $NiCl_2.6H_2O$, and 0.66 g of KI in a suitable volume of distilled water, and then it was completed to 1000 ml with distilled water in a volumetric flask.

- **$MnSO_4$ - $MgSO_4$ Solution (Jacques *et al*., 1999)**

It was prepared by dissolving 0.4 g of $MnSO_4.H_2O$ and 5.0 g of $MgSO_4$ in 1000 ml D.W.

• Methods

2.5 Sterilization Methods (Collins and Lyne, 19^7)

- Culture media (liquid and solid), and solutions were sterilized by autoclaving at 121^{o}C, 15Ib/in^{2} for 15 minutes.
- Glasswares (cylinders, Petri dishes, etc…) were sterilized in an electric oven at 180-200^{o}C for 2 hours.
- Thermolabile components or materials (such as sugars) were sterilized by filtration through Millipore filter paper (0.22μm).

2.6 Soil Samples Collection

Eighteen soil samples were collected from different locations, of Baghdad, and Basra governorates. Samples were taken from just below the soil surface of contaminated soils with hydrocarbons beneath cars fuel stations, oil refineries and other uncontaminated sites.

2.7 Isolation of Bacteria

Four grams of each soil sample was transferred to a container, 20ml of sterile D.W. was added and then heated in a water bath at 80^{o}C for 10 minutes while the content was carefully agitated. After cooling, 0.1 ml of soil sample solutions was spreaded on a nutrient agar plates, and then incubated aerobically at 30^{o}C for 24 hrs (Claus and Berkeley, 1986).

2.8 Screening of Hemolytic Bacteria

Bacterial isolates were plated on blood agar plates (2.3.1.B) and incubated at 30^{o}C for 27hrs. Hemolytic activity was measured as the presence of a definite clear zone around each colony (Bicca *et al*., 1999). Subsequently the concerned *Bacillus* colonies were selected.

2.9 Maintenance of Bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*. (1982) and as follows:

- **Short Term Storage**

Bacterial isolates were maintained for periods of few weeks on the surface of nutrient agar plates. The plates were tightly wrapped in parafilm and stored at 4^{o}C.

- **Medium Term Storage**

Bacterial isolates were maintained in stab culture for period of few months. Such cultures were prepared in screw-capped bottles containing 5-8 ml of nutrient agar medium and stored at 4^{o}C.

- **Long Term Storage**

Bacterial isolates can be stored for many years in nutrient broth medium containing 15% glycerol at freezing temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth phase of bacteria in a screw-capped bottle with final volume 10 ml and stored at -20^{o}C.

2.10 Identification of Bacterial Isolates

According to Claus and Berkeley (1986), Health Protection Agency (2003) and Fritze (2004), the following tests were employed to identify the suspected *Bacillus spp.* Isolates.

2.10.1 Morphological Tests

- **Gram's Stain (Harely and Prescott, 1996)**

A single colony was transferred by a loop to a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with ethanol (95%), and counterstained with safranine, then examined by a microscope.

- **Endospores and Features (Claus and Berkeley, 1986)**

Cultures of different age were used for detection of endospores, endospores position within sporangium, and presence or absence of parasporal crystals by means of phase contrast microscopy. Color, shape, and appearance of colonies were also detected.

- **Motility Test (Collins and Lyne, 1987)**

Hanging drop method was applied, in which a very small drop of liquid bacterial culture was placed in the center of a 16 mm square cover – glass, with the aid of inoculating loop. The cover – glass was inverted over the microslide (well-slide). After adherence of cover- glass to the slide, the hanging drop was suspended in the well, and the slide was examined under microscope.

2.10.2 Biochemical Tests

- **Catalase Test (Atlas *et al*., 1995)**

One drop of H_2O_2 (3%) was added to a loopfull of bacterial culture on a microscope slide, formation of gaseous bubbles indicates a positive result.

- **Oxidase Test (Harely and Prescott, 1996)**

Filter paper was saturated with the substrate (dimethyl-ρ-Phenyl-enediamine dihydrochloride), and then colony of bacteria to be tested was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive test result.

- **Lecithinase Test (Collins and Lyne, 1987)**

The holes which were made within lecithinase medium plates were filled with 50µl of 18 hrs bacterial culture, incubated at 30^{o}C for 48 hrs. Lecithinase-producing colonies were surrounded by zone of opacity.

- **Mannitol Fermentation Test (Collins and Lyne, 1987)**

Tubes of carbohydrate fermentation media (2.3.1) were inoculated with overnight growth, incubated at 30^{o}C for 48 hrs. After incubation, color change was observed to determine the carbohydrate utilization.

- **Citrate Utilization (Atlas *et al*.,1995)**

This test was an indicator for utilization of citrate as a sole carbon source. In this test, slant of Simmon's citrate was inoculated by means of streaking, incubated at 30^{o}C for 5 days. The development of deep blue color indicates positive results.

- **MR-VP Test (Maza *et al*., 1997)**

Tubes of liquid MR-VP media were inoculated with 100 µl of test organism and incubated at 30^{o}C for 48 hrs. Both tests were performed from the same culture, which was divided for testing. Methyl red (MR) test was performed after adding about five drops of MR reagent prepared in (2.4), bright red color indicates a positive results and negative test is yellow, while the Voges-Proskauer (VP) test was performed after adding the VP reagents prepared in (2.4). Developing a pink color indicates positive a result.

2.11 Surface Tension Measurement (Vater *et al*., 2002)

Cultures of the primarily selected isolates were grown in Jacques media (2.3.2.A) at 30^{o}C in a shaker incubator (180 rpm) for 72 hrs, then it was centrifuged (8000 rpm) at 4^{o}C, for 15 min. Surface tension (ST) of the cell- free supernatant was measured by ring method using tensiometer (DuNoüy) (figure 2-1). Before the measurement, tensiometer was calibrated with distilled water (72 mN m^{-1}), chloroform (27.1 mN m^{-1}), and glycerol (64 mN m^{-1}). Then the obtained reading of each sample was recorded at the moment in which surface – immersed ring breaks away from the liquid surface. Surface tension (ST) readings were then recorded.

Figure (2-1) DuNoüy K6 tensiometer used for surface tension measurement.

2.12 Ability of Bacterial Isolates to Produce Biosurfactant

Fifty ml of Jacques *et al*., medium (2.3.2 A) were dispensed in Erlenmyer flasks (250 ml). Each flask was inoculated with 0.5 ml of the fresh bacterial culture (16 hrs old). Inoculated flasks were incubated into a shaker incubator (180 rpm), at 30^{o}C, for 72 hrs. The surface tensions were measured as described previously (2.9).

2.13 Growth Curve of *Bacillus cereus* HI-2 Isolate

The selected *Bacillus cereus* HI-2 isolate was grown in nutrient broth at 30^{o}C in a shaker incubator (180 rpm) for 18 hrs, and then 500ml Erlenmyr flasks containing 100 ml of nutrient broth were inoculated with 1 ml (1%) of the above culture. Flasks were incubated in a shaker incubator (180 rpm) at 30^{o}C for 24 hrs. During this period, samples (2ml) were taken (2 hours intervals) to measure the optical density using spectrophotometer at wave length 600 nm (Rodriguez and Tait, 1983).

2.14 Assay of Emulsification Activity (Gurjar *et al*., 1995)

The emulsification activity (EA) of the crude emulsifier was determined by the method of Johnson *et al*. (1992). Bacterial isolates were grown by inoculating 20 ml of mineral salt medium (2.3.2 B) with 0.2 ml of fresh bacterial culture and incubated in a shaker incubator (180 rpm) at 30°C, for 72 hrs. After centrifugation (4000 rpm) at 5°C; to 10 ml of cell-free broth (crude emulsifier solution) 0.1 ml of dodecane was added and the mixture was shaken vigorously on a vortex mixer for 10 min and left undisturbed. After 1h, the optical density of the oil-in-water emulsion phase was recorded at 610 nm. The optical density was reported as emulsification activity. Blank containing 10 ml of sterile production medium, was used to calibrate the results.

2.15 Optimization of Biosurfactant Production

The optimization experiments was carried out throughout the dispensing of 50 ml of mineral salt medium (2.3.2 B) in 250 ml Erlenmyer flasks, inoculated with 1% of mid-exponential phase culture of the *Bacillus cereus* HI-2 isolate. The flasks were incubated in a shaker incubator (180 rpm) at 30^{o}C. After incubation period, surface tension, and emulsification activity, was measured for each samples in the experiment.

2.15.1 Effect of Carbon Source

Mineral salt medium was supplemented with one of the following carbon sources:

- Carbohydrate source: (Sucrose, date extract) added at a concentration 2% (w/v, v/v) respectively.
- Hydrocarbon sources: (crude oil, kerosene, soybean oil, sunflower oil, ethanol, and burned-engine oil) added at a concentration 2% (v/v).

Flasks were incubated in a shaker incubator (180 rpm) at 30^{o}C for 72 hrs, surface tension (2.11) and emulsification activities (2.14) were measured.

2.15.2 Effect of Carbon Source Concentration

The optimum carbon source (soybean oil) was added at different concentrations (0.5%, 1%, 2%, 3%, 4% and 5% v/v) to the production medium (2.3.2 B). pH was adjusted to 7.0, and then incubated in a shaker incubator (180 rpm) at 30^{o}C for 72 hrs.

Surface tension and emulsification activity were measured and then the optimal concentration was employed later on.

