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Production, purification and characterization of biosurfactant from *Geobacillus thermoleovorans* and studying its antimicrobial and antitumor activity

A Dissertation

Submitted to the Council of College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the degree of Doctorate of Philosophy of Science in Biotechnology

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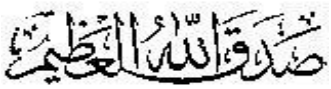

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الاهداء

الى الروح التي كانت قدوةً وفخراً لنا

ابي الغالي

الى من تحمل مشواري واعبائي

زوجي الحبيب

الى التي ساعدتني بما يعجز القلب عن وصفها

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Summary

The ten bacterial isolates used in this study have the ability to utilize crude oil and aromatic compounds. They were isolated in the previous study and considered as a novel group of aromatic hydrocarbon degrading extreme thermophillic bacteria. These isolates were screened for their ability to produce biosurfactant depending on the emulsification index (E24%), surface tension measurement (mN/m) and emulsification activity (E.A) using crude oil as a carbon source. The results showed that all isolates were able to produce biosurfactant and *Geobacillus thermoleovorans* Ir1 (JQ912239) was the most efficient one.

The optimum conditions for biosurfactant production by *G. thermoleovorans* Ir1 (JQ912239) were determined. The results indicated that these conditions are growing this bacterium in mineral salt medium (pH7) containing 1% crude oil as a sole carbon source and 0.3% ammonium chloride as a nitrogen source, incubated in shaker incubator at 60 ºC, with 200 rpm for 10 days.

Biosurfactant was extracted using three methods. The results showed that the extraction with acetone gave maximum biosurfactant activity. The biosurfactant was purified using silica gel column chromatography, three peaks were obtained and gave emulsification activity.

The chemical composition of biosurfactant revealed that it consists of 37.7% lipids, 26.2% carbohydrate and 10.7% protein.

The partial and /or purified biosurfactant of *G. thermoleovorans* Ir1 (JQ912239) was subjected to fourier transform infrared spectrophotometer (FTIR), High Performance Liquid Chromatography (HPLC), gas chromatography (GC)and Nuclear magnetic resonance (NMR) to complete the chemical characterization. The result of FTIR revealed that the biosurfactant contains lipid, carbohydrate and protein. While the HPLC results indicated that the fatty acid components of

biosurfactant were palmitic acid, stearic acid and oleic acid, the carbohydrates were xylose, mannose and maltose. While the amino acids were aspartic acid, glutamic acid and glutamine.

Gas chromatography analysis of the main part (lipid) of biosurfactant showed that it consists the high percentage of palmitic acid methyl ester (C16:0), Stearic acid (C18:0) and Oleic acid (C18:1n9C), and less percentage of other fatty acids.

The 1H Nuclear magnetic resonance spectrum showed that the partial purified biosurfactant consists of two compounds: the main was triglycerides and the second may be attributed to fatty acid. The results of purified biosurfactant (the three peaks which obtained with silica gel column chromatography) revealed that all contain only triglycerides and this study may be the first one which is carried out in Iraq, which elucidates the ability of thermophilic bacteria to produce biosurfactant.

In order to determine the antitumor activity of the purified biosurfactant, three cell lines (MCF7, Hut78 and Jurkat) were exposed to different concentrations of the biosurfactant. The results showed that the effect was dependent on the type of tumor cell line, biosurfactant concentration, and exposure time. The highest effect (most sensitive) was MCF7 cell line and 100 µg/ml caused 66.55 % inhibition of cell line growth after 72hrs. (incubation period).

MCF7 cell line was subjected to cytotoxicity study. The result showed that 50 and 100µg/ml of purified biosurfactant caused a significant decrease of cell count, with significant increase in cell permeability, releasing cytochrome C from mitochondria, nucleus intensity and a significant reduction in mitochondrial membrane potential.

The antimicrobial activity of purified biosurfactant of *G.*

thermoleovorans Ir1 (JQ912239) was applied against some microorganisms, the biosurfactant inhibited the growth of bacteria (*Staphylococcus aureus*, *Streptococcus* sp., *Pseudomonas aeruginosa*) and fungi (*Candida albicans*).

Some physical and chemical properties of biosurfactant were studied. It was found that the biosurfactant was active in a wide range of pH values, thermostable at a high temperatures and stable in a wide range of salt concentrations of NaCl, $CaCl_2$ and KCl.

In an attempt to detect the gene(s) responsible for biosurfactant produced by *G. thermoleovorans*, genomic DNA of this bacterium and *B. subtilis* was extracted and amplified using specific primer for *sfp* gene (coded for biosurfactant of *Bacillus* spp.). The results showed that the amplification product (675bp) was obtained with *B. subtilis* but not with *G. thermoleovorans* Ir1, that means this gene is not responsible for biosurfactant production in *G. thermoleovorans* Ir1.

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List of Abbreviation

Abbreviation	Full Name
Bp	Base pair
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D.W	Distilled water
E24%	Emulsification index
EPS	Exopolysaccharide
ELISA	Enzyme-linked immuonoabsorbent assay
FTIR	Fourier Transform Infrared
GC	Gas chromatography
HPLC	High performance liquid chromatography
LB	Luria – Bertani
MSM	Mineral salts medium
NCBI	National center for biotechnology information
PCR	Polymerase Chain Reaction
pH	concentration of Hydrogen ion
S.T	Surface tension
PTIC	Phenyl isothiocyanate
CMC	Critical micelle concentration
MS	Mass spectroscopy
RT	Retention time
MEL	Mannosylerythritol lipid

CHAPTER ONE

INTRODUCTION

AND

LITERATURES REVIEW

1. Introduction and Literatures review

1.1 Introduction:

Biosurfactant are surface active compounds having both hydrophilic and hydrophobic domains that allows them to exist preferentially at the interface between polar and non-polar media, thereby reducing surface and interface tension (Banat *et al.*, 2010).

Biosurfactant are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi from various substances, including sugars, oils and wastes (Femi-Ola *et al.*, 2015).These molecules comprise complex structures which are grouped either as low (glycolipids and lipopeptides) or high (polymeric biosurfactant) molecular weight compounds (Cameotra *et al.*, 2010). The major classes of biosurfactant include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate surfactants (Cameotra and Makkar, 2004; Salihu *et al.*, 2009).

Recently much attention has been attributed towards biosurfactant over chemically synthesized surfactants due to their ecological acceptance, low toxicity and biodegradable nature, effectiveness at extreme temperatures or pH values and widespread applicability (Mnif and Ghribi, 2015).

During the last decade, biosurfactant have been used as alternatives for synthetic surfactants and are expected to find many industrial and environmental applications such as enhanced oil recovery, crude oil drilling, lubrication, bioremediation of pollutants, foaming, detergency, wetting, dispersing and

solubilization. The application of biosurfactant also increased in cosmetic, health care and food processing industries (Dhasayan *et al*., 2014).

Biosurfactant displayed important biological activities including antimicrobial, insecticidal, immune-modulative and antitumoral activities (Cao *et al*., 2009; Liang *et al.*, 2014).

New trials for cancer treatment have been performed by many researchers in various countries, including Iraq; these trials included using gene therapy, immunotherapy, biological therapy and bacterial byproducts (Al-Saffar, 2010). However, biosurfactant produced by thermophilic bacteria, including *Geobacillus thermoleovorans* as a new trial for cancer treatments and cytotoxic effect have not been tested previously.

Identifying and characterizing new genes involved in the degradation of hydrocarbons and production of surfactants, which have the potential to develop a bioremediation strategy are thus promising and representing an important subject of the research (Oliveira *et al.*, 2015).

Aim of the study:

According to what's mentioned above and because of how rare the studies are about the production of biosurfactant of thermophilic bacteria, only one study reported *G. thermoleovorans* (Feng and Jin, 2009), this study aimed to:

- Screening some isolates of thermophillic bacteria (isolated in the previous study) for their ability to produce biosurfactant and select the most efficient one.
- Optimization of cultural conditions for biosurfactant production by the efficient isolate.

- Extraction and purification of biosurfactant by using appropriate chromatographic methods.
- Characterization and determination the properties of biosurfactant produced by the efficient isolate by using analytical methods (FTIR, HPLC, GC and NMR).

-Study the biological activity of biosurfactant as antitumor and antimicrobial.

1.2 Literatures review

1.2.1 Thermophillic bacteria:

A thermophile is an organism — a type of extremophile — that thrives at relatively high temperatures, between 45 and 122 °C (113 and 252 °F) (Madigan and Martino, 2006; Takai *et al.*, 2008). Many thermophiles are archaea. While thermophilic eubacteria are suggested to have been among the earliest bacteria (Horiike *et al.*, 2009).

Thermophilic bacteria offer crucial advantages over mesophilic or psychrophilic bacteria, especially when they are applied to ex-situ bioremediation processes. Limited biodegradation of hydrophobic substrates caused by low water solubility at moderate temperature conditions can be overcome if the reaction temperature could be increased enough (Kato *et al.,* 2009). Besides their biotechnological importance, thermophilic microorganisms maintain interesting features useful for studying evolution of life. Microorganisms living under extremely high temperature condition, such as hyperthermophilic archaea and hyperthermophilic bacteria, share the cellular mechanisms with not only bacteria but also eukaryotes (Rashid *et al.,* 1995).

One theory suggests that the thermophiles were among the first living things on this planet, developing and evolving during the primordial birthing of the earth when surface temperatures were quite hot. One theory suggested that the thermophiles were among the first living things on this planet, developing and evolving during the primordial birthing days of earth when surface temperatures were quite hot, and thus had been called the "Universal Ancestor" (Doolittle, 1999)

1.2.2 *Geobacillus* :

The members of the Gram-positive endospore-forming bacteria that made up the genus *Bacillus* have been gradually subdivided, during the last few years, into a number of new genera such as *Alicyclobacillus*, *Aneuribacillus*, *Brevibacillus*, *Gracilibacillus*, *Paenibacillus*, *Salibacillus*, *Ureibacillus,* and *Virgibacillus* (Marchant and Banat, 2010). Nazina *et al.* (2001) created the genus *Geobacillus* based around *Bacillus* (now Geobacillus) *stearothermophilu*s DSM22 as the type strain.

The isolation of aerobic highly thermophilic bacteria from various cold soil environments both in Northern Ireland and worldwide (Marchant *et al.,* 2002). Nazina *et al.* (2001) proposed two new species that they had isolated from deep oil reservoirs. Since that original description of the genus with eight species a further nine, from a variety of sources. These include *G. toebii* from composting plant material (Sung *et al.,* 2002), while G. *debilis* from temperate soil environments (Banat *et al*., 2004), also *Geobacillus pallidus* which was originally proposed as *Bacillus pallidus* by Scholz *et al.* (1987) and reassigned by Banat *et al.* (2004), and *G. vulcani* proposed as *Bacillus vulcani* from marine geothermal sources (Caccamo *et al.*, 2000) and transferred to *Geobacillus* by Nazina *et al.* (2004) and *G. tepidamans* (Schaffer *et al*., 2004) in Austria and Yellowstone National Park.

The genus *Geobacillus* was established in 2001 with the following key characteristics: rod shaped cells producing one endospore per cell, cells may be single or in short chains and may have peritrichous flagella. Cells have a gram-positive cell wall structure. Chemo-organotrophs, which are aerobic or facultatively anaerobic using oxygen as the terminal electron acceptor, replaced by nitrate in some species (Marchent *et al.*, 2002).

Geobacillus spp. are obligately thermophillic with a growth range of 37–75ºC and optima of 55–65ºC, and they are neutrophilic with a growth range of pH 6.0 – 8.5 (Nazina *et al.*, 2001). And the vegetative bacilli are large (0.5 ×1.2 µm to 2.5 ×10 µm) and straight. One of the key characteristics of the genus *Geobacillus* is its ability to produce endospores, and in mesophilic bacilli endospores represent a potent survival mechanism under adverse conditions. It is relatively easy to differentiate vegetative cells and spores in mesophilic bacilli through selective killing with heat or chemical agents. This has not proved possible with *Geobacilli* due to the resistance to killing by these agents shown by vegetative cells and active evolution of species still taking place (Marchant and Banat, 2010).

Endospores formation is affected by some factors including the temperature of growth, the pH, aeration, presence of minerals, presence of certain carbon or nitrogen compounds and the concentration of the carbon or nitrogen source, in some circumstances a starvation for phosphorus source, population density, cell cycle (Piggot and Hilbert, 2004; Goesselsberger *et al.*, 2009).

Geobacillus thermoleovorans previously (*Bacillus thermoleovorans*) B23, from a deep-subsurface oil reservoir in Japan (Kato *et al.*, 2001; Nazina *et al*., 2001) Strain B23 effectively degraded alkanes at 70°C with the carbon chain longer than twelve, dodecane. Since tetradecanoate and hexadecanoate or pentadecanoate and heptadecanoate were accumulated as degradation intermediates of hexadecane or heptadecane, respectively, it was indicated that the strain B23 degraded alkanes by a terminal oxidation pathway, followed by β-oxidation pathway (Kato *et al.*, 2009)**.**

1.2.3 Surfactants:

Surfactants are amphiphilic compound consisting of a hydrophobic and a hydrophilic domains. They are active ingredients found in soaps and detergents with the ability to concentrate at the air- water interface and are commonly used to

separate oily materials from a particular media due to the fact that they are able to increase aqueous solubility of non-aqueous phase liquids (NAPLS) by reducing their surface/ interfacial tension at air–water and water–oil interfaces (Yin *et al*., 2009).

Surfactants, widely used for industrial, agricultural, food, cosmetics and pharmaceutical application however most of these compounds are synthesized chemically and potentially cause environmental and toxicology problem due to the recalcitrant and persistent nature of these substances (Makkar and Rockne, 2003).

In English the term surfactant (short for *surface-active-agent*) designates a substance which exhibits some superficial or interfacial activity. In other languages such as French, German or Spanish the word "surfactant" does not exist, and the actual term used to describe these substances is based on their properties to lower the surface or interface tension, e.g. *tensioactif* (French), *tenside* (German), *tensioactivo* (Spanish). This would imply that surface activity is strictly equivalent to tension lowering, which is not absolutely general, although it is true in many cases (Salager, 2002).

Almost all surfactants are chemically derived from petroleum, however, the interest in microbial surfactants has been steadily increasing due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries (Banat, 1995 a,b), and this class of surfactants known as microbial, or biosurfactants, which have some very interesting and complicated structures, although being expensive to produce compared to chemically synthesized surfactants (Lang, 2002).

The main classes of surfactants according to hydrophilic group are (Salager, 2002) table (1-1) :

- Anionic : hydrophilic head is negatively charged.

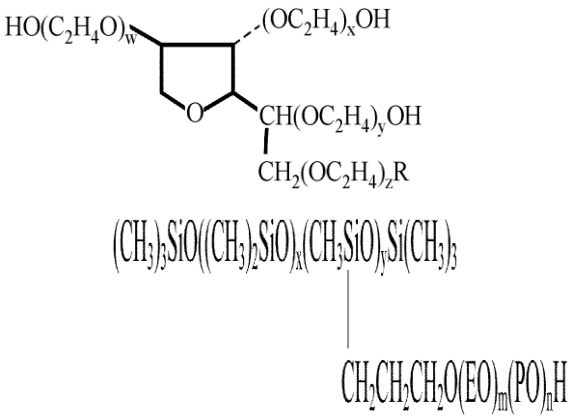
- Cationic : hydrophilic head is positively charged.
- Nonionic : hydrophilic head is polar but not fully charged.
- Amphoteric : Molecule has both potential positive and negative charge depends on pH of the medium.

1.2.4 Biosurfactant:

Biosurfactant are surface active agents of microbial origin (bacteria and fungi), that consiste of a hydrophobic and a hydrophilic domains (Okoliegbe and Agarry, 2012), therefor, they have a unique property of lowering the interfacial tension between two liquids (Sekhon *et al*., 2011) because of theire ability to accumulate between it (Cunha *et al*., 2004). They remain either adherent to microbial cell surfaces or are secreted in the culture broth (Olivera *et al*., 2009; Fathabad, 2011). Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity; higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH, and salinity, and the ability to be synthesized from renewable feed stocks (Fracchia *et al.*, 2012; Silva *et al.*, 2014).

Recently, much attention has been given to biosurfactant due to their broad range of functional properties as in food. In bakery and ice-cream formulations, biosurfactant act by controlling the consistency, slowing staling and solubilizing the flavour oils, while the therapeutic and biomedical applications of biosurfactant act as antimicrobial activity, anticancer activity, anti-human immunodeficiency virus and sperm immobilizing activity, agents for respiratory failure(Gautam andTyagi , 2005), agents for the stimulation of skin fibroblast metabolism(Borzeix and Frederique, 2003), and anti-adhesive agents in surgary (Chakrabarti, 2012).

Table (1-1): The main surfactant classifications (Schramm *et al.*,2003):

Class	Examples	Structures
Anionic	Na stearate Na dodecyl sulfate Na dodecyl benzene sulfonate	CH**3**(CH**2**)**16**COO¯Na_ CH**3**(CH**2**)**11**SO**4**¯Na_ CH**3**(CH**2**)**11**C**6**H**4**SO**3**¯Na_
Cationic	Laurylamine hydrochloride Trimethyl dodecylammonium chloride Cetyl trimethylammonium bromide	CH**3**(CH**2**)**11**NH_Cl_ C**12**H**25**N_(CH**3**)**3**Cl_ CH**3**(CH**2**)**15**N_(CH**3**)**3**Br_
Non-ionic	Polyoxyethylene alcohol Alkylphenol ethoxylate Polysorbate 80 *w* _*x* _ *y* _ *z* = 20 R = (C**17**H**33**)COO Propylene oxide-modified polymethylsiloxane (EO = ethyleneoxy, PO = propyleneoxy)	C*n*H**2***n*_1(OCH**2**CH**2**)***m***OH C**9**H**19**–C**6**H**4** (OCH**2**CH**2**)*n*OH 
Zwitterionic	Dodecyl betaine Lauramidopropyl betaine Cocoamido-2-hydroxypropyl sulfobetaine	C**12**H**25**N_(CH**3**)**2**CH**2**COO_ C**11**H**23**CONH(CH**2**)**3**N_(CH**3**)**2**CH**2**COO_ C*n*H**2***n*_1CONH(CH**2**)**3**N_(CH**3**)**2**CH**2**CH(OH)CH**2**SO**3**_

Although, most biosurfactant are considered to be secondary metabolites, some may play essential roles for the survival of biosurfactant – producing

microorganisms through facilitating nutrients uptake or microbial – host interactions or by acting as biocide agents or promoting the swarming motility of microorganisms and participate in cellular physiological processes of signaling and differentiation (Kearns and Losick, 2003; Das *et al*., 2008). Rhamnolipids, for example, are essential to maintain the architecture of the biofilms and are considered as one of the virulence factors in *Pseudomonas* sp. (Van - Hamme *et al.*, 2006; Arutchelvi *et al.*, 2009).

1.2.5 Bioemulsifier:

Petroleum is a complex mixture of hydrocarbons, organic solvents and heavy metals. Bacteria have designed strategic approaches to overcome the harsh effects of organic solvents and heavy metals in contaminated soil by producing exopolysaccharides / bioemulsifiers, which are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers that stabilize oil-in-water emulsions (Robinson *et al.*, 1996). It can reduce the surface tension, interfacial tension of bacteria and increase the cell surface hydrophobicity of bacteria there by enhancing the dispersal, emulsification and degradation of hydrocarbon pollutants in the contaminated site (Al_ Tahhan *et al*., 2000).

Bioemulsifiers are produced by many microorganisms. *Acenitobacter caicoaceticus* RAG-1 producing emulsan, an extracellular polymeric bioemulsifier has shown great application in oil industry (Fiechter, 1992). While liposan is a bioemulsifier produced by *Candida lipolytica*, primarily composed of carbohydrate (Amaral, 2006).The potential commercial applications of bioemulsifiers include bioremediation of oil-polluted soil and water (Banat *et al.*, 2000; Christofi and Ivshina, 2002), enhanced oil recovery and the formation of stable oil-in-water emulsions for the food and cosmetic industries (Klekner and Kosaric, 1993).

Also the natural role of bioemulsifier is in the formation of biofilms, increasing the bioavailability of water insoluble substrates, regulating the attachment-deatachment of microorganism to and from surface. Bioemulsifiers also have an antimicrobial activity (Ron and Rosenberg, 2001). In addition, bioemulsifiers are involved in cell-to-cell interactions such as bacterial pathogenesis, quorum sensing and biofilm formation, maintenance and maturation. Some biosurfactants/bioemulsifiers enhance the growth of bacteria on hydrophobic water-insoluble substrates by increasing their bioavailability, presumably by increasing their surface area, desorbing them from surfaces and increasing their apparent solubility (Ron and Rosenberg, 2001; Van Hamme *et al*., 2006).

1.2.6 Importantce of bioemulsifier:

Microorganisms produce exopolysaccharide to perform diverse functions such as biofilm formation (Kreft and Wimpenny, 2001), tolerance to hydrocarbons (Aizawa *et al*., 2005), cryoprotectants (Kim and Yim, 2007), shield against antimicrobials (Kumon *et al*., 1994), aggregation (Adav and Lee, 2008), biofouling (Jain *et al*., 2007) and bioleaching of metals (Michel *et al*., 2009). Bioemulsifier minimize health hazards of oil spills by bioremediation of specific microorganisms (Kokare *et al.*, 2007).

1.2.7 Classification of biosurfactant:

Unlike the chemically synthesized surfactants that are generally categorised on the basis of the type of polar group present, biosurfactant are in general classified chiefly by their chemical composition and microbial origin.

Rosenberg and Ron (1999) suggested that biosurfactant could be divided into low molecular mass molecules that efficiently lower surface and interfacial tension, and large molecular- mass polymers that are more efficient as emulsion-

stabilizing agents. The major classes of low – mass surfactants include glycolipids, lipopeptides and phospholipids whereas high – mass surfactants include polymeric and particulate surfactants, that classified according to their structure: glycolipids, lipopeptides, fatty acids, phospholipids and polymeric biosurfactant. While Healy *et al*. (1996) group biosurfactant into four main categories namely, glycolipids, phospholipids, lipoproteins / lipopeptides and polymeric biosurfactant. A further way to classify microbial surface active compound is by the nature of hydrophilic part of the surface active compound such as the carboxylate group of fatty acid, the glycerol of the glycerolipids, the carbohydrate of glycolipids (Cooper and Zajic, 1980). While other distinguished between different location of surface active compound in term which either adhere to cell surface or are extracellulary in the growth medium (Kadhim *et al*., 2008). Lastly, the biosurfactant can be divided into different kinds according to the organism produced it, as shown in table (1-2).

1.2.8 Types of biosurfactant:

There are many types of biosurfactant that produced by various microorganisms, the following are some of the various types of biosurfactant:

1.2.8.1 Glycolipid biosurfactant:

The most known biosurfactant are glycolipids, which are carbohydrates in combination with long-chain aliphatic acids or hydroxyl aliphatic acids, the linkage is by means of either ether or an ester group.

Table (1-2): Major biosurfactant classes and microorganisms involved (Chakrabarti, 2012).

Surfactant class	Microorganism
Glycolipids :	
Rhamnolipids	*Pseudomonas aeruginosa*
Trehalose lipids	*Rhodococcus erithropolis* *Arthobacter sp.*
Sophorolipids	*Candida bombicola, C. apicola*
Mannosylerythritol lipids	*C. antartica*
Cellobiolipids	*Ustilago zeae, U. maydis*
Lipopeptides :	
Surfactin/iturin/fengycin	*Bacillus subtilis*
Viscosin	*P. fluorescens*
Lichenysin	*B. licheniformis*
Serrawettin	*Serratia marcescens*
Surface-active antibiotics:	
Gramicidin	*Brevibacterium brevis*
Polymixin	*B. polymyxa*
Antibiotic TA	*Myxococcus xanthus*
Fatty acids/neutral lipids :	
Corynomicolic acids	*Corynebacterium insidibasseosum*
Fatty acids	*C. lepus*
Neutral lipids	*Nocardia erythropolis*
Phospholipids	*Acinetobacter sp.* *Corynebacterium lepus* *Thiobacillus thiooxidans*
Polymeric surfactants:	
Emulsan	*Acinetobacter calcoaceticus*
Alasan	*A. radioresistens*
Lipomanan	*C. lipolytica* *C. tropicalis*
Particulate biosurfactants :	
Vesicles and fimbriae	*A. calcoaceticus*
Whole microbial cells	*Cyanobacteria* (variety of bacteria)

The fatty acid component usually has a composition similar to that of phospholipids of the same microorganisms (Chen *et al*., 2007), while the carbohydrate moiety is consist of mono- , di- , tri – and tetrasaccharides which include glucose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate (Veenanadig *et al*., 2000). Glycolipid biosurfactant consists of many type as follow:

1.2.8.1.1 Rhamnolipids:

Are exoproducts of the opportunistic pathogen *P. aeruginosa* (Abdel-Mawgoud *et al.,* 2010). They composed of one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid usually, but other fatty acids may be found depending on the *Pseudomonas* species or growth conditions (Desai and Banat, 1997).

Rhamnolipids from *Pseudomonas* spp. have been demonstrated to lower the interfacial tension against *n*-hexadecane, they also emulsify alkanes and stimulate the growth of *P. aeruginosa* on hexadecane. Also two unusual rhamnolipids, designated myxotyrosides A and B, have been isolated from a *Myxococcus* sp., they have a rhamnose unit linked to tyrosine and hence to a fatty acid (Ohlendorf *et al*., 2008).

1.2.8.1.2 Trehalolipids:

Several structural types of microbial trehalolipid biosurfactant have been reported. These are known to be produced by microorganisms belonging to mycolates group, such as Mycobacteria, Corynebacteria, *Arthrobacter*, *Nocardia*, *Gordonia* and specially *Rhodococcus*.

Most of the trehalose lipids synthesized by this group are bound the cell envelope and are produced mainly when microorganisms are grown on hydrocarbons probably as strategy to overcome the low solubility of hydrocarbons and enhance their transport. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation (Desai and Banat, 1997). They are composed of trehalose (is a non-reducing disaccharide).

Most of the trehalose lipids synthesized by this group are bound to the two glucose units which linked together either to mycolic acids in the *Mycobacterium* and most species of *Corynebacterium* and *Nocardia* (Gautam and Tyagi, 2006) or to corynomycolic or nocardomycolic in the case of rest species of *Corynebacterium* and *Nocardia* (Shimakata and Minatogawa, 2000).

1.2.8.1.3 Sophorolipids:

These biosurfactant are a mixture of at least six to nine different hydrophobic sophorosides. Consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid. They are produced mainly by yeasts such as *T. bombicola*, *T.petrophilum* and *T. apicola* (Cooper and Paddock, 1984).

Torulopsis petrophilum produced sophorolipids on water-insoluble substrates such as alkanes and vegetable oils. The sophorolipid, which were chemically identical to those produced by *T. bombicola*, did not emulsify alkanes or vegetable oils. Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents (Desai and Banat, 1997).

1.2.8.2 Fatty acids biosurfactant:

Biosurfactant under this category are produced from alkane as a result of microbial oxidations (Okoliegbe and Agarry, 2012). These fatty acids are either straight chain acids, or complex fatty acids containing OH groups and alkyl branches such as corynomucolic acids (Kretschner *et al*., 1982). But the most active saturated fatty acids in lowering surface and interfacial tensions are in the range of C12-C14 because the hydrophilic or lipophilic balance of fatty acids is related to the length of the hydrocarbon chain (Rosenberg and Ron, 1999).

1.2.8.3 Phospholipids:

They form major components of microbial membranes, however, their level can be increased greatly when certain hydrocarbon degrading bacteria or yeast were grown on alkane substrate.

The quantitative production of phospholipids has also been detected in some *Aspergillus* spp. and *Arthrobacter* strain AK-19 and *T. thiooxidans* and *P. aeruginosa* 44T1, accumulate up to 40 to 80 % (wt/wt) of such lipids when cultivated on hexadecane and olive oil(Desai and Banat, 1997).

1.2.8.4 Particulate biosurfactant:

Surface activity in most hydrocarbon- degrading micro-organisms are attributed to several cell surface constituents, which includes extracellular membrane vesicles partition hydrocarbons forming micro emulsion which play an essential role in alkane uptake by microbial cells (Okoliegbe and Agarry, 2012). There are other structures such as M protein and lipoteichoic acid in group A *Streptococci*, protein A in *S. aureus*, layer A in *A. salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *B. brevis* spores and thin fimbriae in *A. calcoaceticus* (Singh *et al.,* 2011).

The vesicles of *Acinetobacter* sp. strain HO1-N with a diameter of 20–50 nm are composed of protein, phospholipids and lipopolysaccharide (Muthusamy *et al.*, 2008).