2.15.3 Effect of Nitrogen Sources

Mineral salts medium containing 0.1% of different nitrogen sources such as peptone, tryptone, urea, ammonium nitrate, and ammonium chloride, was added to determine the optimal nitrogen source for biosurfactant production. pH

was adjusted to 7.0, then flasks were incubated in a shaker incubator (180 rpm) at 30^{o}C for 72 hrs. The surface tension and emulsification activity were measured, and the selected optimal concentration was employed later on.

2.15.4 Effect of Nitrogen Source Concentration

The optimal nitrogen source (NH_4NO_3) which was shown better results than others was added in a gradual concentrations 0.05%, 0.1%, 0.2%, 0.4%, and 0.6% to the production medium, pH was adjusted to 7.0 and incubated in a shaker incubator (180 rpm) at 30^{o}C for 72 hrs. Surface tension and emulsification activity were estimated. The optimal concentration was employed in the later experiments.

2.15.5 Effect of Phosphate Concentration

Different concentrations of KH_2PO_4 (0.1, 0.5, 1.0, 1.5, 2.0 g/l) were examined to determine the optimum phosphate concentration required to be added to the production medium. After the pH adjustment to 7.0, flasks were incubated in a shaker incubator (180 rpm) at 30^{o}C for 72 hrs. Both of surface tension and emulsification activity were measured, and the obtained optimal phosphate concentration was employed in the later experiments.

2.15.6 Effect of Medium pH

The production medium was adjusted in different pH values (5, 6, 7, 8 and 9) to determine the suitable value. Then cultures were incubated in a shaker incubator (180 rpm) at 30^{o}C for 72 hrs. Surface tension and emulsification activity were measured, and then the optimal pH value was employed in the later experiments.

2.15.7 Effect of Temperature

The parameters which were determined for the biosurfactant best production previously were carried out in this experiment. Medium was inoculated, and incubated at different temperatures 25, 30, 35, and 40°C for 72 hrs. Optimal temperature was subsequently employed, depending on surface tension and emulsification activity measurements.

2.15.8 Effect of Incubation Period

According to the previously performed optimization for the biosurfactants production, flasks containing the optimum medium were incubated in a shaker incubator (180 rpm) at the optimum temperature for 24, 48, 72, and 96 hrs. Biosurfactant production was evaluated every 24 hrs depending on surface tension and emulsification activity measurement.

2.16 Partial Purification and Characterization of Biosurfactant Produced by *Bacillus cereus* HI-2

2.16.1 Isolation and Purification of Biosurfactant (Noudeh *et al.*, 2003)

Bacillus cereus HI-2 isolate was grown by inoculating 200 ml of optimal production medium (dispensed in 1000 ml Erlenmyer flask) with 0.2 ml of mid-log phase bacterial culture and incubated in a shaker (180 rpm), at 30^{o}C, for 48 hrs. Bacterial cells were removed from the liquid culture by centrifugation at 10000 rpm for 25min at 10^{o}C; then the supernatant (crude biosurfactant) was acidified by adding 6 N HCl.

At pH 2.0, a flocculated precipitate was formed and collected by centrifugation (10000 rpm), at 10^{o}C for 20 min. The precipitate was dried under vacuum in a dissicator, and kept overnight at 4^{o}C. The crude product was resuspended in dichloromethane followed by over night stirring; suspension was

filtered through Whatman No.1 filter paper to remove the coarse impurities. The filtrate was then extracted twice with equal volumes of distilled water (pH 8.0) with stirring for 20min. after this period, it was left 3h in a separating funnel to allow the two phases to separate. The aqueous phases containing the biosurfactant were collected and acidified to pH 2.0 by 6 N HCl. The biosurfactant, which was precipitated in the form of white to yellowish crystals was recovered by centrifugation 10000 rpm, for 15 min, dried under freeze dryer and weighed to determine the yield.

2.16.2 Detection Tests

A. Molisch's Test (Sadasivam and Manickam, 1996)

Two drops of Molisch's reagent (2.4) was added to about 2 ml of purified biosurfactant solution and mixed well, then the tube was inclined and bout 1 ml of concentrated sulphric acid was added along the sides of the tube. Appearance of red-violet ring at the junction of the two liquids indicates it contain sugar moiety.

B. Biuret Test (Sadasivam and Manickam, 1996)

To 2ml of purified biosurfactant solution 2 ml of 10% NaOH was added and mixed well. Then two drops of 0.1% $CuSO_4$ solution was added. Observation of violet or pink color indicates presence of peptide bond.

2.16.٣ Characterization

2.16.3.1 FTIR Analysis

Nature and chemical structure of the purified biosurfactant was examined using the Fourier transformed infrared spectroscopy (FTIR) in order to characterize the chemical nature of a compound. FTIR spectrometry, an advanced type of infrared (IR) spectrometry, will give the functional groups that are found in the compound in order to propose a possible chemical structure of the test compound.

Chapter Three

Results

and

Discussions

Results and Discussions

3.1 Isolation and Identification of Biosurfactant-producing *Bacillus* spp.

3.1.1 Isolation

Eighteen soil samples were collected from different hydrocarbon contaminated and uncontaminated areas of soils during the period from 12 November 2003 to 1 February 2004.

Sixty-three bacterial isolates were isolated as shown in table (3-1). The obtained isolates (63) were suspected to be *Bacillus* spp. according to the isolation method which was proper for the detection and selective isolation of *Bacillus* genus (Claus and Berkeley, 1986) also because this genus is a common soil organism and the members ability of this genus in production of biosurfactants is well documented (Georgious *et al*., 1992; Rouse *et al*., 1994; Shafi and Khanna, 1995; Banat, 1995a).

Previous observations indicated that the biosurfactant producers were thought to be restricted to contaminated soil where conditions would select for microbes with an enhanced ability to utilize hydrocarbons (Rouse *et al*., 1994; Willumsen and Karlson, 1997; Volkering *et al*., 1998). Additionally, *B. licheniformis* BAS 50, has been isolated form a deep oil well, and produced a biosurfactants when cultured on a variety of substrates (Yakimov *et al*., 1995). However, a small production have been demonstrated in unimpacted soils and may be a reflection of the other roles biosurfactants play in a soil ecosystem, functioning as biocides, fungicides, and nutrient transport molecules (Banat, 1995a and 1995b; Lin, 1996; Jennings and Tanner, 2000). Therefore, detecting a

biosurfactant producer population from uncontaminated soils was not necessarily surprising.

Table (3-1) Soil origin, types and numbers of biosurfactant-producing *Bacillus* spp.

Region	Types of Soil Sample	Number of Samples	Number of Isolates	Number of Biosurfactant Producers
1. Al-Hussein City	Uncontaminated	3	8	4
2. Al-Doorah Oil Refinery	Contaminated	4	18	15
3. Cars Fuel Stations (Baghdad)	Contaminated	4	14	3
4. Industrial Area (Basrah)	Contaminated	2	3	0
5. AL-Jadryia (Al-Nahrain University Fields)	Uncontaminated	3	11	6
6. Al-Hussein City	Contaminated	2	9	6
Total	—	18	63	34

In order to determine the ability of the bacterial isolates to produce biosurfactant, all isolates (63) were grown on blood agar plates. Results indicated that 34 isolates shown haemolytic activity β-haemolysis (Figure 3-1). So that haemolytic activity was regarded as indicator for biosurfactant production and used as a rapid method for bacterial screening (Brenheimer and Avigade, 1970; Banat, 1995a; Lin, 1996).

Figure (3-1) Haemolytic activity of biosurfactant producer isolates (*Bacillus* spp.).

3.1.2 Identification of Bacterial Isolates

The collected isolates (63) were identified depending on morphological and biochemical characteristics. Morphologically, the bacterial isolates revealed different colonial appearance, so they were showed differences in translucence, opaqueness, or more or less whitish or cream colored. The colonies of all isolates were varying in size and some of them were mucoid. Prior investigations showed that colony diameter depends largely on the number of colonies developing on the plate as well as on the concentration of nutrients and the amount of nutrient agar poured into the Petri dish (Claus and Berkeley, 1986).

Microscopic examination of the bacterial isolates showed that their cells occurred singly or in chains, spore forming, Gram-positive rods, and spore shapes were ellipsoidal, either central or terminal. These results were indicators of *Bacillus* spp., also the whole isolates were examined to produce Catalase, and gave a positive results. It was demonstrated that most species of *Bacillus* genus was catalase producers (Claus and Berkeley, 1986).

Furthermore, the selected efficient biosurfactant producer isolate that gave a minimum surface tension measurement (Table 3-3), was subjected to additional biochemical and physiological tests to be determined at species level. Results shown in (Table 3-2) indicated that the examined isolate was belonged to *Bacillus cereus*, which was subsequently symbolized as *B. cereus* HI-2 isolate, and this result was in agreement with Claus and Berkeley, (1986); Holt *et al*. (1994) and Fritze, (2004). The ability of *B. cereus* for biosurfactant production was revealed by Jennings and Tanner, (2000) and Tuleva *et al*. (2005).

Table (3-2) Morphological and phenological characteristics of the *Bacillus* HI-2 isolate.

Characteristic	*Bacillus* HI-2
Cell shape	R
Parasporal Crystals	–
Motility	+
Gram stain	+
Catalse	+
Oxidase	–
Vogas-Proskauer	+
Methyl Red	–
Acid from D-Mannitol	–
Egg-Yolk Lecithinase	+
β-haemolysis	+
Citrate Utilization	+

R: Cell form rods; +: positive; –: negative.