1.2.8.5 Lipopeptide biosurfactant:

A large number of cyclic lipopetides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *B. brevis* and *B. polymyxa*, possess remarkable surface-active properties (Okoliegbe and Agarry, 2012). These consist of a lipid attached to a polypeptide chain. Other examples are orinithine lipids, Iturin with surfactin being the best studied (Muthusamy *et al.*, 2008).

Biosurfactant are produced from several species of the genus *Bacillus* that can be classified into three families (Ongena and Jacques, 2008):

- Lipopeptides of the surfactin family
- Lipopeptides of the iturin family
- Fengycins and various lipopeptides.

The lipopeptide surfactin produced by *B. subtilis*, is one of the most powerful biosurfactant. It lowers the surface tension from 72 to 27 mN/m (Jazeh *et al.*, 2012) figure (1- 1).

1.2.8.6 Polymeric biosurfactant:

Most of these biosurfactant are polymeric heterosaccharide containing proteins. The best studied polymeric biosurfactant are emulsan, liposan, mannoprotein and polysaccharide protein complexes (Okoliegbe and Agarry, 2012), which are mainly produced by *A. calcoacetius*, *C. lipolytica*, *S. cerevisiae*, *S. malanogramma*, *U. maydis* and *Pseudomonas* Spp. (Singh *et al.*, 2011).

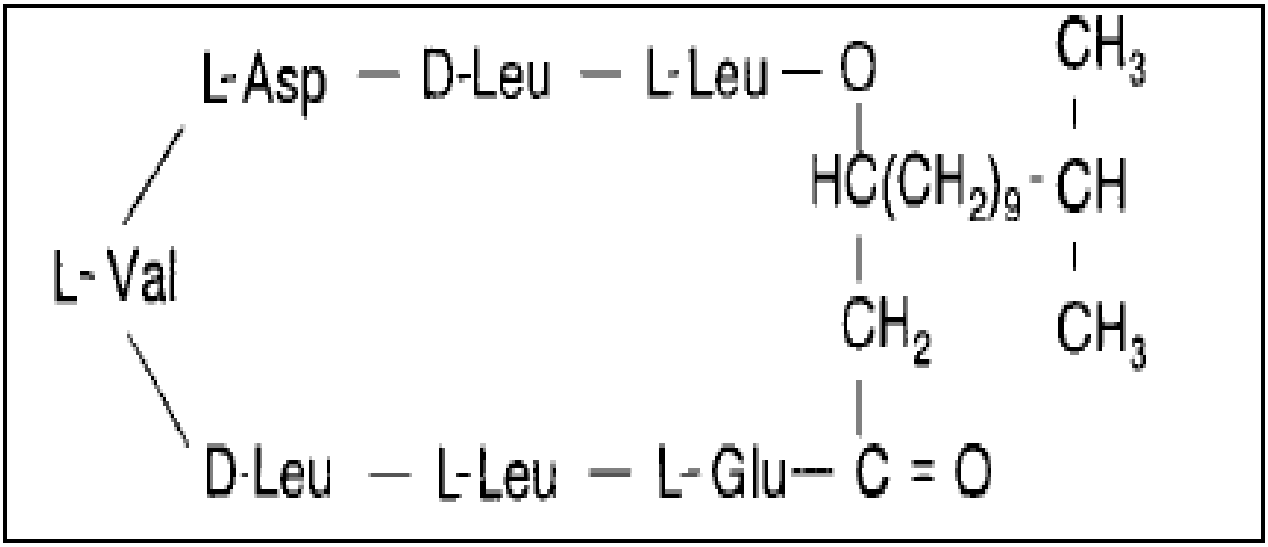


Figure (1-1): Structure of cyclic lipopeptide surfactin produced by *B. subtilis* (Muthusamy *et al*., 2008).

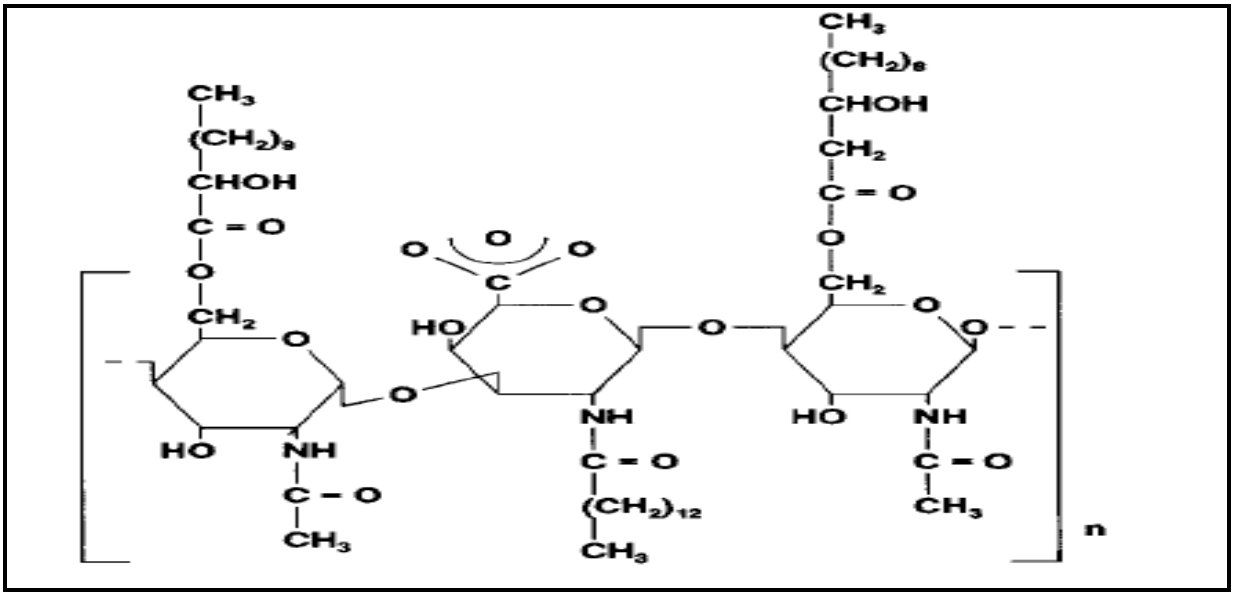


Figure (1-2): Emulsan Structure , produced by *A.calcoaceticus*, in which fatty acids are linked to a heteropolysaccharide backbone (Desai and Banat, 1997).

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion play an important role in alkane uptake by microbial cells (Mukherjee *et al*., 2006; Monteiro *et al*., 2007).

Acintobacter. calcoaceticus RAG-1 produces a potent polyanionic amphipathic heteropolysaccharide bioemulsifier called emulsan (Dasai and Banat, 1997) figure (1-2).

Geobacillus pallidus was able to grow on various hydrocarbons and produce bioemulsfier with complex carbohydrates, lipid and protein (Zheng *et al.*, 2011).

1.2.9 Methods used to detect biosurfactant producing microorganisms:

There are many methods used to screen and detect potential biosurfactant producing microorganisms. These methods were as follows:

1.2.9.1 Drop collapse test:

The drop collapse test was performed to screen the biosurfactant production. It is one of the qualitative methods.

Crude oil was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24 hrs. The supernatant containing biosurfactant was transferred to the oil-coated well regions and a drop size was observed after 1 min with the help of a magnifying glass. The result was considered to be positive when the diameter of the drop was increased by 1mm from that which was produced by distilled water already taken as the negative control (Youssef *et al.*, 2004).

1.2.9.2 Blood haemolysis test:

The biosurfactant producing isolate can be detected by blood heamolysis test through fresh single colonies of the isolated cultures that were taken and streaked on blood agar plates. These plates were incubated for 48 to 72 hrs. at 37 °C. The plates were then observed and the presence of a clear zone around the colonies

indicated the presence of biosurfactant producing organisms (Anandaraj and Thivakaran, 2010).

1.2.9.3 Emulsification index:

The emulsification index was carried out using petroleum (Jazeh *et al*., 2012), five ml of hydrocarbon i.e., petrol was taken in a test tube to which 5 ml of cell free supernatant obtained after centrifugation of the culture, was added and vortexed for two minutes to ensure a homogenous mixing of both the liquids.

The emulsification index was observed after 24 hrs. and it was calculated by using the formula:

E24% = total high of the emulsified layer / total high of the liquid layer X 100.

The calculations were done for all the cultures individually and their emulsification activities were compared with each other

1.2.9.4 Emulsification activity:

The biosurfactant activity can determined by measuring the emulsification activity, 0.5 ml of cell free supernatant was added to 7.5 ml of Tris-Mg buffer and 0.1 ml of dodecanese and mixed with a vortex for 2 min. The tubes were left for 1 hr. and absorbency was measured at 540 nm. Emulsification activity was defined as the measured optical density, blank was Tris-Mg, dodecanese and mineral salt broth without culture (Patel and Deasi, 1997)**.**

1.2.9.5 Surface tension:

Surface tension measured by a du Nouy ring-type tensiometer is one of the simplest techniques used. Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions.

Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m, and addition some kind of surfactant lowers this value. 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 (Satpute *et al*., 2010).

1.2.10 Recovery and purification of biosurfactant:

Most of the biosurfactant can be easily recovered from the culture medium by using a combination of traditional techniques (Sen and Swaminathan, 2005).

Generally, a number of impurities are often co-extracted during the extraction along with several structural types of the target biosurfactant, which are produced in varying quantities. These may need to be evaluated by separating and removing the impurities.

The selection of a method for purification and recovery of surfactants depends on the nature of their charge, solubility characteristics, whether the product is intracellular or extracellular, also on the economics of recovery and downstream processing, physicochemical properties of the desired biosurfactant (Shaligram and Singhal, 2010)**.**

Different method used for biosurfactant/Bioemulsfier purification, include precipitation, adsorption–desorption, ion exchange chromatography, centrifugation, crystallization, filtration and precipitation, foam fractionation, isoelectric focusing, solvent extraction, ultrafiltration, dialysis (Satpute *et al.*, 2010).

Many precipitation procedures are used such as, acetone precipitation which has been used by several workers, to purify biosurfactant (Rosenberg *et al*., 1979), while Phetrong *et al.* (2008) found that precipitation of emulsifier from *A. calcoaceticus* subsp. *Anitratus* SM7 with ethanol was the most efficient method,

also acid precipitation method is easy, inexpensive and readily available to recover crude biosurfactant such as surfactin, lipopeptides, glycolipids.

Acid hydrolysis is carried out by using concentrated HCl to bring down the pH, so biosurfactant becomes insoluble at lower pH (Mukherjee *et al.,* 2006). Some biosurfactants molecules can adsorb and desorb from Amberlite XAD 2 or 16 polystyrene resins and therefore, this interaction is used for the purification of biosurfactants. This process offers good examples of continuous recovery of BS from fermentation broth as well as from concentrated foam, through an in situ method that avoids end product inhibition (Satpute *et al.,* 2010).

The foam fractionation is the technique, in which foam is allowed to overflow from the bioreactor through a fractionation column, resulting in a highly concentrated product, due to the outstanding features of this technique, such as high effectiveness, high purity of product, low space requirements and environmentally friendly. There are a number of reports that presented foam fractionation as one of the most efficient methods in biosurfactant recovery (Noah *et al*., 2002; Chen *et al*., 2006; Sarachat *et al*., 2010).

Separation of biological molecules using ultrafiltration membrane systems has been very popular. Biosurfactant molecules are also characterized by their ability to form micelles or miceller aggregates at concentrations higher than the critical micelle concentration (CMC) and hence can easily retained by high molecular weight cut-off ultrafiltration membranes (Chtioui *et al*., 2005).

In addition, column chromatography is a relatively inexpensive method that can be used to purify biosurfactant. Itoh *et al.* (1971) used this technique for the excessive carbon sources such as fatty acids and other impurities that are coextracted with the glycolipids can be removed, while Kiran *et al.* (2009) used column chromatography with a step wise elution for the purification of biosurfactant.

1.2.11 Techniques used for biosurfactant characterization:

1.2.11.1 Fourier transform infrared spectrophotometer (FTIR):

The activity of biosurfactant depends on their structural components, the types of hydrophilic and hydrophobic groups and their spatial orientation (Bonmatin *et al*., 1994). Surfactin, lichenysin and rhamnolipids have been characterized by the FTIR technique (Das *et al.,* 2008). Also the Alkyl, carbonyl, ester compounds of biosurfactant are detected clearly (Tuleva *et al*., 2002). FT-IR spectra of the purified bioemulsifier, which exhibited the typical feature characteristics of heteropolysaccharide, in which abroad band was observed around 3428 cm and a weak C–H stretching band at 2923 cm which attributed to the characteristic for O–H content, typical of polysaccharide.

1.2.11.2 High Performance Liquid Chromatography:

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. HPLC is used to determine the amount of a specific compound in a solution. In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase. The differences in interaction with the column can help separate different sample components from each other (Kupiec, 2004). Since the compounds have different motilities, they exit the column at different times, they have different retention times, Rt.

The retention time is the time between injection and detection. There are numerous detectors which can be used in liquid chromatography. It is a device that senses the presence of components different from the liquid mobile phase and converts that information to an electrical signal. For a qualitative identification one must rely on matching retention times of known compounds with the retention

times of components in the unknown mixture (Brown *et al*., 1997; Holler and Saunders, 1998). High performance liquid chromatography (HPLC) is not only appropriate for the complete separation of different biosurfactant, but can also be coupled with various detection devices (UV, MS, evaporative light scattering detection, ELSD) for identification and quantification of biosurfactant (Heyd *et al*., 2008). *S. marcescens* can cause biodegradation for palmarosa oil (green oil); the compounds have been identified by HPLC, the HPLC of palmarosa oil shows three peaks, it indicates that oil contains three compounds (Mohanan *et al*., 2007).

1.2.11.3 Analysis of biosurfactant by gas chromatography:

Gas chromatography (GC), is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture (Pavia *et al*., 2006).

The chemical analysis uses gas chromatography (GC) and mass spectroscopy (MS): This consists of a GC column and a mass interface. The ionization source is electron impact or chemical. The mass analyzer magnetic sector has a quadrapole, iron trap, time of flight and mass detector. It is provided with the software MS facility which acts as a gas chromatograph. MS measures the molecular weight of a compound. Separate peaks arising in the GC column and enter in MS. The heat transfer line keeps the compound in the gaseous phase as they enter in the MS. In the ionization chamber, and under a high voltage, the filament is heated up and

provides an electron source. Peaks get transferred to a mass analyzer via focused charged plates and they focus the ions (Satpute *et al*., 2010).