3.2 Detection of Biosurfactants Producer Isolates

Potentially biosurfactant producer isolates (34 isolates) were examined for their ability in production of biosurfactants which was detected by the surface tension lowering of cell-free supernatant of each isolate.

Results (Table 3-3) showed that all the isolates were capable in minimizing the surface tension but in different degrees, so that, the isolates referred HC-5, HG-1, HI-1, HI-2, HJ-1, HK-2, HL-4, HO-3 and HR-1 were seems to be efficient in lowering the surface tension (ST) to less than 50 mN m^{-1}, although they were isolated from different areas. The minimum surface tension value 45 mN m^{-1} was obtained by HI-2 as compared with the control (65 mN m^{-1}), as a consequence of this result, it was subsequently studied.

Table (3-3) Surface tension of culture supernatants of β-haemolytic *Bacillus* spp. grown in Jacques medium containing 2% sucrose with shaking (180 rpm), at 30°C for 72 hrs.

Isolate	Symbol	Surface Tension (mN m^{-1})	Isolate	Symbol	Surface Tension (mN m^{-1})
Control*	–	65	*Bacillus sp.*	HJ-1	47
Bacillus sp.	HC-3	51	*Bacillus sp.*	HJ-2	51
Bacillus sp.	HC-4	52	*Bacillus sp.*	HJ-3	55
Bacillus sp.	HC-5	49	*Bacillus sp.*	HJ-5	51
Bacillus sp.	HD-2	53	*Bacillus sp.*	HK-2	47
Bacillus sp.	HE-2	59.5	*Bacillus sp.*	HK-3	51
Bacillus sp.	HF-1	54	*Bacillus sp.*	HL-4	46
Bacillus sp.	HF-2	50	*Bacillus sp.*	HN-1	59
Bacillus sp.	HG-1	48.5	*Bacillus sp.*	HN-2	59
Bacillus sp.	HH-1	58	*Bacillus sp.*	HN-4	50
Bacillus sp.	HH-2	56	*Bacillus sp.*	HO-2	52
Bacillus sp.	HH-3	54	*Bacillus sp.*	HO-3	48
Bacillus sp.	HH-4	54	*Bacillus sp.*	HO-4	59
Bacillus sp.	HH-5	55	*Bacillus sp.*	HP-1	60
Bacillus sp.	HI-1	46	*Bacillus sp.*	HQ-2	58.5
Bacillus sp.	HI-2	45	*Bacillus sp.*	HQ-4	54
Bacillus sp.	HI-3	53	*Bacillus sp.*	HR-1	49
Bacillus sp.	HI-5	55			

Control*: The medium without carbon source.

It was shown that reduction of surface tension is considered as a selection criterion for biosurfactant-producing capacity of microorganisms in a liquid medium (Cooper *et al*., 1981; Carrillo *et al*., 1996; Randhir, 1997). Due to the efficiency of surface-active compounds produced by the *Bacillus* spp. a number of studies were conducted towards this field (Arima *et al*., 1968; Bernheimer and Avigad, 1970; Nakano *et al*., 1988; Morikawa *et al*., 1992).

Furthermore, the low-molecular weight biosurfactants (Glycolipids, lipopeptides) were found to be more effective in lowering the interfacial and surface tensions (Banat, 1995a; Lin, 1996; Cameotra and Makkar, 1998).

3.3 Growth Curve of *B. cereus* HI-2

When the OD_{600} reading versus time was plotted, the curve was generated in an attempt to specify the growth curve of this isolate. Results showed in figure (3-2) refers that a little cell division occurred during the first 4 hrs, and this period called the lag phase. During the period of 6 to 12 hrs, a rapid cell division was occurred and because of continuous doubling in cell number this period of growth was called the logarithmic (log) phase. Within the period of 14 to 22 hrs, the culture entered the stationary phase as a result of nutrient consumption leading to a slower rate of cell division. Then the culture enters the death phase within 24 hrs. It has been mentioned that most strains have entered cell death phase within 24 hrs (Stephenson, 2003).

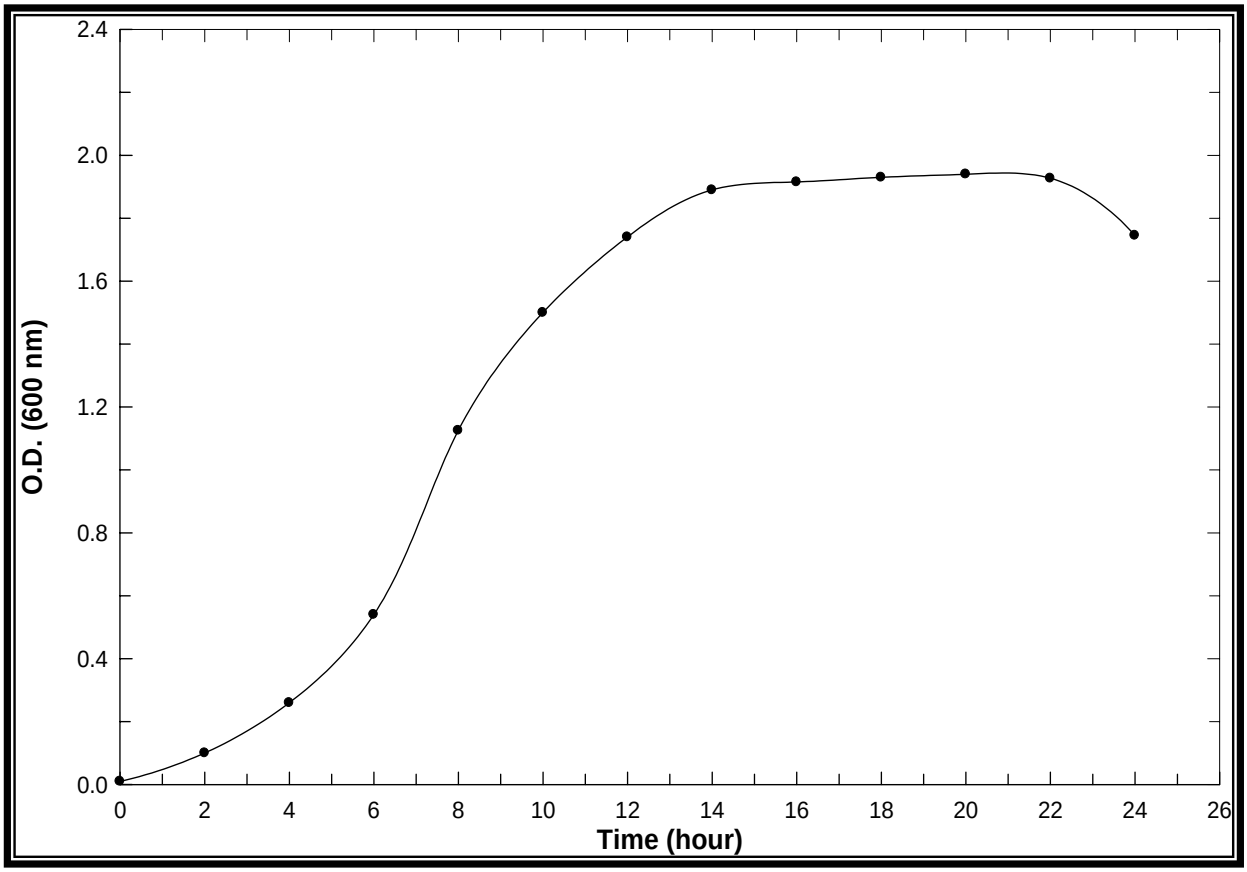


Figure (3-2) Growth curve for *B. cereus* HI-2 grown in nutrient broth (pH 7) with shaking (180 rpm) at 30°C.

3.4 Optimization of Biosurfactant Production from *B. cereus* HI-2

3.4.1 Effect of Carbon Source

Effect of carbon source on the production of biosurfactant was elucidated in figure (3-3). It has been shown, depending on the obtained results of surface tension and emulsification activity, that the growth on water-insoluble organic substrates (soybean oil and sunflower oil) were accompanied by an obvious changes in surface tension and emulsification activity which was seems to be better than the obtained results from water-soluble organic substrates (glucose, date juice and ethanol) and these findings agreed with previous study showed

that during growth of *B. subtilis* 22BN on n-hexadecane, there was a drop from 71 to 38.3 mN m^{-1} within 24 hrs of cultivation (Christova *et al*., 2004).

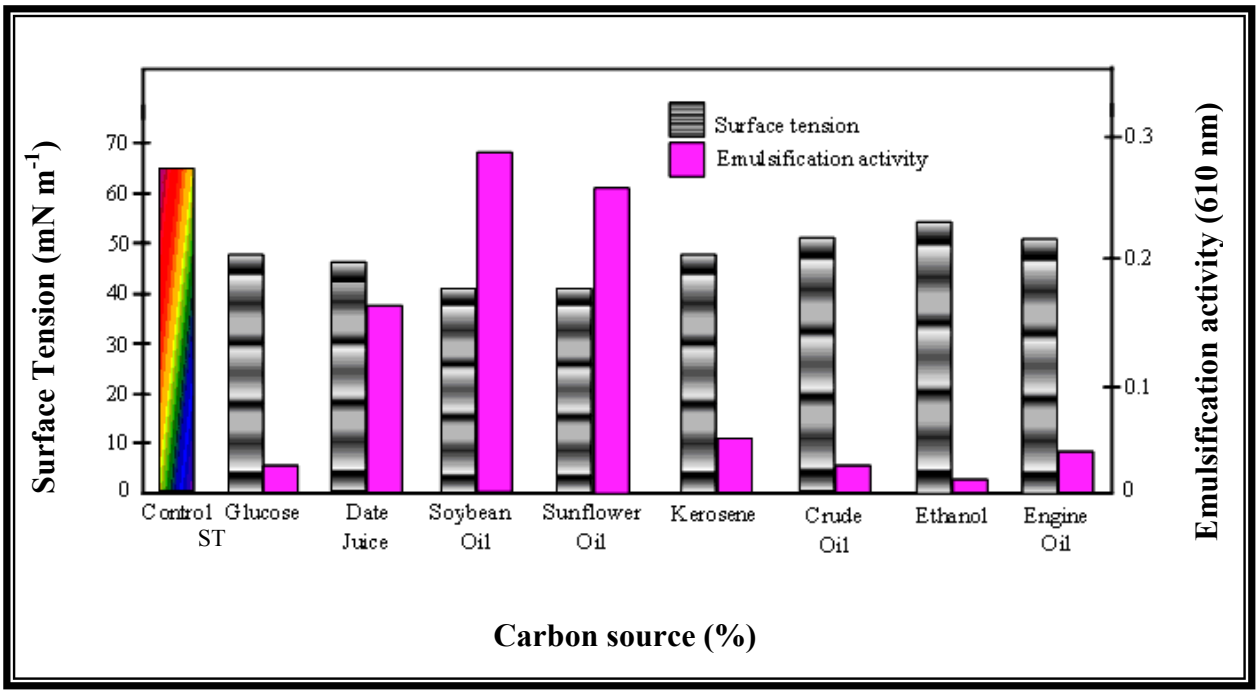


Figure (3-3) Effect of carbon source (2%) on biosurfactant production from *B. cereus* HI-2, grown at 30°C in a shaker incubator (180 rpm) for 72 hrs.

Hydrocarbon-degrading microbes produce a variety of biosurfactants as part of their cell surface or as molecules released extracellularly (Sar and Rosenberg, 1983; Burd and Ward, 1997; Makkar and Cameotra, 2002). While biosurfactant production comes form growth on water-soluble substrates was low due to the simplicity and bioavailability of these substrates (Banat *et al*., 1991). Therefore, Tuleva *et al*., (2005) found that the *B. cereus* 28 BN grew well and produced effective biosurfactants in the presence of n-alkanes, naphthalene, crude oil and vegetable oils.

Results (Figure 3-3) showed that the higher emulsification activity and the lower surface tension values obtained with soybean oil and sunflower oil, but the highest emulsification activity 0.29 and the lowest surface tension 40 mN m^{-}

was achieved with soybean oil and as a consequence of these results, soybean oil was subsequently used.

Gurjar *et al*., (1995) patently showed that maximum emulsification activity was found to be culture density-associated in crude oil-containing medium. Furthermore, the reduction of surface tension and emulsifying activity do not necessarily correlate (Willumsen and Karlson, 1997).

3.4.2 Effect of Carbon Source Concentration

Different concentrations (0.5%, 1%, 2%, 3%, 4% and 5%) of soybean oil, which was chosen as the best carbon source for biosurfactant production, were used to grow the *B. cereus* HI-2 in order to determine the optimum concentration. Results shown in figure (3-4) indicates that the gradual increasing of carbon source concentration accompanied by a dropping of surface tension and increasing in emulsification activity, which was in turn an indicator of biosurfactant production, then after, these dramatic changes in surface tension and emulsification activity reached to its better values 38.6 mN m^{-1} and 0.5 respectively at a concentration of 4% , and then higher surface tension coincided with lower emulsification activity obtained beyond this concentration and these results were comparable with a previous studies (Persson *et al*., 1988; Robert *et al*., 1989; Saifour *et al*., 2004).

Also Kosaric, (2001) was mentioned that hydrocarbon concentration play a role in biosurfactants is synthesized by microorganisms grown on water immiscible hydrocarbons (Abu-Ruwaida *et al*., 1991). Negative effect of higher carbon source concentration may reflect the toxic effect to the producing organisms (Duvnjak and Kosaric, 1985; Johnson *et al*., 1992).

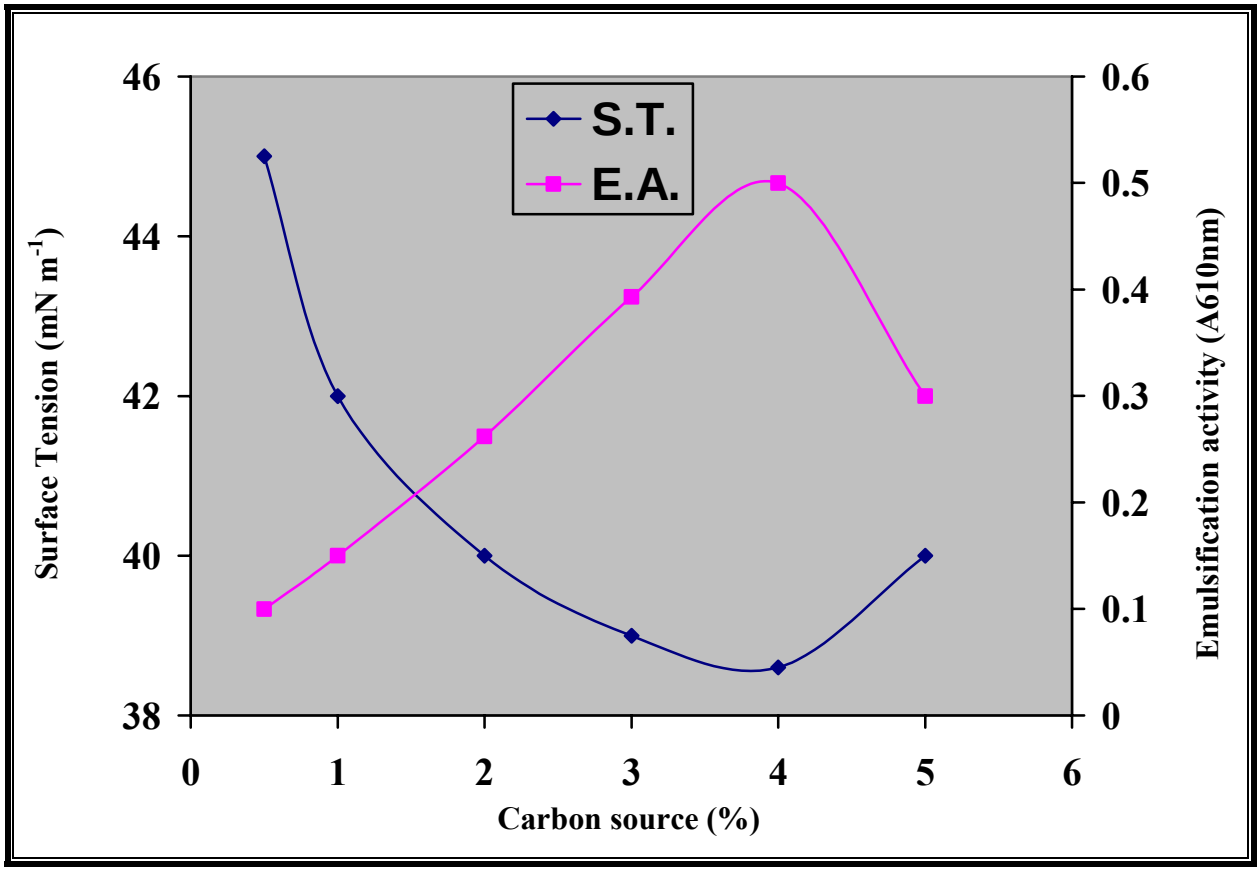


Figure (3-4) Effect of carbon source (Soybean Oil) concentration on biosurfactant production form *B. cereus* HI-2, growth at 30°C in a shaker incubator (180 rpm) for 72 hrs.

3.4.3 Effect of Nitrogen Source

The obtained results mentioned in figure (3-5) varied due to the effects of different nitrogen sources on biosurfactant production. The lowest surface tension (38 mN m^{-1}) and the efficient emulsification activity (0.52) was obtained by using ammonium nitrate as a nitrogen source in production medium, therefore it has been chosen in the subsequent experiments. Such effect was also noticed with other nitrogen sources, in which urea gave a surface tension measurement 40 mN m^{-1} and emulsification activity (0.26) whereas the other sources gave relatively lower results.