1.2.11.4 Nuclear magnetic resonance (NMR) for biosurfactant analysis:

Nuclear magnetic resonance (NMR) is a spectroscopic technique that detects the energy absorbed by changes in the nuclear spin state. NMR spectroscopy is the only method that allows the determination of three-dimensional structures of proteins molecules in the solution phase. The principle of NMR spectroscopy in which the atomic nuclei with odd mass numbers has the property spin, this means they rotate around a given axis. The nuclei with even numbers may or may not have this property. A spin angular momentum vector characterizes the spin. The nucleus with a spin is in other words a charged and spinning particle, which in essence is an electric current in a closed circuit, well known to produce a magnetic field. The magnetic field developed by the rotating nucleus is described by a nuclear magnetic moment vector, which is proportional to the spin angular moment vector. The strength of the applied magnetic field has a significant impact on the quality of the recorded spectra. The NMR spectrum was used to show the exopolysaccharide (Kumar *et al*., 2004). While Smyth *et al*. (2010) used NMR methodology to analyses glycolipid biosurfactant.

1.2.12 The main applications of surface active compounds:

1.2.12.1 The antitumor activity:

The problems of systemic toxicity and drug resistance in cancer chemotherapy urge the continuing discovery of new anticancer agents. It explore the specific anticancer activity from microbial metabolites to find new compounds. One of the most thrilling results that have been recently reported for biosurfactant its potential

ability to act as anti-tumour agents interfering with some cancer progression processes (Rodrigues, 2011; Fracchia *et al.*, 2012).

Chiewpattanakul *et al.* (2010) mentioned that monoolein biosurfactant from *E. dermatitidis* has the most prominent antiproliferative effect against the cervical cancer (HeLa) and leukemia (U937) cell lines in a dose-dependent manner, while Kim *et al*. (2007) evaluated the anti tumer activity of lipopeptid biosurfactant (surfactin) on the human colon carcinoma cell line LoVo, they found that the lipopeptide has a strong growth inhibitory activity by inducing apoptosis and cell cycle arrest.

Furthermore Duarte *et al.* (2014) indicated that the apoptosis of breast cancer cell line is due to the disturbance of the cellular fatty acid composition by lipopeptides.

In addition viscosin, an effective surface-active cyclic lipopeptide, recovered from *P. libanensis* M9-3, inhibited of the metastatic prostate cancer cell line PC-3M without visible toxicity effects (Saini *et al.*, 2008).

The reactive oxygen species (ROS) and Ca^{2+} have an impact on mitochondria permeability transition pore (MPTP) activity, and MCF-7 cell apoptosis induced by surfactin. Surfactin initially induce the ROS formation, leading to the MPTP opening accompanied with the collapse of mitochondrial membrane potential which leads to an increase in the cytoplasmic Ca^{2+} concentration. In addition, cytochrome C has been released from mitochondria to cytoplasm through the MPTP which activated caspase-9, eventually inducing apoptosis (Cao *et al*., 2011).

In addition MEL was also reported to markedly inhibit the growth of mouse melanoma B16 cells in a dose-dependent manner. Moreover, MEL exposure stimulated the expression of differentiation markers of melanoma cells, such as tyrosinase activity and the enhanced production of melanin, which is an indication that MEL triggered both apoptotic and cell differentiation mechanisms.

Isoda *et al.* (1995) investigated the biological activities of seven extracellular microbial glycolipids, including MEL-A, MEL-B, polyol lipid, rhamnolipid, SL and succinoyl trehalose lipids STL-1 and STL-3, except for rhamnolipid, all the other glycolipids tested induced cell differentiation instead of cell proliferation in the human promyelocytic leukaemia cell line HL60.

The serratamolide AT514, cyclic depsipeptide from *S. marcescens*, belonging to the group of serrawettins, has also been reported to be a potent inducer of apoptosis of several cell lines derived from various human tumors and B-chronic lymphocytic leukemia cells, it primarily involvs the mitochondria-mediated apoptotic pathway and interference with Akt/NF-kB survival signals (Matsuyama *et al.*, 2010).

1.2.12.2 Antimicrobial activity:

One useful property of many biosurfactant was their antimicrobial activity (Rahman and Ano, 2009). Some biosurfactant are a suitable alternative to synthesized medicines and may be used as safe and effective therapeutic agents, as they have a strong antibacterial, antifungal and antiviral activity (Irfan *et al*., 2015).

The biosurfactant was produced by marine *B. circulans* that had a potent antimicrobial activity against Gram-positive and Gram-negative pathogenic and semi-pathogenic microbial strains including multidrug resistance (MDR) strains (Das *et al*., 2009). Also Rodrigues *et al.* (2004) evaluated the antimicrobial activity of two biosurfactant obtained from probiotic bacteria, *L. lactis* 53 and *S. thermophilus* A, against a variety of bacterial and yeast strains isolated from explanted voice prostheses.

The rhaminolipids are highly valued for their antimicrobial activity with lesser toxicity when compared to their chemical counterparts (Kulakovskaya, 2003; Haba *et al*., 2003).

As well as the MEL-A and MEL-B produced by *C. antarctica* strains have also been reported to exhibit antimicrobial action against Gram-positive bacteria (Kitamoto *et al.*, 1993).

Similarly bioactive fractions from the marine *B.circulans* biosurfactant had an antimicrobial action against various Gram-positive and Gram negative pathogenic and semi-pathogenic bacteria including *M. flavus, B. pumilis, M. smegmatis, E. coli, S. marcescens, P. vulgaris, C. freundii, P. mirabilis, A. faecalis, A. calcoaceticus, B. bronchiseptica, K. aerogenes* and *E. cloacae* (Das *et al*., 2008).

Fernandes *et al*. (2007) investigated the antimicrobial activity of biosurfactant from *B. subtillis* R14 against 29 bacterial strains, they demonstrated that lipopeptides have a broad spectrum of action, including antimicrobial activity against microorganisms with multidrug-resistant profiles. *B. pumilis* cells were found to produce pumilacidin A, B, C, D, E, F and G, which exhibited antiviral activity against HSV-1 and inhibitory activity against H+, K+-ATPase, and were also found to be protective against gastric ulcers, probably through the inhibition of microbial activity contributing to these ulcers (Naruse *et al.*,1990).

The production of an extracellular, low molecular weight, protease-resistant thermostable glycolipid fungicide from the yeast *P. fusiformata* (Ustilaginales), this fungicide was active against >80 % of the 280 yeast and yeast-like species tested under acidic conditions (pH 4.0) at 20–30 ºC (Golubev *et al.*, 2001). The cellobiose lipid flocculosin isolated from *P. flocculosa*, was shown to display in vitro antifungal activity against several pathogenic yeasts, associated with human mycoses, including *C. lusitaniae, C. neoformans, T. asahii* and *C. albicans* (Mimee *et al.*, 2005). And Nielsen *et al*., (1999) reported viscosinamide, a cyclic

depsipeptide, to be a new antifungal surface-active agent produced by *Pseudomonas fluorescens*, with different properties compared with the biosurfactant viscosin, known to be produced from the same species and shown to have an antibiotic activity .

The appearance of the antiviral activity of some lipopeptides therefore may take place as a result of the viral lipid envelope and capsid disintegration due to ion channels formation, with consequent loss of the viral proteins involved in virus adsorption and/or penetration (Jung *et al.*, 2000; Seydlová and Svobodová, 2008).

In addition the high molecular weight biosurfactant the massetolides A–H, and cyclic depsipeptides, were isolated from the *Pseudomonas* species, derived from a marine habitat, and found to exhibit in vitro antimicrobial activity against *M. tuberculosis* and *M. avium*-intracellulare (Gerard *et al*., 1997).

1.2.12.3 Antiadhesion:

Biosurfactant have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites, making them useful for treating many diseases as therapeutic and probiotic agents.

Pre-coating vinyl urethral catheters by running the surfactin solution through them before inoculation with media resulted in a decrease in the amount of biofilm formed by *S. typhimurium*, *S. enterica*, *E. coli* and *P. mirabilis* (Rodrigues *et al*., 2004).

Heinemann *et al*. (2000) showed that *L. fermentum* RC-14 releases surface-active components that can inhibit adhesion of uropathogenic bacteria, including *E. faecalis.*

The pulmonary surfactant is a lipoprotein complex synthesized and secreted by the epithelial lung cells into the extracellular space, where it lowers the surface tension at the air–liquid interface of the lung and represents a key factor against

infections and inflammatory lung diseases (Wright, 2003). The important feature that the biosurfactant obtained from *S. thermophilus* A was more effective against *R. dentocariosa* GBJ 52/2B, which is one of the strains responsible for valve prosthesis failure (Rodrigues *et al*., 2006a).

Precursors and degeneration products of sphingo lipid biosurfactant were found to inhibit the interaction of *S. mitis* with buccal epithelial cells and *S. aureus* with nasal mucosal cells (Bibel and Aly, 1992).

1.2.12.4 Food application:

In addition to other application of biosurfactant, food line have been given many enhancements by decrease surface and interfacial tension, thus facilitating the formation and stabilization of emulsions. In addition to control the aggregation of fat globules, stabilization of aerated systems, improvement of texture and shelf-life of products containing starch, modification of rheological properties of wheat dough and improvement of constancy and texture of fat-based products (Shoeb *et al*., 2013). They are also utilized as fat stabilizers and anti spattering agent during cooking of oil and fats (Kosaric, 2001).

The improvement in the stability of dough, volume, texture and conservation of bakery products is obtained by the addition of rhamnolipid surfactants (Van Haesendonck and Vanzeveren, 2004). Moreover L-Rhamnose which already has an industrial application as precursor of high-quality flavor components like furaneol is obtained by hydrolyzing rhamnolipid surfactants produced by *P. aeruginosa* (Linhardt *et al*., 1989).

1.2.12.5 Environmental applications:

Bioremediation, dispersion of oil spills, enhanced oil recovery and transfer of crude oil are some examples of environmental applications of biosurfactant (Shafiei *et al.,* 2014).

Heavy crude oil recovery, facilitated by microorganisms, was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery (MEOR), although there were not many reports on productive microbial enhanced oil recovery project using biosurfactant and microbial biopolymers. In MEOR processes, the microbes are applied for the enhanced recovery of oil from the oil reservoirs and can be considered as applied processes of in situ bioremediation (Van Hamme *et al.,* 2003).

Emulsification properties of biosurfactant make them potentially useful tools for oil spill pollution-control by enhancing hydrocarbons degradation in the environment (Bertrand *et al.,* 1994). The biosurfactant are involved in bioremediation in two ways: by increasing the surface area of hydrophobic water insoluble substrate and by increasing the bioavailability of hydrophobic water insoluble substances. The bioremediation of some contaminated sites are limited due to the low water-solubility of many hydrocarbons, which reduce their availability to micro-organisms. It has been assumed that surfactants can be used to enhance the bioavailability of hydrophobic compounds (Atlas and Cerniglia, 1995).

Thermophilic and halophilic bacteria capable of the living at 80 to 110 ˚C under anaerobic conditions hold a promise to be used in the system (Margesin and Schinner, 2001), and the respective isolates potentially useful for microbial enhanced oil recovery have been described (Yakimov *et al.*,1997).

Marchant *et al*. (2002) examined the ability *Geobacillus* isolates in soil and oil contamination remediation throughout the utilization of a wide range of alkanes. Also biosurfactant have many other application as shown in table (1-3).

1.2.13 Biosynthesis of biosurfactant :

Amphiphilic structure, the hydrophobic moiety is either a long-chain fatty acid, a hydroxy fatty acid, or a-alkylb- hydroxy fatty acid, and the hydrophilic moiety may be a carbohydrate, carboxylic acid, phosphate, amino acid, cyclic peptide, or alcohol. Two primary metabolic pathways, namely, hydrocarbon and carbohydrate, are involved in the synthesis of their hydrophobic and hydrophilic moieties, respectively. The pathways for the synthesis of these two groups of precursors are diverse and utilize specific sets of enzymes. In many cases, the first enzymes for the synthesis of these precursors are regulatory enzymes (Hommel and Ratledge, 1993).

Kinetics of biosurfactant production can be grouped in four main types (Desai and Banat, 1997).

The first type is the production under growth-limiting conditions; this has been extensively demonstrated in *P. aeruginosa* with an overproduction of biosurfactant in limitation of nitrogen and production of bioemulsifier by *C. tropicalis* IIP-4 and the synthesis of the glycolipid by *Nocardia* strain SFC-D seems to follow this pattern too.

The second one is a growth- associated production in which there is a relationship among growth, substrate utilization and biosurfactant production. This behaviour has been observed in the production of rhamnolipids by some strains of *Pseudomonas* and in the biosynthesis of biodispersan.

Table (1-3) Industrial applications of chemical surfactants and biosurfactant (Muthusamy *et al*., 2008).

Industry	Application	Role of surfactants
Petroleum	Enhanced oil recovery	Improving oil drainage into well bore; stimulating release of oil entrapped by capillaries; wetting of solid surfaces; reduction of oil viscosity and oil pour point; lowering of interfacial tension; dissolving of oil
	De-emulsification	De-emulsification of oil emulsions; oil solubilization; viscosity reduction, wetting agent
Environmental	Bioremediation	Emulsification of hydrocarbons; lowering of interfacial tension; metal sequestration
	Soil remediation and flushing	Emulsification through adherence to hydrocarbons; dispersion; foaming agent; detergent; soil flushing
Food	Emulsification and de-emulsification	Emulsifier; solubilizer; demulsifier; suspension, wetting, foaming, defoaming, thickener, lubricating agent
	Functional ingredient	Interaction with lipids, proteins and carbohydrates, protecting agent
Biological	Microbiological	Physiological behaviour such as cell mobility, cell communication, nutrient accession, cell–cell competition, plant and animal pathogenesis
	Pharmaceuticals and therapeutics	Antibacterial, antifungal, antiviral agents; adhesive agents; immunomodulatory molecules; vaccines; gene therapy; microbubble preparation
Agricultural	Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypovirulence
Bioprocessing	Downstream processing	Biocatalysis in aqueous two-phase systems and microemulsions; biotransformations; recovery of intracellular products; enhanced production of extracellular enzymes and fermentation products
Cosmetic	Health and beauty products	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agent, mediators of enzyme action

The productions of glycoprotein AP-6 by *P. fluorescens* surface-active agent by *B. cereus* IAF 346, and biodispersan by *Bacillus* sp. strain IAF-343 are all examples of growth-associated biosurfactant production (Persson *et al.*,1988).

Emulsan-like substance accumulates on the cell surfaces during the exponential phase of growth and is released into the medium when protein synthesis decreases (Shabtai and Gutnick, 1985).

The third production is by resting or immobilized cells is a type of biosurfactant production in which there is no cell multiplication. The cells nevertheless continue to utilize the carbon source for the synthesis of biosurfactant and mannosylerythritol lipid production by *C. antarctica* (Kitamoto *et al.*,1993), sophorolipid production by *T. bombicola.*

The fourth the production is done with precursor supplementation in which the addition of biosurfactant precursors to the growth medium causes both qualitative and quantitative changes in the product. Similarly, increased production of biosurfactant containing different mono-, di-, or trisaccharides was reported to occur in *A. paraffineus* DSM 2567(Li *et al.*, 1984).

1.2.14 Genetics of biosurfactant biosynthesis:

There are numerous reports on the isolation of mutants deficient in biosurfactant production with a contaminant loss in the ability to grow on water-insoluble substrates (Gervasio *et al.*, 2009).

Ochsner *et al*. (1994) have extensively studied the genetics of rhamnolipid biosynthesis in *P. aeruginosa*. The rhI ABR gene cluster was found to be responsible for the synthesis of RhIR regulatory protein and a rhamnosyl transferase, both essential for rhamnolipid synthesis, but in the *E. coli* active rhamnosyl transferase was synthesized but rhamnolipid were not produced (Ochsner *et al.*, 1995).

The organization of the biosynthetic gene cluster of surfactin was published in the early 1990s by different researcher groups (Cosmina *et al*., 1993; Fuma *et al*., 1993). It suggested the involvement of three chromosomal genes (*sfp, srf A,*and *comA*) in biosynthysis of the surfactin production suggested (Nakano *et al*., 1992; Nakano and Zuber, 1993).

The detailed knowledge of the genetics of microbial surfactant production should be used to produce organisms giving higher production with better product characteristics. The molecular genetics of biosynthesis of alasan and emulsan by *Acinetobacter* species and of the fungal biosurfactant such as mannosylerythritol lipids (MEL) and hydrophobins have been deciphered (Das *et al.,* 2008).

Rusansky *et al.* (1987) described the isolation and partial characterization of an oil-degrading microorganism, *A. calcoaceticuis* RA57 was found to contain four plasmids. Evidence referred that one of the plasmids, pSR4 (20 kilobases [kb]), is required for optimal growth on crude oil in liquid culture. While Pines and Gutnick, (1986) demonstrated that the mutant *A. Calcoaceticus RAG1* strain l lacks its ability to grow on crude oil.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials and Methods:

2.1 Materials:

2.1.1 Equipments and Apparatus:

The following equipments and apparatus were used throughout this study:

Equipment	Company (origin)
Agarose gel tank with power supply	BioRad - Germany
Autoclave	GallenKamp - England
Balance	Ohans - France
Cooling centrifuge	Hettich - Germany
Compound light microscope	Olympus - Japan
Distillator	GFL – Germany
Eppendrof centrifuge	Eppendorf - Germany
Elisa reader	Awareness - USA
Fourier Transform Infra –Red	Shimadzu - Japan
Fluorescence microscope	Olympus
Flask of tissue culture :plastic disposable	Iwaki – Japan
Freeze –Dryer (lyophilizer)	Labcon – USA
Gas chromatography	Shimadzu
Hot plate with magnetic stirrer	Gallenkamp
HPLC	Shimadzu
Incubator	Gyromax – USA
Incubator with CO_2 supply	Thermo electron corporation - USA
Laminer air flow	Heraeus – Germany
Micropipettes	Eppendorf - Germany

96 Well microtiter plate, 6 well plate	BD falcon – USA
Millipore filter paper unit	Millipore corp - USA
Nanodrop spectrophotometer	Bio rad
Nuclear magnetic resonance	Bruker – UK
Oven	Memmert - Germany
pH-meter	Crison – Spain
Sensitive balance	Sartorius - Germany
Shaker incubator	GallenKamp
Tensiometer	Kruss – Germany
Thermo cycle T-5000	Biometra – England
Spectrophotometer	Aquarius – UK
UV- transiluminator	Ultraviolet products - USA
Water bath	Memmert - Germany

2.1.2 Chemicals:

The following chemicals were used in this study:

Material	**Company(Origin)**
Agar-agar	Hi media - India
Acetic acid	Riedel-Dehaeny - Germany
Acetone	Sigma – USA
Agarose	Sigma
Alcohol	BDH - England
Ammonium chloride- NH_4Cl	Sigma
Ammonium sulphate- $(NH_4)_2SO_4$	Sigma
Ammonium nitrate (NH_4NO_3)	Sigma

Boric acid	Sigma
Bovine serum albumin	BDH - England
n-Butanol	BDH
Calcium chloride- $CaCl_2$	Sigma – USA
Chloroform	Sigma
Coomassie Brilliant Blue G-250	Sigma
Copper sulphate pentahydrate $CuSO_4$.$5H_2O$	Sigma
Crude oil	Al- Durra refinery - Iraq
Diethyl ether	GCC - Germany
Dimethyle- α-naphthylamine	Sigma
Dipotassium hydrogen phosphate (K_2HPO_4)	BDH
Disodium hydrogen phosphate (Na_2HPO_4)	BDH
Ethyl acetate	Sigma
Ethylene diamine tetra acetic acid (EDTA)	Sigma
Fructose	Sigma
Glucose	Sigma
Hexane	Sigma
Hydrochloric acid	Sigma
Hydrogen peroxide (H_2O_2)	BDH
Iodine	BDH
Magnesium sulphate($MgSO_4$, $MgSO_4.4H_2O$)	Sigma

Manganese sulphate ($MnSO_4$.4 H_2O, $MnSO_4$. $7H_2O$)	Sigma
Manganese chloride monohydrate($MnCl.H_2O$)	Sigma
Methanol	Sigma
Peptone	Sigma
Phenol	Riedel-Dehaeny - Germany
Phosphate buffer saline(PBS)	Sigma
Phosphoric acid	Sigma
Potassium dihydrogen phosphate(KH_2PO_4)	BDH
Potassium chloride (KCl)	BDH
Potassium nitrate (KNO_3)	BDH
Sillica gel 60	BDH
Sodium chloride (NaCl)	Sigma
Sodium nitrate ($NaNo_3$)	BDH
Sulfanilic acid	Sigma
Sulfuric acid (H_2SO_4)	Analar – England
Sun flower oil	Commercialy
Tris-HCL	BDH
Tris-base	BDH
Trypan blue	Sigma
Urea	Sigma
Vanillin	Sigma
Yeast extract	Sigma

2.1.3 Reagents and Solutions:

2.1.3.1 Phosphate buffer saline (PBS 1 %):

It was prepared by dissolving 10 gm of PBS in 1 liter of sterilized D.W.

2.1.3.2 Catalase reagent:

Hydrogen peroxide (H_2O_2) 3 % was prepared for detection the catalase production (Atlas *et al.*, 1995).

2.1.3.3 Oxidase reagent:

One gm of tetra methyl – p – phenylenediamine dihydrochloride was dissolved in 100 ml of distilled water (D.W) and kept in dark bottle at 4 °C (the reagent should be used fresh). This reagent was used for detection the oxidase production (Benson, 2002).

2.1.3.4 Nitrate test reagent (Atlas *et al*., 1995).

This reagent consists of two solutions:

Solution A: it was prepared by adding 0.8 gm of sulfanilic acid to 5 N acetic acid up to 100 ml.

Solution B: it was prepared by adding 0.5 gm of dimethyle- α-naphthylamine to 5 N acetic acid up to 100 ml.

Equal volumes of solution A and solution B were immediately mixed before use.

2.1.3.5 Trace element solution (Kadhim *et al*., 2008):

It was prepared by dissolve the following component in D.W.:

Component	Weight(gm)
$ZnSO_4.7\ H_2O$	2.32
$MnSO_4.4H_2O$	1.78
$CuSO_4.5H_2O$	1
Boric acid	0.56
EDTA	1
$NiCl_2.6H_2O$	0.004
KI	0.66

All components were dissolved in 950 ml of D.W, then volume was completed to 1000 ml in volumetric flask, pH was adjusted to 7 and sterilized by filtration.

2.1.3.6 Tris – Mg solution:

Composed of 20 mM (Tris - HCl, pH 7) and 10 mM ($MgSO_4$) (Bach *et al.*, 2003).

2.1.3.7 Tris – HCl solution (0.1 M) and (0.2 M):

* For preparing 0.1 M of Tris – HCl, 7.88 gm was dissolved in 100 ml then complet the volume to 500 ml with D.W, pH was adjusted to 7.5.

* Tris – HCl (0.2 M) was prepared by dissolving 4.73 gm of Tris –HCl in 100 ml then complet the volume to 150 ml with D.W, pH was adjusted to 7.5 (Maneerat and Dikit, 2007).

2.1.3.8 Tris – base solution (0.2 M):

This buffer was prepared by dissolving 1.211 gm of Tris-base in 10 ml then complet the volume to 50 ml with D.W, pH was adjusted to 8, 9 and 10 (Maneerat and Dikit, 2007).

2.1.3.9 Salts solutions:

Different salts included: NaCl, KCl and $CaCl_2$ were prepared at different concentrations (2%, 4%, 8%, 12%, 16% and 20%), by dissolving these salts in 0.1 M of Tris-HCl (pH 7).

2.1.3.10 Solutions for lipid estimation:

The following solutions were used for estimation the total lipid (Kaufmann and Brown, 2008):

- **Sulfuric acid (98 %).**
- **Vanillin – phosphoric acid reagent :**

It was prepared by dissolving 600 mg vanillin in 100 ml deionized hot water, then 400 ml of 85 % phosphoric acid was added and stored in dark.

2.1.3.11 Solutions for protein estimation (Bradford, 1976):

- **Bovine serum albumin (BSA) stock solution :**

It was prepared by dissolved 1 mg of bovine serum albumin in 1 ml of D.W to obtain final concentration 1000 µg /ml (stock solution). Different concentrations of BSA (0-130 µg/ml) were prepared from the stock solution for making standard curve.

- **Bradford reagent:**

It was prepared by dissolving 100 mg Coomassie Brilliant Blue (G-250) in 50 ml of 95 % ethanol, then 100 ml of 85 % (w/v) phosphoric acid was added. The volume

was completed to 1 L with sterile D.W., when the dye was completely dissolved, filtered through Whatman #1 paper just before use.

2.1.3.12 Solutions for carbohydrate estimation:

The following solutions were used for estimation of total carbohydrate by phenol – sulfuric acid method (Dubois *et al.*, 1956):

- **Stock solution of glucose:**

This solution was prepared by dissolving 100 mg of glucose in 100 ml of sterile D.W., to obtain a final concentration of 1000 µg/ml. Different concentrations of glucose (0-1000 µg/ml) were prepared to obtain the standard curve of glucose.

- **Phenol solution (5 %):**

A weight of (5) gm of phenol crystal was dissolved in 10 ml of D.W., the volume was completed to 100 ml.

- **Sulfuric acid (98 %).**

2.1.3.13 Heat inactivated fetal bovine serum:

It was prepared according to Wang *et al.* (2007) by incubating fetal bovine serum in the water bath at 56 ºC for 30 min.

2.1.4 Primer:

The following primer was used in this study:

Primer name		Primer sequence 5'----3'	Uses	References
Sfp	F	ATGAAGATTTACGGAATTTA	*Bacillus*	Sekhon *et al.* (2011)
Sfp	R	TTATAAAAGCTCTTCGTACG		

2.1.5 Culture media:

2.1.5.1 Ready made culture media:

Culture media	Company (Origin)
Muller – Hinton broth	BDH –England
Muller –Hinton agar	BDH
Nutrient agar	Fluka- Germany
Nutrient broth	Fluka

These media were prepared as recommended by the manufacturing company and sterilized by autoclaving at 121 ºC for 15 min.

2.1.5.2 Laboratory prepared media:

- **Luria-Bertani (LB) medium (Nazina *et al*., 2001).**

This medium was prepared by dissolving tryptone (10 gm), yeast extract (5 gm) and NaCl (5 gm) in 950 ml D.W., pH was adjusted to 7, the volume was completed to 1L, sterilized by autoclaving, cooled to 45 ºC then 1 ml of (0.1 M) $MnCl.H_2O$ (autoclaved separately) was added.

- **Nitrate medium :**

This medium was composed of 5 gm peptone supplemented with 0.2 gm KNO_3 in 1L of D.W., pH was adjusted to 7, and distributed in tubes, then sterilized by autoclaving (Atlas *et al*., 1995).

- **Mineral salt medium (MSM):**

Chemically defined medium was prepared according to the method described by Yakimov *et al.* (1995) with modification:

Component	Weight (gm)
NH_4Cl	4
NaCl	4
KH_2PO_4	3
Na_2HPO_4	6
$MgSO_4$	0.1

These components were dissolved in 950 ml distilled water, and 2.5 ml trace element solution prepared as in item (2.1.3.5) was added, pH was adjusted to 7, and the volume was completed to 1000 ml then sterilized by autoclave. This medium was used to detect the ability of bacterial isolates to produce biosurfactant.

- **RPMI 1640 medium(Sigma- USA):**

This medium was enriched with 10 % heat inactivated fetal bovin serum (item 2.1.3.13),100U/ml penicillin and 100 µg/ml streptomycin.

- **EMEM medium(Thermo- USA):**

It contains the following supplements: 10 % of fetal bovine serum, 1 mM of sodium pyruvate, 1X non-essential amino acids, 100 units/ml of penicillin and 100 µg/ml streptomycin. This medium was used for the cytotoxicity test.

2.1.6 Kits used in this study:

- **Extraction of genomic DNA:**

The genomic DNA was extracted from different bacterial isolates using Wizard Genomic DNA purification Kit (Promega, USA). It consists of the following:
Cell Lysis Solution, Nuclei Lysis Solution, RNase Solution, Protein Precipitation Solution, DNA Rehydration Solution.

- **PCR master mix (2 X):**

It was supplied by Promega company, USA. The solutions were composed of the following:

PCR master mix (2 X) (Promega)
DNA polymerase (5 U/µl)
DNTPs
PCR reaction buffer
$MgCl_2$

- **Cell titer 96 non- radioactive cell proliferation assay (MTS assay, promega-USA):**

- A weight of 1.90 mg /ml of tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5 (3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS].
- 300 µM of an electron coupling reagent (phenazine ethosulfate) PES.
- Dissolving in Dulbecco´s phosphate- buffered saline (pH 6.0).