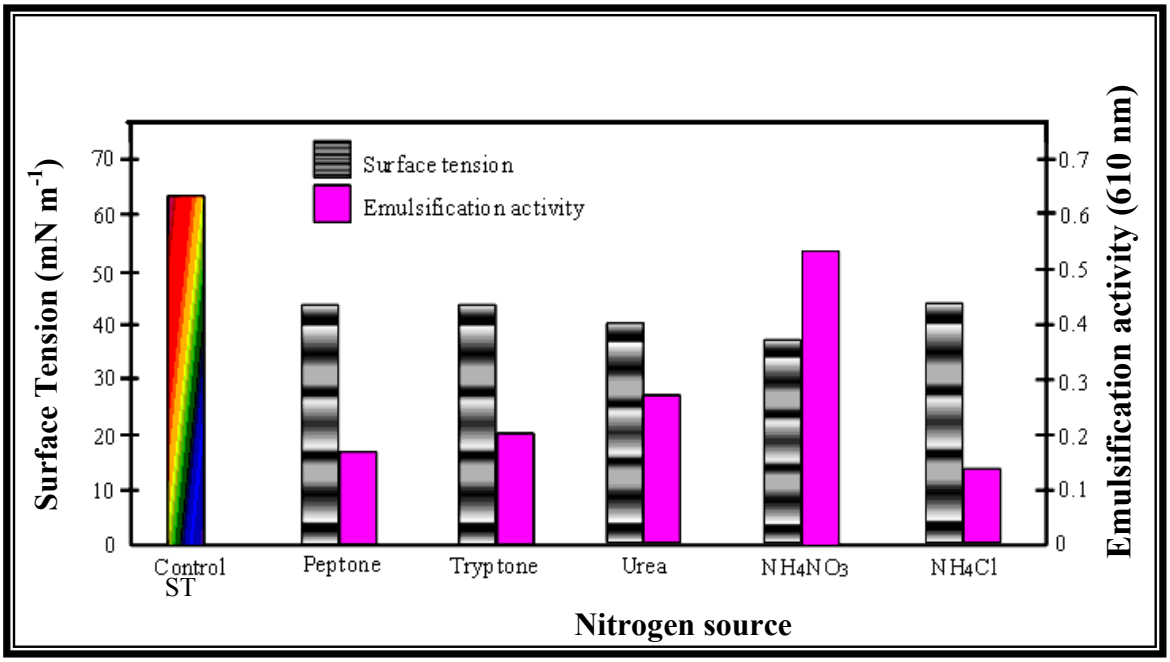


Figure (3-5) Effect of nitrogen source on biosurfactant production from *B. cereus* HI-2, grown with 4% soybean oil, at 30°C in a shaker incubator (180 rpm) for 72 hrs.

It was shown that the ammonium carbonate (NH_4HCO_3), and ammonium nitrate (NH_4NO_3) which was used in Cooper's medium were positively affected biosurfactant production (Yakimov *et al.*, 1995; Kim *et al.*, 1997). Another study showed that better production was obtained in the medium containing nitrate as the nitrogen source (Guerra-Santos *et al.*, 1984). In addition, Makkar and Cameotra (1998), showed that maximum amount of biosurfactant was obtained when nitrate ions were supplied as the nitrogen source.

3.4.4 Effect of Nitrogen Source Concentration

The production medium was supplemented with different concentrations of ammonium nitrate (NH_4NO_3) ranging between 0.05 and 0.6% to determine the optimum concentration required from nitrogen compounds. Results in figure (3-6) showed that using 0.4% of ammonium nitrate as a nitrogen source in production medium enhance the ability of *B. cereus* HI-2 in biosurfactant

production , as a result of surface tension decreasing and emulsification activity increasing of the culture filtrate to 38 mN m^{-1} and 0.53 respectively.

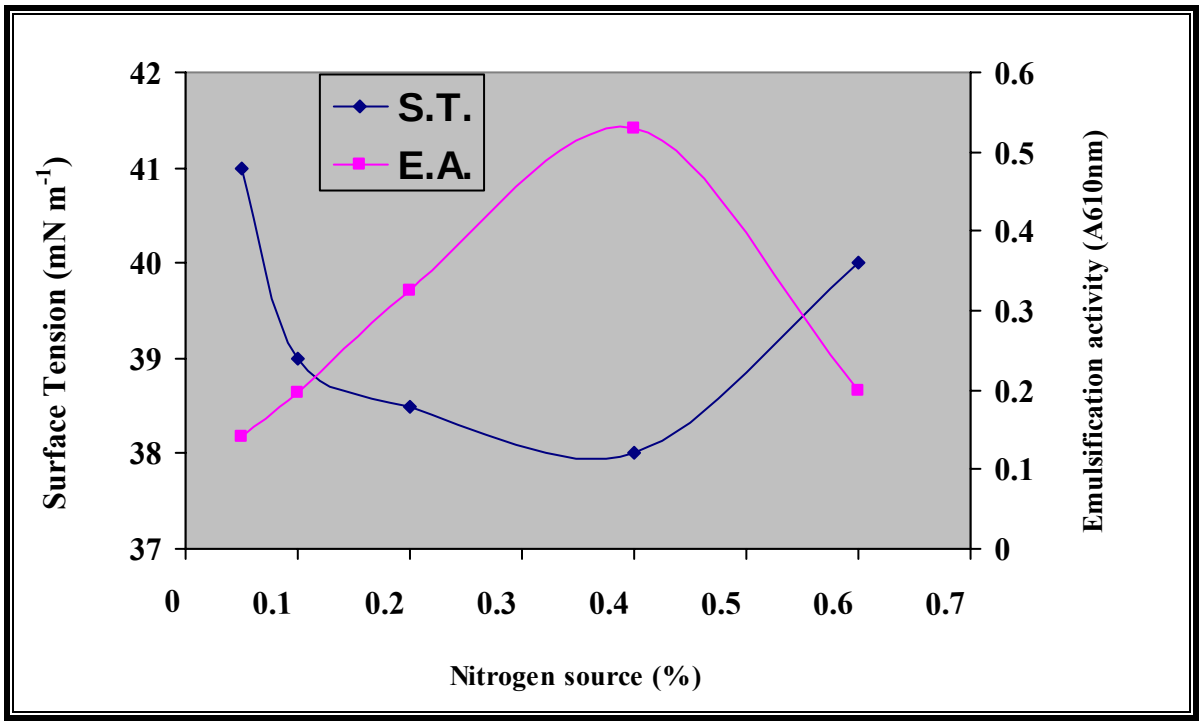


Figure (3-6) Effect of nitrogen source [NH_4NO_3] on biosurfactant production form *B. cereus* HI-2, grown with 4% soybean oil, at 30°C in a shaker incubator (180 rpm) for 72 hrs.

A slightly better production was obtained at this concentration, as indicated by the minimum surface tension and the maximum emulsification activity reading of the cultures broth as compared with results of the other concentrations of NH_4NO_3. Whereas the surface tension was increased to 41 mN m^{-1} coincided with a lowering in emulsification activity to 0.14 at a concentration of 0.05% of NH_4NO_3 referring to that a less amount of bioemulsifiers were produced by the locally isolated *B. cereus* HI-2. As a consequence of these results, 0.4% of ammonium nitrate was used in the later optimization experiments.

In other study it was demonstrated that above 0.4% $NaNO_3$, biosurfactant (rhamnose) was no longer detected; while at increasing N limitations, there was

a shift in the metabolism which was manifested by decreasing of both biomass concentration and surface tension, so they were patented that an optimum carbon-to-nitrogen ratio was of 181 (Guerra-Santos *et al*., 1984).

3.4.5 Effect of Phosphate Concentration

Phosphate represents another important factor influencing the bacterial metabolism. To evaluate the effect of the phosphate concentration on biosurfactant production from the *B. cereus* HI-2, the production medium was supplemented with different concentrations of KH_2PO_4 ranging between 0.1 to 2 g/l. Results represented in figure (3-7) indicated that the maximum production of biosurfactant was obtained at a concentration of 0.5 g/l of KH_2PO_4, at this concentration surface tension of culture filtrate decreased to 36 mN m^{-1} while the emulsification activity increased to 0.68. Other results obtained from this figure showed that using concentration of 1 g/l of KH_2PO_4 gave a dramatic decrease in surface tension with nearly effective emulsification activity. At a concentration of 2 g/l, a slightly increased emulsification activity obtained with increased surface tension reading. Based on these results, the subsequent experiments were carried out at a concentration of 0.5 g/l.

In other study it was demonstrated that optimum surfactin production from *B. subtilis* S499 obtained at 1.9 g/l of KH_2PO_4 (Jacques *et al*., 1999). Another study showed that when the media of higher carbon-to-phosphate ratios were applied, a decrease in biosurfactant concentration occurred (Guerra-Santos *et al*., 1984).

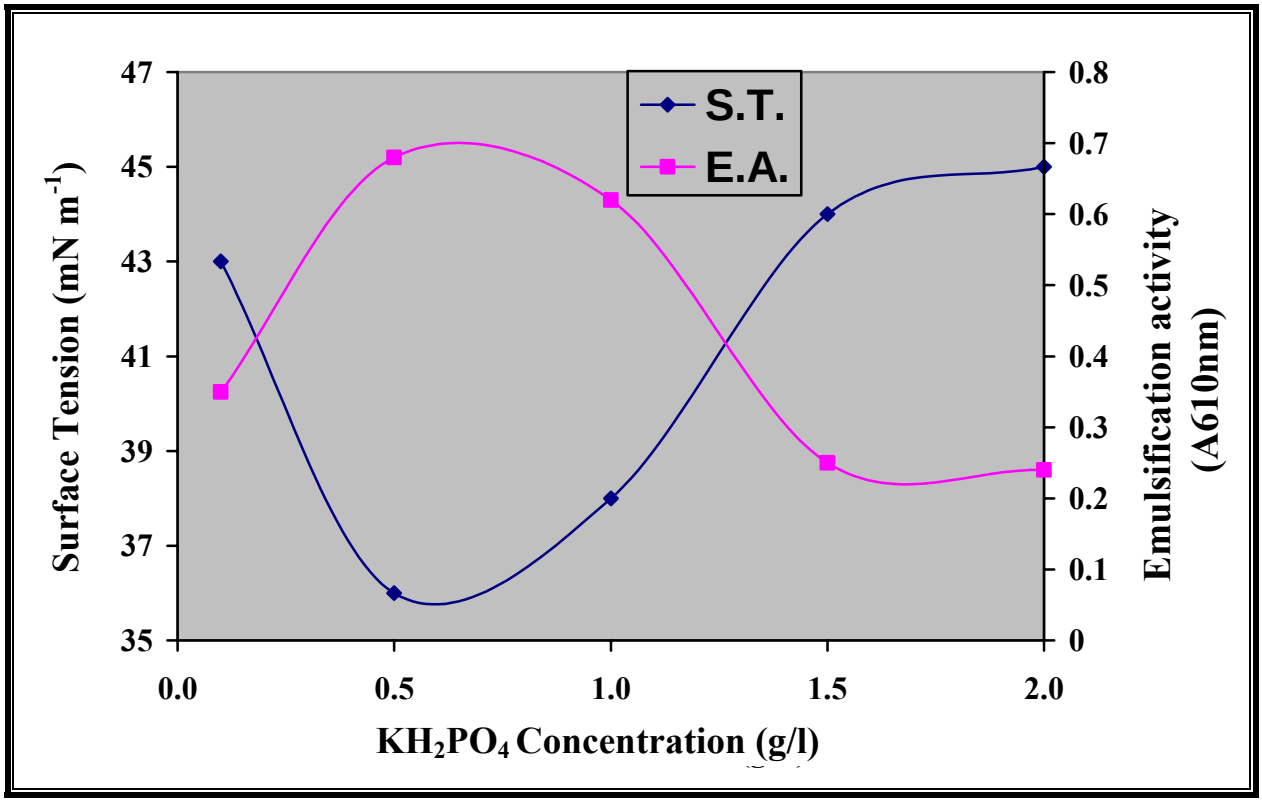


Figure (3-7) Effect of KH_2PO_4 on biosurfactant production form *B. cereus* HI-2, grown with 4% soybean oil, 0.4% NH_4NO_3, at 30°C in a shaker incubator (180 rpm) for 72 hrs.

3.4.6 Effect of pH

The production medium was prepared at different pH values ranged between 5 to 9 in attempt to determine the optimum pH required for biosurfactant production from *B. cereus* HI-2. The obtained results as shown in figure (3-8) elucidated that an efficient production occurred at pH 7 which was indicated by the minimum surface tension (35 mN m^{-1}) and the maximum emulsifying activity (0.70). Within the pH range between 5 to 7 an increase in emulsification activity was coincided with dramatic decrease in surface tensions; while at pH 8, again the surface tension increased to 39 mN m^{-1} while the emulsification activity was decreased to 0.61, and then at pH 9 a slight emulsification activity (0.28) was reached with an increase in the surface tension (42 mNm^{-1}).

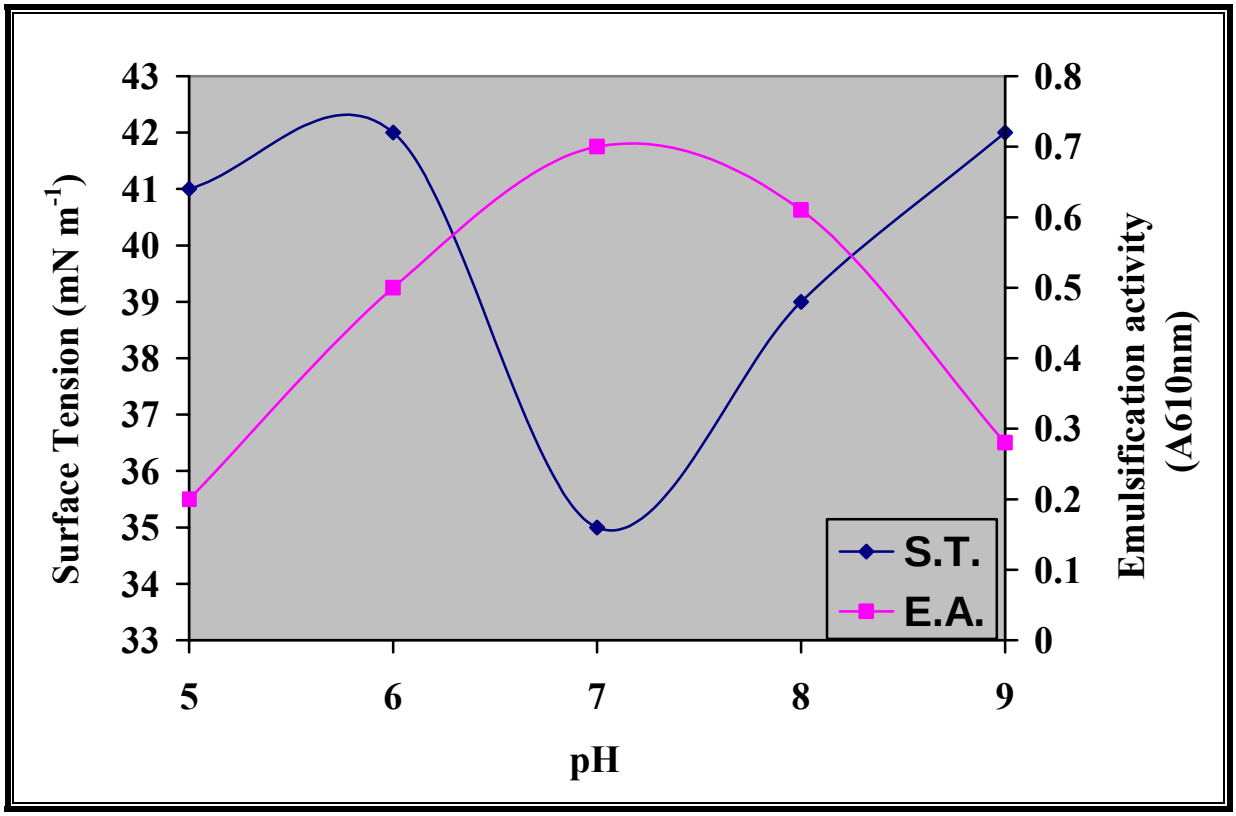


Figure (3-8) Effect of pH on biosurfactant production form *B. cereus* HI-2, grown with 4% soybean oil, 0.4% NH_4NO_3, 0.5 g/l KH_2PO_4, at 30°C in a shaker incubator (180 rpm) for 72 hrs.

It was found that the type, quality and quantity of biosurfactant produced are influenced by culture conditions such as pH (Karanth *et al.*, 1999). The obtained results were in accordance with other studies concerning the pH effect on the production. Jacques *et al.*, (1999), described an optimum pH ranging between 6.5 to 7.0 for surfactin production by *B. subtilis* S499. Other study refereed that the initial pH of 8.0 was optimum for biosurfactant production by *B. subtilis* C9 (Kim *et al.*, 1997).Whereas the optimum pH for bioemulsifier production from *B. stearothermophilus* VR-8 and *B. subtilis* 22BN was found to be 7.2 (Gurjar *et al.*, 1995; and Christova *et al.*, 2004).

3.4.7 Effect of Temperature

The *B. cereus* HI-2 was grown and incubated at different temperatures ranging between 25 to 40°C to determine the optimum temperature for biosurfactant production. Results shown in figure (3-9) pointed out that the minimum surface tension (34 mN m^{-1}) and better emulsification activity (0.71) was noticed at 35°C, which was suggested as the optimum temperature for biosurfactant production. Relative results of surface tension (35 mN m^{-1}) and emulsification activity (0.70) were recorded at 30°C, whereas, at 40°C a low emulsification activity (0.55) and higher surface tension (38 mN m^{-1}) was obtained. As a consequence of these results, subsequent experiment, were carried out at a temperature of 35°C.