- **Multiparameter Cytotoxicity kit (Thermo- USA) :**

- **Kit contents:**

Contents	Volume
Cytochrome C Primary Antibody	75 µl
DyLight ™ 649 Conjugated Goat-Mouse Antibody	72 µl
Mitochondrial Membrane Potential Dye	1 µl
Permeability Dye	25 µl
Hoechst Dye	30 µl
Wash Buffer (10 X Dulbeccos PBS)	100 ml
Permeabilization Buffer (10 X Dulbecco's PBS with 1% Triton X-100)	100 ml
Blocking Buffer (10 X)	85 ml
Thin Plate Seal Assembly	7/ pack

- **Solutions for multiparameter cytotoxicity test:**

The following solutions were prepared for cytotoxicity test:

Solution	Preparation
A- 1X Wash Buffer	20 ml of 10X Wash Buffer was added to 180 ml ultrapure water, buffer was stored at 4 °C for up to 7 days.
B- Fixation Solution	3 ml of 16% paraformaldehyde solution was added to 9 ml of 1X wash buffer just before used.
C-1XPermeabilization Buffer	1.5 ml of 10X Permeabilization Buffer was added to 13.5 ml of the 1X wash buffer. This buffer was stored at 4 °C for up to 7 days
D- 1X Blocking Buffer	5 ml of 10X Blocking Buffer was added to 44 ml of 1X wash buffer. This buffer was stored at 4 °C for up to 7 days.
E- Primary Antibody Solution	15 µl of the Cytochrome C Primary Antibody was added to 6 ml of 1X blocking buffer. Solution was prepared just before each assay.

F- Secondary Antibody/ Staining Solution	0.6 µl of Hoechst Dye and 12 µl of the Dye Light 649 Goat Anti-Mouse were added to 6 ml of 1X Blocking Buffer. Solution was prepared just before each assay.
G- Live Cell Staining Solution	117 µl of DMSO was added to the Mitochondrial Membrane Potential Dye to make a 1 mM stock solution. Then add 2.1 µl of Permeability Dye and 21 µl of Mitochondrial Membrane Potential Dye were added to 6 ml complete medium pre-warmed to 37 °C.

2.1.7 Microorganisms:

The following microorganisms were used in this study:

Microorganisms	Source
Geobacillus thermoleovorans Ir1 (JQ912239)	Department of Biotechnology, Al-Nahrain University
Anoxybacillus rupiensis Ir2 (JQ912240)	
Anoxybacillus rupiensis Ir3 (JQ912241)	
Anoxybacillus sp.	
Staphylococcus aureus	
Streptococcus sp.	
Pseudomonas aeruginosa	
Proteus mirabilis	
E. coli	
Aspergillus niger	
Penicillium	
Candida albicans	
Six non identified thermophillic bacteria (9SM, 12SM, 13SM, 14SM, 21SM, 34SM)	

2.1.8 Cell lines:

Different cell lines were used in this study:

Cell lines	Source
Breast cancer(MCF7)	Department of biotechnology /Ioannina University, Greece.
Jurkat cells	
Hut 78 cells	
Normal Kidney (HEK)	

2.2 Methods:

2.2.1 Sterilization methods (Atlas *et al.*, 1995):

Three methods of sterilization were used

- **Moist heat sterilization (autoclaving):-**

Media and solutions were sterilized by autoclaving at 121 ºC (15 Ib/in2) for 15 minutes.

- **Dry heat sterilization**

Electric oven was used to sterilize glassware at 160 ºC for 3 hrs. and 180 ºC for 2 hrs.

- **Membrane sterilization (filtration)**

Millipore filtering was used to sterilize heat sensitive solutions by millipore filter paper (0.22 µm in diameter).

2.2.2 Maintenance of bacterial isolates (Green and Sambrook, 2012):

Maintenance of bacterial isolates was performed as follow:

- Short term storage:

Bacterial isolates were maintained for few weeks on different agar slants, they were tightly wrapped with parafilm, and then stored at 4 ºC**.**

-Medium- term storage:

Bacterial isolates were maintained as stab cultures for months. Such cultures were prepared in small screw- capped bottles containing 2-3 ml of LB, nutrient agar the vials were wrapped with parafilm and stored at 4 °C.

-Long term storage:

Single colonies were cultured in nutrient broth and incubated for 24 hrs., at 55 °C, then 8.5 ml of bacterial culture was mixed with 1.5 ml of glycerol, and stored for a long time at -20 °C.

- **Preservation in silica gel (Al - Azawi, 1982):**

A little amount of silica gel was sterilized in autoclave, and dried in oven at 60-70 °C for 24 - 48 hrs., The sterilized silica gel was inoculated with 0.1 – 0.2 ml of bacterial culture and stored at room temperatures in parafilm wrapped eppendrof tubes.

2.2.3 Identification of bacterial isolates:

2.2.3.1 Microscopic and morphological examination:

Morphological features of bacterial isolates grown on LB, nutrient agar medium were studied included shape, size, margin, color, arrangement and other features. On the other hand microscopic examination was done by transferred a loop full of single colony to clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with 95 % alcohol, and counterstained with safranine, then examined by a microscope (Atlas *et al.*, 1995).

2.2.3.2 Biochemical tests:

- **Catalase test** :

A loop full of bacterial growth on nutrient agar was placed on a clean glass slide, mixed with a drop of 3 % H_2O_2 (2.1.3.2) and the result was reported. The appearance of bubbling indicates a positive catalase test (Prescott, 2002).

- **Oxidase test :**

Filter paper was saturated with oxidase reagent (2.1.3.3), a colony of bacteria was transferred on the filter paper with a sterile wooden applicator stick. An immediate change of the color to deep blue indicates a positive result (Benson, 2002).

- **Nitrate reduction test :**

A single colony of each bacterial isolate was used to inoculate 5 ml of nitrate media (2.1.5.2), and then test tubes were incubated at 55 ºC for 24 hrs. After incubation, 0.1 ml of the test reagent (2.1.3.4) was added to each tube. The immediate formation of red color indicates a positive result (Atlas *et al*., 1995).

2.2.4 Screening the bacteria for biosurfactant production:

2.2.4.1 Biosurfactant production:

To determine the capability of bacterial isolates to produce biosurfactant, 50 ml of mineral salt medium (2.1.5.2) was despised in 250 ml Erlenmeyer flasks. The flasks were sterilized, and then 1% of crude oil (sterilized by tyndrazation) was added as carbon source. The flasks were inoculated with 1% of fresh bacterial growth (18 hrs.) and incubated under shaking (180 rpm) at 55 °C for 7 days. Then, the cultures were centrifuged at 4 °C, 10000 rpm, for 15 min. Production of biosurfactant was investigated in cell –free supernatant.

2.2.4.2 Determination of biosurfactant compounds:

- **Determination of emulsification activity (E.A):**

The emulsification activity was determined by taking 0.5 ml of the cell free supernatant from cultured mineral salt medium, and added to 7.5 ml of Tris-Mg buffer (2.1.3.6) plus 0.1 ml of dodecane, then mixed with vortex for 2 min. The tubes were left for 1 hr.

The absorbency was measured at 540 nm. Emulsification activity was defined as the measured optical density, while the blank was Tris-Mg, dodecane and mineral salt broth without culture (Sifoure *et al*., 2007)**.**

- **Determination of emulsification index (E 24 %):**

One ml of cell free supernatant was added to 1 ml of sun flower oil (equal volumes v/v), mixed with vortex for 2 min., and left for 24 hrs. at room temperature, The height of emulsifier layer was measured.

The emulsification index was given as a percentage of the height of the emulsified layer (mm) to the total height of the liquid column (mm) multiplied by 100 (Tabatabaee *et al*., 2005).

- **Surface tension (S.T):**

The surface tension reduction was determined by a K6 tensiometer using the du Nouy ring method. A volume (20 ml) of cell free supernatant was placed into 50 ml clean glass beaker then left to equilibrating for 15 min.

Before measuring, the tensiometer must calibrate with D.W (72 mN/m). The tensiometer is an instrument incorporating aprecision microbalance, platinum wire ring with a defined geometry and precision mechanism to vertically move sample liquid in a glass beaker. The ring hanging from the balance hook is first immersed

into the liquid surface and then carefully pulled up, the micro balance records the force applied on the ring while pulling through the surface. The surface tension is the maximum force needed to detach the ring from the liquid surface. Between each pair of measurement, the platinum wire ring was rinsed three times with water, three times with acetone and allowed to dry (Abouseoud *et al* ., 2008; Sekhon *et al.*, 2011).

2.2.5 Optimization condition for biosurfactant production:

Optimization experiments were done in an Erlenmyer flask containing 50 ml of mineral salt medium (2.1.5.2) with 1 % of crude oil inoculated with 1 % of fresh bacterial culture (*G. thermoleovurance* Ir1 (JQ912239)). The flasks were incubated in a shaker incubator (180 rpm) at 55 ºC. After incubation period, emulsification index and surface tension were measured.

2.2.5.1 Effect of carbon source:

Different carbon sources (fructose, glucose, manitol, sucrose, diesel, date extract, crude oil and sunflower oil) were used to determine the optimum source for biosurfactant production, each of these sources was added to the medium in a concentration 1 % separately. Then, pH was adjusted to 7.0, and inoculated with bacterium then incubated in shaker incubator (180 rpm) at 55 °C for 7 days.

Emulsification index and surface tension were measured and the optimal carbon source was employed later on.

2.2.5.2 Effect of carbon source concentration:

Different concentrations (0.25%, 0.5%, 0.75%, 1%, 2%, 3% and 4 %) of crude oil were used to grow the bacterium in order to determine the optimum

concentration for biosurfactant production. After the pH adjustment to 7.0, flasks were incubated in shaker incubator (180 rpm) at 55 °C for 7 days.

2.2.5.3 Effect of temperature:

In order to determine the optimum temperature for biosurfactant production, mineral salt medium (pH 7), after inoculated with bacterium flasks were incubated in a shaker incubator (180 rpm) at different temperatures (40, 50, 55, 60, 65 and 70 °C) for 7 days, Then the optimal temperature was subsequently employed.

2.2.5.4 Effect of different nitrogen sources:

Different nitrogen sources ($(NH_4)_2SO_4$, NH_4Cl, yeast extract, KNO_3, NH_4NO_3 and urea) were used to determine the optimum condition for the biosurfactant production by the bacterial strain. These nitrogen sources were added to the mineral salt medium in a concentration 0.4 %, and pH was adjusted to 7.0. Then after inoculation the flasks were incubated in a shaker incubator with 180 rpm at 60 ºC for 7 days. Optimal nitrogen source was selected and employed later on.

2.2.5.5 Effect of nitrogen source concentration:

The optimal nitrogen source (NH_4Cl) was added in gradual concentration (0.05%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5%) to the mineral salt medium. pH was adjusted to 7.0, then inoculated with the bacterial strain and incubated in shaker incubator (180 rpm) at 60 °C for 7 days. Then the optimal concentration was employed.

2.2.5.6 Effect of pH:

Mineral salt medium was adjusted with different pH values (5, 6, 6.5, 7, 8 and 9) to determine the suitable value. The flasks after inoculation with bacterial strain were

incubated in a shaker incubator with 180 rpm at 60 ºC for 7 days. And the better pH value was employed in later experiment.

2.2.5.7 Effect of aeration:

Different rpm values (120, 150, 180, 200 and 220 rpm) were examined to determine the optimum shaking required to obtain the high biosurfactant activity. The flasks after inoculated with bacterial strain were incubated at 60 °C for 7 days and the optimal aeration was used in a later experiment.

2.2.5.8 Effect of incubation period:

Mineral salts media broth contain an optimum carbon concentration source and nitrogen source with pH 7 was inoculated with bacterial strain and incubated at 60 ºC with shaking at 200 rpm for (2–11) days, after each incubation period E24% and surface tension were estimated.

2.2.6 Extraction of biosurfactant compound:

One litter of mineral salt medium (pH7) containing crude oil (1%) as carbon source, and ammonium chloride (0.3%) as a nitrogen source, inoculated with 1% of fresh culture of *G. thermoleovorans* Ir1(JQ912239) and incubated in a shaker incubator (200 rpm) at temperature 60 ºC for 10 days.

After incubation, supernatant of bacterial culture was obtained by centrifugation at 10000 rpm for 15 min at 4 °C. The supernatant subjected to extraction with different methods as follows:

1- **Extraction with Acetone**: biosurfactant was extracted by precipitating metabolic cell-free liquid with acetone 1:1 (v/v) and allowed to stand for 24 hrs. at 4 °C, and then it was centrifuged (4000 rpm) for 15 min, at 5 °C. The

supernatant was discarded and the isolated biosurfactant was submitted to dialysis against deionized water for 72 hrs., at 5 °C, and was subjected to change every 3 hrs. The biosurfactant was collected and freeze dried (Jara *et al.*, 2013).

2- **Extraction with Chloroform – methanol**: The supernatant was acidified by adding 6 N HCL to pH 2 and incubated over night at 4 °C, a flocculated precipitate was formed and collected by centrifugation (10000 rpm), at 4 °C for 10 min. Then extracted with chloroform – methanol (2:1 v/v). The aqueous layer at the bottom of the separation funnel was removed and the emulsifier layer was collected in a glass petri dish and dried in an oven (40 – 45 °C)(Tahzibi *et al*., 2004; Sifour *et al*., 2007; Das *et al.*, 2008).

3- **Extraction with diethyl ether:** Equal volumes of cell free supernatant and diethyl ether were mixed in a separating funnel. The aqueous layer was removed and the emulsifier layer was collected in a sterilized glass petri dish and dried in oven (40 – 45 °C) (Nadaling *et al*., 2000).

2.2.7 Purification of biosurfatant compound:

A portion of the crude extract was re- dissolved in distilled water and purified from the remain impurities by dialyzed with dialysis membrane (12 kDa cut-off) for 24 hrs., by using distilled water and change every 3 hrs. (Joshi *et al.*, 2008).

For further purification of biosurfactant column chromatography with fallowing dimension (2.5*40) cm was used, filled with silica 60 gel. It was packed tightly by a continuous flow of hexane, then the column was washed with hexane. Sample dissolved in 6 ml chloroform was loaded in column until majority of the solvent is absorbed. Then eluted the column with sequential polar solvent: hexane, chloroform, ethyl acetate, methanol and collected the fraction (3 ml) for each.

All eluted fractions were collected and tested for their emulsification activity as previously described (2.2.4.2) (Thanomsub *et al.*, 2004).