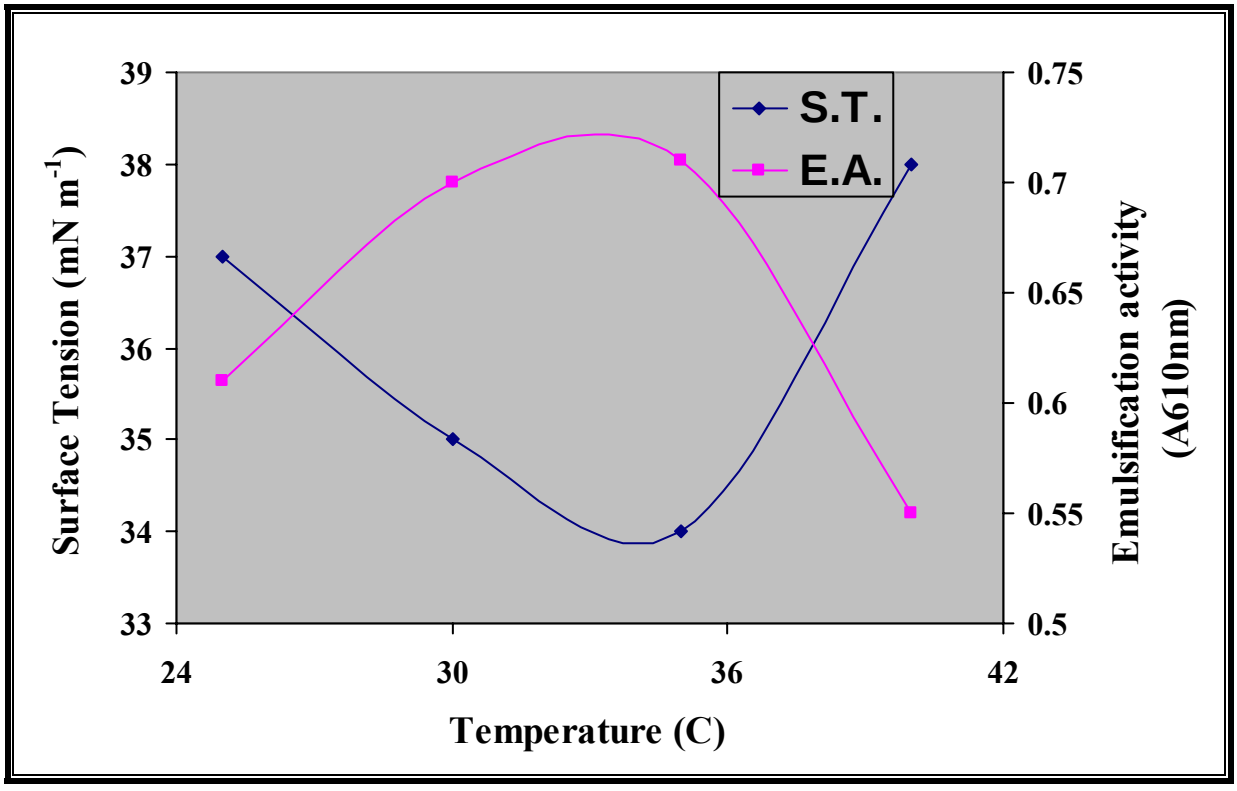


Figure (3-9) Effect of temperature on biosurfactant production form *B. cereus* HI-2, grown with 4% soybean oil, 0.4% NH_4NO_3, 0.5 g/l KH_2PO_4, pH 7,at 30°C in a shaker incubator (180 rpm) for 72 hrs.

In a previous study concerning biosurfactant (lipopeptid) production from *B. licheniformis* BAS it was found that the optimum temperatures for production was between 35 and 45^{o}C (Yakimov *et al.*, 1995). Other study pointed out that *B. subtilis* was able to produce surfactants at 37^{o}C (Noudeh *et al.*, 2003) and at 42^{o}C by *B. licheniformis* JF-2 (Lin *et al.*, 1993). Jacques *et al.*, (1999) noticed that an optimum temperature 30^{o}C for high- surfactant production by *B. subtilis* S 499 was 30^{o}C, when it was grown aerobically with sucrose as a carbon source, and also 28 ±1^{o}C has been described for biosurfactant production by newly isolated *B. subtilis* 22BN during growth on n-hexadecane as a carbon source (Christova *et al.*, 2004).

3.4.8 Effect of Incubation Period

The optimum period required for biosurfactant production from the locally isolated *B. cereus* HI-2 under the previously monitored culture conditions was determined as represented in figure (3-10). The measurements were made within constant intervals. After 24 hrs of incubation a change in surface tension (40 mNm^{1}) was observed coincided with a low emulsification activity (0.24).

Within 48 hrs surface tension was dramatically decreased to 36 mN m^{-1} and emulsification activity increased to 0.62, depending on the growth curve (figure 3-2) of this isolate, the stationary phase has been reached after 15 hours. Results indicated in figure (3-10) showed that the maximum production of biosurfactant was obtained after 72 hrs of incubation, after this period the surface of cell-free broth decreased to 34 mN m^{-1} while the emulsification activity increased to 0.72. Finally, after 96 hrs again the surface tension (38 mN m^{-1}) increased coincided with a slight decrease in emulsification activity.

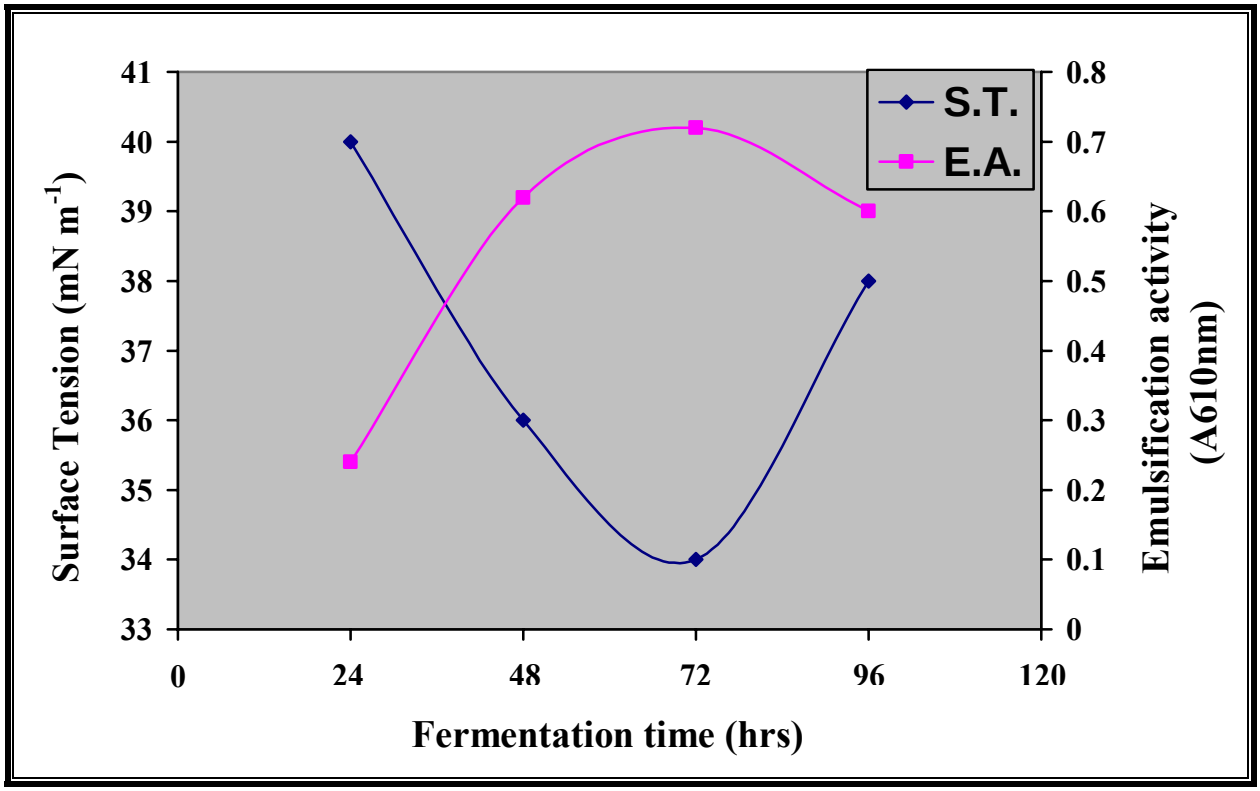


Figure (3-10) Effect of incubation periods on biosurfactant production form *B. cereus* HI-2, grown with 4% soybean oil, 0.4% NH_4NO_3, 0.5 g/l KH_2PO_4, pH 7, in a shaker incubator (180 rpm) at 30°C.

In a previous study, it was found that, growth was associated production of biosurfactant from several microorganisms (Duvnjak and Kosaric, 1985; Johnson *et al.*, 1992). It was also found that surfactin S1 produced during the exponential phase of *B. subtilis* S49, while iturin A and fengycins produced during the stationary phase (Jacques *et al.*, 1999). Gurjar *et al.*, (1995), demonstrated that in *B. stearothermophilus* VR-8, the emulsification activity increased with cell mass up to 24 hrs, and after 30 hrs there was a sharp decrease in emulsification activity and culture density. Growth of *B. subtilis* 22BN on naphthalene was accompanied by decreasing in surface tension from 70 mN m^{-1} within 4 days of cultivation (Christova *et al.*, 2004).

Additionally, it has been mentioned that maximal bioemulsifeir production occurs when the cultures have progressed well into the stationary phase (Ron and Rosenberg, 2001).

3.5 Partial Purification and Characterization of Biosurfactant Produced by *B. cereus* HI-2.

3.5.1 Isolation and Purification of Biosurfactant

The surface-active compound was isolated from the cell-free culture supernatant of *B. cereus* HI-2 by acid precipitation method which was followed as a purification step. The obtained results indicated that biosurfactant was finally achieved in the form of white precipitate. Subsequently, the dried precipitate was weighed to determine the yield which it was 0.454 gram per liter of culture medium.

In other study it was found that effective isolation of sufactin produced from B. subtilis was performed by acid precipitation and the yield was equal to 40-50 mg/l of white precipitate (Arima *et al*., 1968). Also it has been mentioned that effective purification of biosurfactant from *B. licheniformis* JF-2 can be achieved either by acid precipitation with concentrated HCl at pH 2.0 or by XAD-2 adsorption chromatography and further purification was achieved by reverse-phase HPLC (Lin *et al*., 1994).

3.5.2.1 Biochemical Detection Tests

A. Molisch's Test

This test was followed in an attempt to determine whether the sugar moiety is present in the tested molecules or not. Negative result obtained from this test indicates that this product possibly a lipopeptide. This result was potentially agreed with the major researches that demonstrated most of bioemulsifiers belong to *Bacillus* spp. were free of rhamnose, trehalose or sophorose sugars, with some exception in case of *B. subtilis* 22BN (Christova *et al*., 2004). Other studies concerning biosurfactants produced by *B. cereus* 28BN and *Bacillus* sp. referred that it was mainly a glycolipid compound. (Tuleva *et al*., 2005; Tabatabaee *et al*., 2005).

B. Biuret Test

The presence of peptide bonds in the structure of biosurfactant was determined throughout this sensitive test to evaluate if it was a lipopeptide compound (Arima *et al*., 1968). Result of this test gave a violet or pink color was observed indicating a positive result and the examined compound was a lipopeptide molecules. The current result was agreed with previous studies which were mentioned that the majority of biosurfactants belong to *Bacillus* sp. were identified as a lipopeptide compounds (Arima *et al*., 1968; Bernheimer and Avigad, 1970; Lin *et al*., 1994; Yakimov *et al*., 1995).

3.5.3 Characterization

3.5.3.1 FTIR Analysis

The purified biosurfactant was also subjected to the Fourier Transformer Infra Red Spectroscopy in an attempt to give relatively an idea about the chemical structure of this compound.

IR spectrum shown in figure (3-11) of the isolation fraction in CH_2Cl_2 showed the following characteristics bands:-

1. Broad band at 3398 cm^{-1} resulting from the C-H stretching mode suggest the presence of an aliphatic chain $CH_3(CH_2)_n$.
2. Band at 1641.3 cm^{-1}, which may be due to the stretching mode of N-C bond present in amide group  .

From the above IR spectral data and the biochemical tests it can be concluded that the structure of biosurfactant produced by the locally isolated *B. cereus* HI-2 was potentially a lipopeptide. These patterns of absorption were closely relative to those of surfactin and lichenysin B elucidated in other study (Lin *et al*., 1994). Depending on the IR and NMR spectra with a variety of analytical techniques, detailed information about the already known biosurfactants were studied by a number of investigators which have

demonstrated that lipopeptide nature of these compounds (Arima *et al*., 1968; Berheimer and Avigad, 1970; Jenny *et al*., 1991; Lin *et al*., 1994; Jacques *et al*., 1999).

Vater *et al*. (2002), has patented that the *B. subtilis* C-1 was able to produce three lipopeptide complexes, the surfactin, the iturins, and the fengycins.

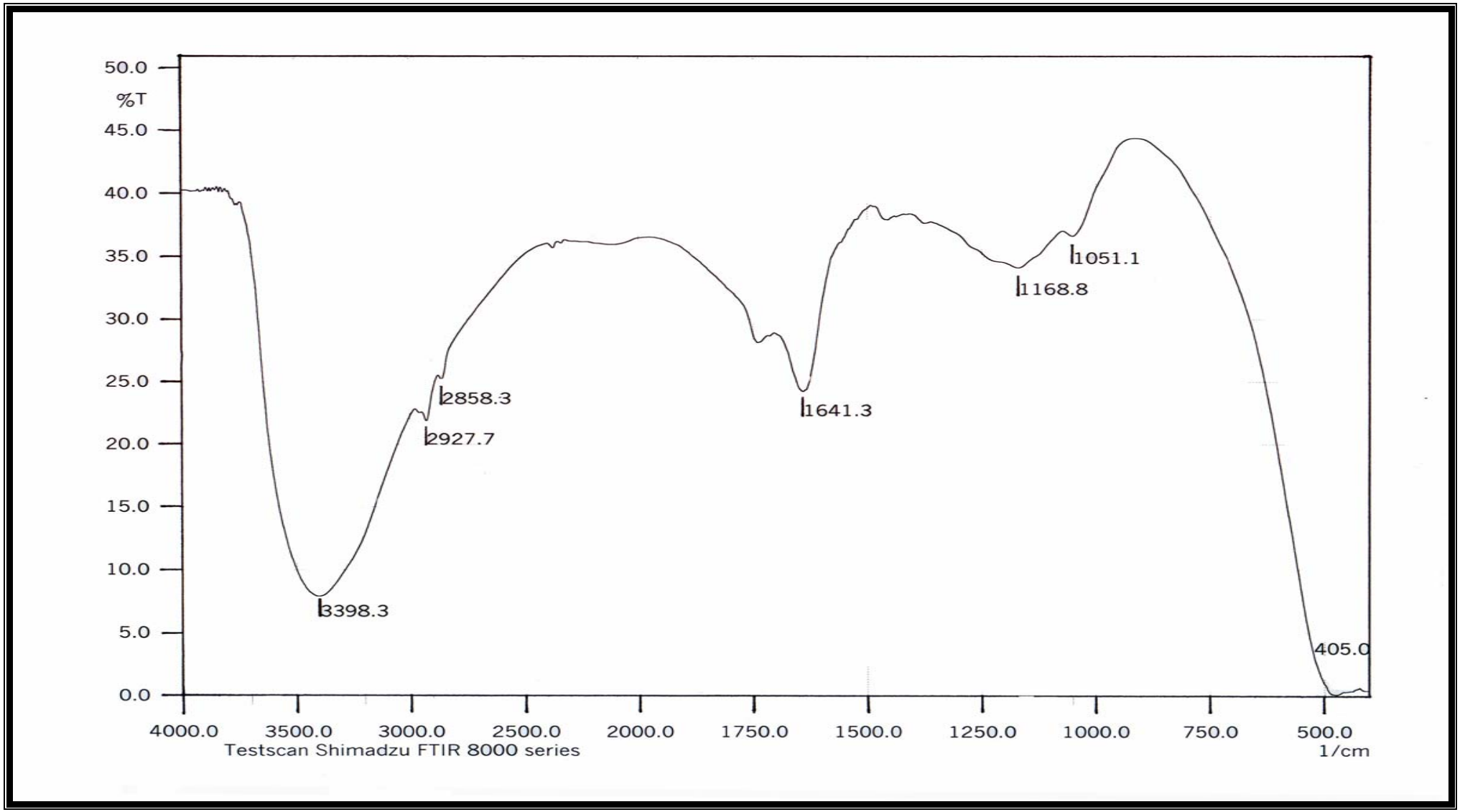


Figure (3-11) FTIR spectroscopy of purified biosurfactant isolated from *Bacillus cereus* HI-2.

Chapter Four

Conclusions

and

Recommendations

Conclusions and Recommendations

4-1 Conclusions

1. Ability of locally isolated *Bacillus* spp. in biosurfactants production, among them *Bacillus cereus* HI-2 was the best in this field.

2. Ability of *B. cereus* HI-2 in biosurfactant production was enhanced under optimum conditions (soybean oil (4%), NH_4NO_3 (0.4%), KH_2PO_4 (0.5%), pH 7, 35°C, for 72 hrs).

3. The produced biosurfactant was easy to purify from cell-free broth, by using acid precipitation method.

4. According to FTIR spectroscopy results and other biochemical tests, biosurfactant produced by locally isolated *B. cereus* HI-2 was a lipopeptide compound in nature.

4-2 Recommendations

1. Genetic study on the locally isolated *Bacillus cereus* HI-2 to determine the genetic elements responsible for biosurfactant production.

2. Mutagenesis of *B. cereus* HI-2 to develop it's ability in biosurfactant production using physical and chemical mutagens.

3. Examine the biological activity of biosurfactant produced by *B. cereus* HI-2, as antimicrobial, antiviral and antitumor agent.

4. Determine the amino acid sequence and finally the chemical structure of lipopeptide biosurfactant produced by *B. cereus* HI-2 using amino acid analyzer, NMR and GC-Mass spectrometry techniques.

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الخلاصة

عُزلت 63 عزلة من البكتريا العائدة لجنس *Bacillus spp.* من 18 عينة تربة ملوثة وغير ملوثة بالمركبات الهيدروكاربونية والمشتقات النفطية المختلفة من بغداد والبصرة، وقد درست قابلية العزلات اعلاه على انتاج المستحلبات الحيوية بقياس فعالية تحلل الدم على وسط اكار الدم الصلب، وقد اشارت النتائج الى قابلية 34 عزلة من الـ 63 عزلة على انتاج المستحلبات الحيوية. ولغرض انتقاء العزلة الاكفأ في انتاج المستحلبات الحيوية مُحصت تلك العزلات بقياس الشد السطحي (ملي نيوتن / متر) لرائق المزارع البكتيرية الخالي من الخلايا بعد تنميتها على وسط الاملاح المعدنية المدعم بمادة السكروز كمصدر وحيد للطاقة ثم شُخصت العزلة البكتيرية الاكفأ في انتاج المستحلبات الحيوية واظهرت نتائج التشخيص انها تعود الى جنس *Bacillus cereus* HI-2.

تم تعيين الظروف المثلى لانتاج المركبات الفعالة سطحياً من العزلة المحلية *Bacillus cereus* HI-2 والتي تضمنت استخدام زيت فول الصويا 4% (حجم/حجم) مصدرا كاربونياً، ونترات الامونيوم 4 غم/لترمصدرا نتروجينياً، وفوسفات البوتاسيوم ثنائية الهيدورجين 0.5 غم/لتر مصدراً فوسفاتيا، وبرقم هيدروجيني 7، وبدرجة حرارة 35م لمدة 72 ساعة في الحاضنة الهزازة بسرعة 180 دورة/دقيقة. جمعت المركبات الفعالة سطحياً والمنتجة من قبل تلك العزلة بعد تنميتها تحت الظروف المثلى للانتاج وتم تنقية المنتج بطريقة الترسيب الحامضي وقدرت كميته بـ 0.454 غم/لتر.

تم التحري عن طبيعة المركب الفعال سطحياً المنتج من العزلة المحلية *Bacillus cereus* HI-2 باجراء بعض الاختبارات الكيموحيوية والطيفية باستخدام تنقية FTIR وقد اشارت النتائج الى ان المركب المنتج هو عبارة عن ببتيد دهني (Lipopeptide).

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كلية العلوم

انتاج المركبات الفعالة سطحياً من عزلة محلية لبكتريا *Bacillus cereus*

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

حسام الدين محمد كاظم

بكلوريوس تقانة احيائية جامعة النهرين
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