2.2.8 Chemical composition of biosurfactant compound:

2.2.8.1 Estimation of the lipid concentration:

The lipid concentration of partial purified biosurfactant was determined according to Kaufmann and Brown (2008) as follows:

- One mg of commercial vegetable oil was dissolved in 1 ml of chloroform (1000µg/ml).
- The standard curve was prepared by preparing a serial amount of lipid (50-600µg).
- The previous solution was added as the following 50, 100, 200, 400 and 600 µl separately to each glass tube in triplicate.
- These glass tubes were placed in a heating block at 90-110 ºC to evaporate the solvent.
- Two hundred microliter of sulfuric acid (98%) was added to each tube separately and heated for 10 min at 90-110 ºC.
- Vanillin reagent was prepared as (2.1.3.10) was added to 5 ml level and mixed.
- The tubes were removed from heating block and allowed to cool.
- The absorbance at 625 nm was measured.
- The lipid amount of unknown sample was treated in the same manner by dissolving 1 mg of biosurfactant in 1 ml of chloroform with mixing and the lipid was determined according to the standard curve (figure 2-1).

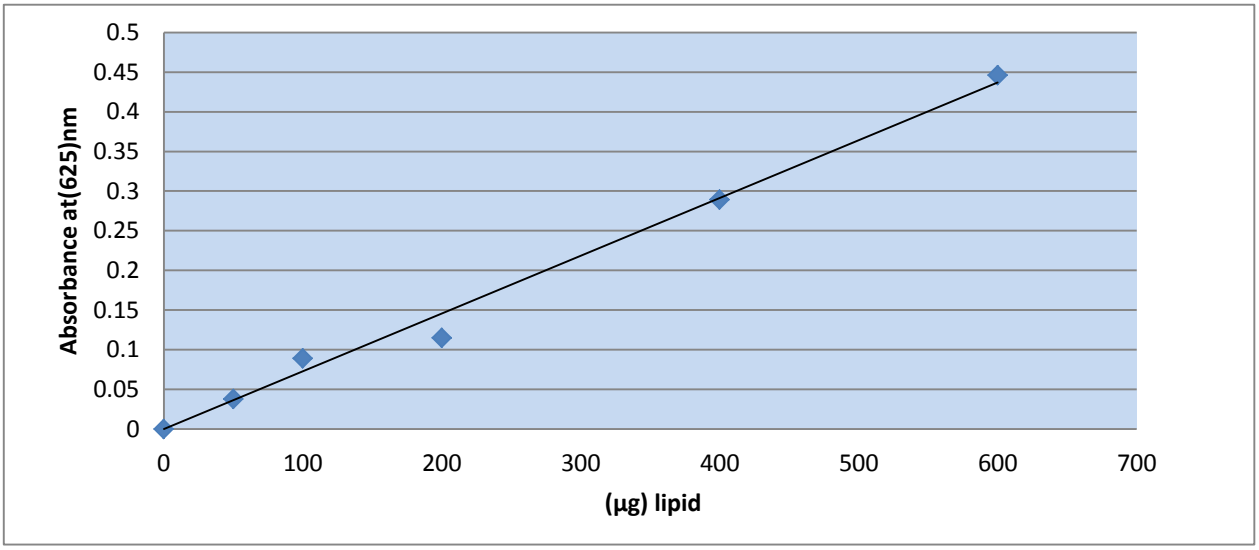


Figure (2-1): Standard curve of lipid determining by Kaufmann and Brown (2008) method.

2.2.8.2 Estimation of the protein concentration:

Bradford method was used for estimation of the protein concentration of partial purified biosurfactant depending on bovine serum albumin (BSA) standard curve (Bradford, 1976) as follows:

- A volume of 200 µl of different concentrations (0 -130 µg /ml) of BSA was prepared from stock solution of bovine serum albumin (1 mg/ml) (2.1.3.11) table (2-1).
- Eight hundred microliters of Bradford reagent (2.1.3.11) was added to each tube .
- The solution was vortexed and left to stand for 10 min.
- The optical density (O.D) was measured at 595 nm.
- The curve was drown to show the relationship between the optical density (O.D) and the protein concentration to obtain the standard curve for the BSA, figure (2-2).

The unknown protein samples were treated in the same manner by dissolving 1 mg of biosurfactant in 1 ml of Tris – HCl by mixing with magnetic stirrer and the protein concentration was determined according to the standard curve.

Table (2-1): Preparation of different bovine serum albumin concentrations:

Tube No.	Volume of BSA solution(µl)	Volume of D.W (µl)	Final concentrations (µg /ml)
1*	0	200	0
2	2	198	10
3	4	196	20
4	6	194	30
5	8	192	40
6	10	190	50
7	12	188	60
8	14	186	70
9	16	184	80
10	18	182	90
11	20	180	100
12	22	178	110
13	24	176	120
14	26	174	130

Tube (1*) represents the blank solution.

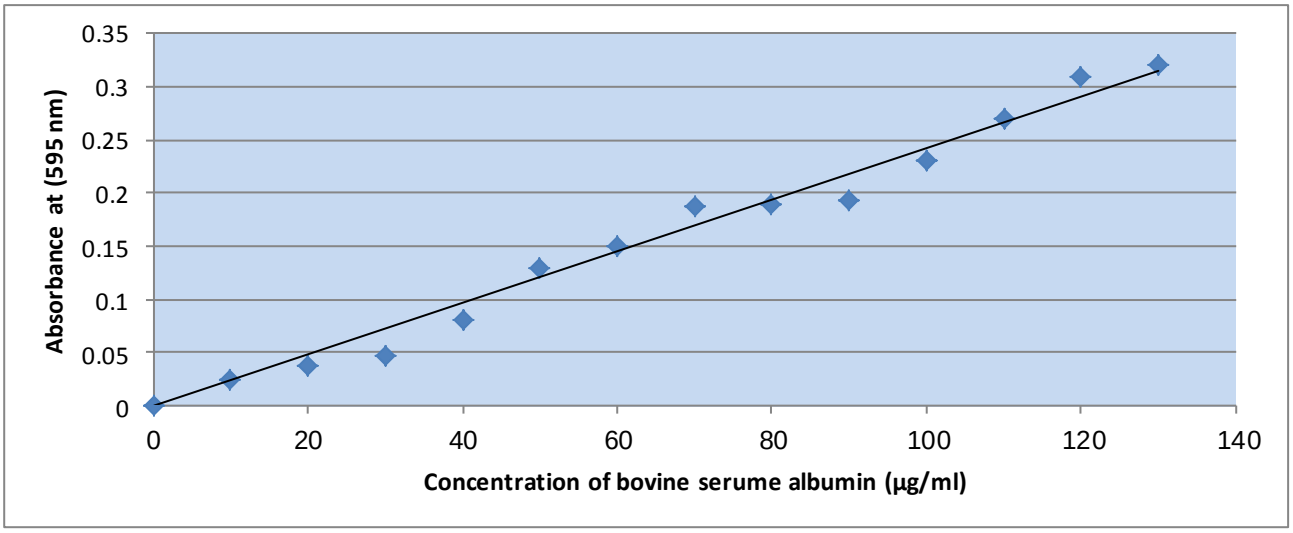


Figure (2-2): Standard curve of Bovine Serum Albumin (BSA) by Bradford method for determination protein concentration of biosurfactant produced by *G. thermoleovorans*.

2.2.8.3 Estimation of the carbohydrate concentration:

The carbohydrate content of the partially purified biosurfactant sample was determined by the preparation of glucose standard curve (Dubois *et al*., 1956) as follow:

- Different concentrations ranged from (0-1000 µg /ml) were prepared from glucose stock solution (2.1.3.12). The final volume was 1 ml, as shown in table (2-2).
- One ml of phenol solution (2.1.3.12) was added to each tube with shaking.
- Five ml of concentrated sulphuric acid (98%) was added to the mixture, mixed well and left to cool at room temperature.

Table (2-2): Preparation of different glucose concentrations.

Tube No.	volume of Sugar solution(ml)	Volume of D.W (ml)	Final concentrations (µg /ml)
1*	**0**	**1**	**0**
2	**0.1**	**0.9**	**100**
3	**0.2**	**0.8**	**200**
4	**0.3**	**0.7**	**300**
5	**0.4**	**0.6**	**400**
6	**0.5**	**0.5**	**500**
7	**0.6**	**0.4**	**600**
8	**0.7**	**0.3**	**700**
9	**0.8**	**0.2**	**800**
10	**0.9**	**0.1**	**900**
11	**1**	**0**	**1000**

Tube (1*) represents the blank solution.

- The absorbency (O.D) was measured for each tube at 490 nm.
- The curve of glucose was demonstrated the relationship between absorbency (O.D) and glucose concentration as shown in the figure (2-3).
- The unknown samples used for carbohydrate estimation were prepared by dissolving 1mg of biosurfactant powder in 1ml of Tris – Mg or Tris – HCl with mixing with a magnetic stirrer.

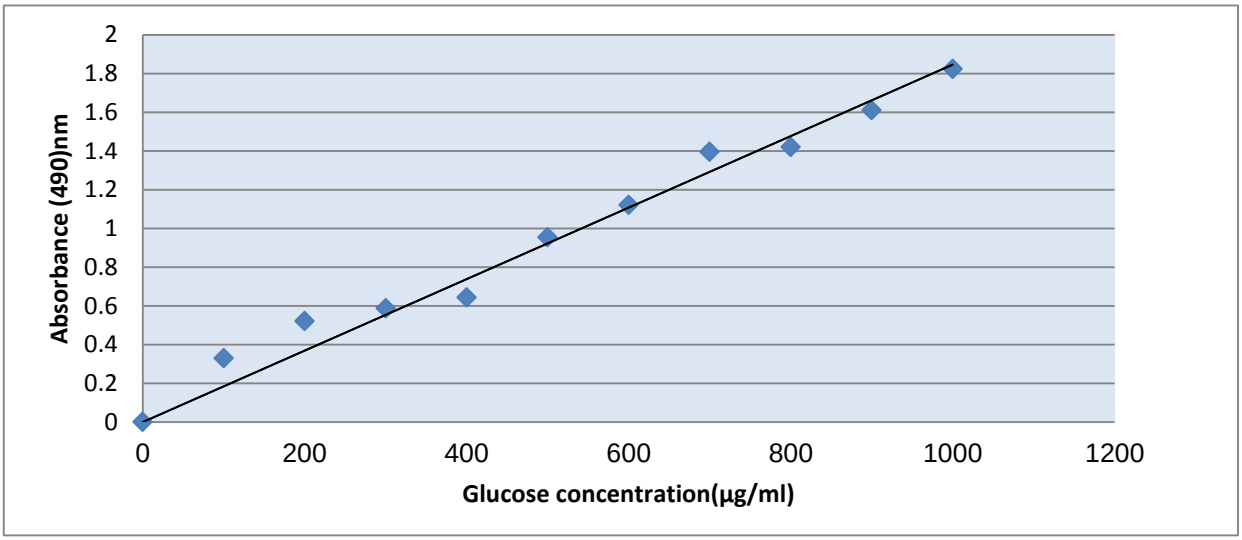


Figure (2-3): Standard curve of glucose by Dubois *et al*. method for glucose determination.

2.2.9 Characterization of partial purified biosurfactant compound:

2.2.9.1 Analysis with Fourier Transform Infra Red:

The basic functional groups of the partial purified biosurfactant from *G. thermoleovorans* (JQ912239) were analyzed qualitatively by Fourier Transform Infra red (FTIR) (Aparnaa *et al.*, 2012).

Fourier transform infra red spectroscopy of the biosurfactant sample was obtained by using (shimadzu) spectrophotometer, in which the 2 mg of partial purified sample was mixed with 150 mg KBr and pressed into a tablet form in a dry atmosphere.

The FT-IR spectra measurement was done in the frequency range of 400–4000 wavenumbers (cm^{-1}) with resolution of 2 cm, 50 scans (Yalçın and Çavuşoğlu, 2010).

2.2.9.2 Analysis with High Performance Liquid Chromatography (HPLC):

The partial purified biosurfactant was analyzed into main components by using the HPLC:

1- Fatty acids:

The main components (fatty acids) within the sample were detected by using HPLC as follow:

- **Preparation of sample:**

The 100 mg of partial purified biosurfactant mixed with 20 ml ionized water, then the fat separation was carried out according to ISO-IDF (2001), to the previous mixture 20 ml of (0.1M) ammonium hydroxide was added. Follow with the addition of 20 ml from the following mixture (50:50 v/v), n- pentane and diethyl ether, then taking the extracted fatty acid.

- **Tested the sample:**

Ten microliter of previously prepared solution was injected in C-8DB column, 3 µm particle size, the test was done by using acetonitrile:tetrahydrofuran (THF): 0.1% of phosphoric acid in THF(50.4: 21.6: 28v/v) as a mobile phase, with a flow rate of 1.5 ml/min at 40 ºC and UV detector at 215 nm, then the observed retention time for each fatty acid was determine and compared to the standard retention time respectively.

2- Amino acids:

- **Treatment the sample:**

In order to prepare the sample for protein analysis by HPLC, the partial purified biosurfactant sample was hydrolyzed with 6 N HCL at 110 ºC for 24 hrs., (Huang *et al*., 2012), then the samples were derivatization by mixing 10 µl of each of standard and biosurfactant sample separately with 10 µl of PTIC reagent and after 1 min, 50 µl of (0.1 M) sodium acetate was added. The samples were shaked and agitated in ultrasonic bath for 10 min, the extract was filtered on disposable filters (0.2 µm).

- **Tested the sample**:

The amino acids were determined by injected 20 µl of each standard and previously treated samples on the shimpack XR-ODS (3 um particle size) column, with 1 ml/min a flow rate and UV detector at 254 nm gradient were formed between two solvents.

- Solvent A: 5 % methanol in 0.1 N sodium acetate buffer.
- Solvent B: methanol, linear gradient from 0-20 minutes.

The data were processed and analyzed by RC-6A data processing, and the concentration for each compound was quantitatively determined by comparison the peak area of the standard to the sample.

3-Carbohydrates:

- **Sample preparation:**

In order to detect the sugars components of the partial purified biosurfactant, sample was hydrolyzed with (1N) HCL and incubated at 90 ºC for 3 hrs., then the solution was filtered through a single use 0.22µm filter.

- **Tested the sample**:

A volume of 20 µl from the above solution sample and the standard were injected separately in an anion exchange shimpack A1 column, 3 µm particle size, and NaOH (15 mM) spiked with 1mM barium acetate used as a mobile system, and run at a flow rate of 1.5 ml/min with RD-6A refractive index detector. The temperature was adjusted to 40 ºC. Under these conditions, the observed retention time for each sugar was determined and compared to the standard retention time respectively (Cataldi *et al*., 2000).

2.2.9.3 Analysis of the lipid part with Gas chromatography (GC):

Lipids were analyzed to their fatty acids components using gas chromatography (GC) (Zheng *et al.*, 2011). Fatty acids composition was investigated as follows. Acid methyl ester was prepared by dissolving 10 mg of partial and purified biosurfactant with 1 ml of sulpheric acid - methanol at 90 ºC for 15 hrs., and 1 ml of hexane was added with mixing, then hexane phase was taken after evaporated the sulfuric acid . To the hexane phase, 1 ml of D.W was added with mixing. The fatty acid methyl ester was extracted with hexane and subjected to an analysis with GC, by using helium as carrier gas on a Shimadzu 17-A GC equipped with an fused silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness).

2.2.9.4 Analysis the lipid part with NMR:

Lipid part of partial and purified biosurfactant was analyzed with 1H NMR spectrum by using Bruker Avance-II 500 spectrometer at 298 k, 500 MHz.

A weight of (20 mg) of partial and purified biosurfactant was dissolved in 600 µl of the CDCL3. Then the mixture was mixed in vortex for 2 min and placed in ultrasonic bath for 15 min. The mixture was centrifuged for 15 min at 4000 rpm, 25 ºC. The supernatant was collected and placed in an NMR tube. The sediment was allowed to settle in order to have a better NMR spectrum.

2.2.10 Effect of biosurfactant on tumor cell line:

2.2.10.1 Cell lines:

Different mobile and attach cell lines (2.1.8) were used to show the inhibitory effect of the purified biosurfactant.

2.2.10.2 Preparation of cells for MTS assay:

- Cells were regenerated from cryogenic.
- Cells were recovered by a rapid thawing at 37 ºC in water bath and centrifuged at 800 rpm for 10 min at a room temperature, then resuspended in culture medium (2.1.5.2).
- Cells were splitted to T-25 three tissue culture flasks.
- Flasks were incubated in a humidified atmosphere with 5 % CO_2 and 95 % air at 37 ºC.
- Cells were grown for 24-36 hrs., and whenever the cells reached high growth under microscope, the media was changed with fresh one until the number of cells reached approximately 4-6 million in each plate.

2.2.10.3 Harvest and count cells (Wang *et al.*, 2007):

The two cell line Hut78 and jurkate were subcultured routinely every 24-36 hrs., to maintain cell culture in an exponential growth in the RPMI 1640 medium (2.1.5.2). Then they were incubated in a humidified atmosphere with 5 % CO_2 and 95 % air at 37 ºC. The flask was investigated under a microscope daily until the cells reach the optimum growth. While the (MCF7) and kidney cells line were subcultured routinely whenever the cell in the flask formed a confluent monolayer. The cells were harvested by washing the flasks twice with PBS (2.1.3.1) followed by detaching cell from flask surface with adding 2 ml trypsin solution for each flask and kept for about 10 min at 5 % CO_2 and 95 % air at 37 ºC until the cell rounded up. Trypsin suspension was inactivated by adding 10 ml cold RPMI media and transferred into blue cap centrifuge tube, then centrifuged at 200 rpm for 10 min. The cell pellet was diluted with RPMI medium and cell were counted with hemacytometer. Cells were splitted to T-25 three tissue culture flasks.

2.2.10.4 Preparation testing plate (96-well):

The 96 well microtiter plate were seeded with a cell line for cell viability study (MTS) and incubated for 24-72 hrs., to a final concentration of 20000 cells per well according to the cell's counting. Then they were treated with different concentrations of biosurfactant (10, 20, 30, 40, 50, 60, 100 µg/ml) dissolved with RPMI media (2.1.5.2) and 0.1 % DMSO, then incubated from 24 -72 hrs., at 37 ^{o}C.

2.2.10.5 Adding MTS dye to the 96-well plate:

MTS dye solution was added with the volume 20 µl to each of 96 well microtiter plates that contain 100 µl of treated cells or blank. The plate was incubate with an aluminum foil at 37 ºC for 1–4 hrs. in a humidified 5 % CO_2 atmosphere, and record the absorbance at 490 nm at every 30 minutes within the incubation period.

The controls and test compounds were assayed for each concentration. The data of the optical density taken from plate reader were then subjected to analysis by using the cell inhibition rate (%) and calculated according to Wang *et al*. (2007):

Cell inhibition rate (%) = [(average absorbance of control cell – average absorbance of treated cell) /average absorbance of control cells)] x100.

Data were analyzed with ANOVA- test.

2.2.11 Multiparameter Cytotoxicity Assay:

In order to determine the cytotoxic effect of biosurfactant many assays were done (Vivek *et al*., 2008) as follows:

2.2.11.1 Cell Preparation:

- MCF7 cells were splited when they reached 90% confluenced at a dilution of 1:4. Cells at a passage number ≤ 10 were used.
- MCF7 cells were harvested by trypsinization, diluted into EMEM complete medium (2.1.5.2) and cell density was determined. Cells were diluted to 7.5×10^4 cells/ml in EMEM complete medium.
- The cell suspension (100 µl) was added to each well of a 96-well microplate to achieve 7500 cells/well.
- Cells were incubated overnight at 37 ºC in 5 % CO_2.

2.2.11.2 Treatment of cell within 96-well microplate:

- A volume 25 µl of doxorobin at a concentration of 50 µg/ml and purified biosurfactant at concentration 50, 100 µg/ml were added separately to cells. Cells were incubated at 37°C for 24 hours.
- Live cell staining solution (50 µl) (2.1.6G) was added to each well and the cells were incubated at 37 ºC for 30 minutes.
- The medium and the staining solution were gently aspirated and 100 µl/well of fixation solution (2.1.6 B) was added. The plate was incubated for 20 minutes at room temperature.
- The fixation solution previously prepared was gently aspirated and 100 µl/well of 1X wash buffer (2.1.6A) was added.
- Wash buffer was removed and 100 µl/well of 1X permeabilization buffer (2.1.6C) was added. The plate was incubated for 10 minutes at room temperature and protected from light.
- Permeabilization buffer was aspirated and plate was washed twice with 100 µl/well of 1X Wash buffer.

- Wash buffer was aspirated and 100 µl of 1X blocking buffer (2.1.6 D) was added and the plate was incubated for 15 minutes at room temperature.
- Blocking buffer was aspirated and 50 µl/well of primary antibody solution (2.1.6E) was added. Plate was incubated for 60 minutes, protected from light at room temperature.
- Primary antibody solution was aspirated and the plate was washed three times with 100 µl/well 1X Wash Buffer.
- Wash buffer was aspirated and 50 µl/well of Secondary antibody/staining solution (2.1.6 F) was added. The plate was incubated for 60 minutes, protected from light at room temperature.
- Secondary antibody/staining solution were aspirated and plate was washed three times with 100 µl/well of 1X Wash Buffer.
- Wash buffer (100 µl/well) was added.
- The plate was sealed and evaluated on the Array Scan HCS Reader.

2.2.12 Antimicrobial activity of biosurfactant:

The antimicrobial activity of biosurfactant was examined against some bacteria and fungi:

It was evaluated by an agar disc diffusion method (Rodrigues *et al.*, 2006b). The bacteria and fungi (2.1.7) were cultured in Muller Hinton broth and incubated over night at 37, 28 ºC, serial dilutions were prepared. Samples (0.1 ml) from appropriate dilutions of each of bacterial and fungal strain were swabbed on the plates of Muller Hinton ager, and wells were made with cork borer.

Different concentrations (100, 75, 50, 25 mg/ml) of purified biosurfactant was prepared, and one hundred microliter of each concentration was added into wells.

For the bacteria the plates were incubated at 37 °C for 24 hrs., while the fungi were incubated at 28 °C for 5 days. The clear zone marked the antimicrobial activity of biosurfactant. And calculated to determine the actual zone diameter (Yalcin and Ergene, 2009).

2.2.13 Determination of some physical properties of biosurfactant:

The following physical properties of biosurfactant were studied:

2.2.13.1 Determination colour and solubility of biosurfactant:

Colour and the solubility of biosurfactant produced by *G. thermoleovorans* Ir1 (JQ912239) in different solvents (water, buffers, chloroform, methanol and butanol) were studied.

2.2.13.2 Determination of melting point:

The sample must be in a fine powder form to fill a capillary tube with it, the open end of the capillary was pressed gently into the substance (biosurfactant) several times. The powder was then pushed to the bottom of the tube by repeatedly pounding the bottom of the capillary against a hard surface, and then the sample were put in the heat block and as the temperature increased, the sample was observed to determine when the phase change occurs from solid to liquid. The operator or the machine recorded the temperature range starting with the initial phase change temperature and ending with the completed phase change temperature. The temperature range determined could then be averaged to gain the melting point of the sample being examined.

2.2.14 Determination the effect of some chemical factors on biosurfactant activity:

2.2.14.1 Biosurfactant concentration for emulsification of sunflower oil:

Different concentrations of dried biosurfactant were prepared by dissolving 2, 3, 4, 5, 6, 7, 8 and 9 mg in 1 ml of 0.1 M Tris-HCl (2.1.3.7) (wt /v) with vortex, pH was adjusted to 7 and 1 ml of sunflower oil was added, E24% (2.2.4.2) was measured.

2.2.14.2 Effect of temperature :

To determine the stability of the biosurfactant with different temperatures. Glass tubes containing 7mg biosurfactant (2.2.14.1) in 1 ml of 0.1M tris-HCl (2.1.3.7) were exposed to different temperatures (20, 40, 60, 80, 100 and 120 ºC) for 30 minutes, then they were cooled and E24% with sun flower was measured (Maneerat and Dikit, 2007).

2.2.14.3 Effect of some mineral salts:

To investigate the effect of salt concentration and type, different salts (NaCl, KCl and $CaCl_2$) were used at concentrations of 2% , 4%, 8 %, 12 %, 16% and 20%. Then 7 mg of biosurfactant was dissolved in each concentration of salt, mixed well, left for 30 min., and E24% was measured with a sun flower oil(Amiriyan *et al* ., 2004).

2.2.14.4 Effect of pH:

In order to determine the stability of biosurfactant in different pH values, the pH of the biosurfactant was adjusted to different values (2, 3, 4, 5, 6, 7, 8, 9 and 10) using tris-HCl (2.1.3.7) or Tris-base (2.1.3.8). Then the activity of biosurfactant was determined, by dissolved 7 mg of biosurfactant in 1 ml of different pH solutions for 30 min., the E24% with sunflower was measured (Maneerat and dikit, 2007).

2.2.15 Detection of gene(s) coded for biosurfactant production:

2.2.15.1 Extraction of genomic DNA:

Genomic DNA was isolated from *G. thermoleovorans* Ir1 (JQ912239) and *B. subtilis* according to the kit (2.1.6) as follows:

- One milliliter of an overnight culture was added to a 1.5 ml microcentrifuge tube.
- The cells suspension was centrifuged at 13000 g for 2 min. And the supernatant was removed.
- Nuclei lysis solution (600 µl) was added. Pipetted it gently until the cells were resuspended, and incubated at 80 °C for 5 min to lyse the cells; then cooled at room temperature.
- RNase Solution (3 µl) was added to the cell lysate. Then, the tubes were inverted 2–5 times to mix. The tube was incubated at 37 °C for 15–60 min. Cool the sample at room temperature.
- Protein precipitation solution (200 µl) was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate.
- The sample was incubated on ice for 5 min. and centrifuged at 13000 g for 3 min.
- Supernatant containing the DNA was transfered to a clean 1.5 ml microcentrifuge tube containing 600 µl of isopropanol.
- The tube was mixed gently by inversion until the thread-like strands of DNA formed a visible mass, then centrifuged at 13000 g for 2 min.
- The supernatant was carefully poured off and the tube was drained on a clean absorbent paper. 600 µl of 70 % ethanol was added and the tube were gently inverted several times to wash the DNA pellet.
- The tube was centrifuged at 13000 g for 2 min., carefully aspirate the ethanol.

- One hundred microliter of DNA rehydration solution was added to the tube and the DNA was rehydrated by incubation at 65 ºC for 1hrs.

2.2.15.2 Measurement of DNA concentration and purity:

In order to determine the concentration and the purity of the DNA, a Nanodrop spectrophotometer was used, 1µl of DNA solution was measured according to the nanodrop spectrophotometer manual, then the absorbency at 260 and 280 nm was measured after calibration with D.W. or TE buffer at 260 nm and 280 nm respectively (Green and Sambrook, 2012).

2.2.15.3 Amplification of biosurfactant gene(s):

Genomic DNA (template) of *G. thermoleovorans* Ir1 (JQ912239) and *B. subtilis* were used to amplified *Spf* gene of *Bacillus subtilis* by using a specific primer for this gene (2.1.4) (Sekhon *et al*., 2011). The PCR reaction was performed by adding the following:

PCR reaction	50µl Rxn
2x PCR master mix solution	25µl
Template DNA(genomic DNA)	2 µl
Primer (F:10 pmol/µl)	1µl
Primer (R: 10 pmol/µl)	1 µl
Deionized distilled water	21µl
Total reaction volume	50µl

Amplification was carried out according to the following conditions:

cycling condition:	Temp.	Time	Cycle No.
Initial denaturation	94 ºC	3 min	1
Denaturation	94 ºC	30 sec	35
Annealing	55 ºC	30 sec	
Extension	72 ºC	1 min	
Final extension	72 ºC	10 min	1

2.2.15.4 Agarose gel electrophoresis (Green and Sambrook, 2012):

Agarose gel (0.7-1.5 %) was utilized to detect the genomic DNA bands and PCR products. The gel was run horizontally in 1 X TBE buffer. Electrophoretic buffer was added to cover the gel. Samples of DNA were mixed with a loading buffer (1:10 v/v) and loaded into the wells and run for 1-3 hours at 5 V/cm, and then agarose gel was stained with an ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 min. DNA bands were visualized by UV transilluminator. The gel was de-stained using distilled water for 30- 60 min. to get rid of background before photographing of DNA bands.

CHAPTER THREE

RESULTS AND DISCUSSION

3. Results and discussion

3.1 Bacterial isolates:

The ten bacterial isolates used in this study were isolated in the previous study and considered as a novel group of aromatic hydrocarbon degrading extreme thermophillic bacteria. All these isolates belonged to the family *Bacillaceae*, and four of them were molecularly identified, and reported for the first time as carbazole utilizing bacteria (Al-Jailawi *et al*., 2013).

Mahdi (2013) referred that these ten isolates could produce biosurfactant compounds when they utilize crude oil, that is why these isolates were chosen for this study.

All isolates were subjected to morphological, biochemical and physiological tests in order to confirm their identification. Results showed that these isolates were gram positive with variable shape from the short to the long rod (figure 3-1). They were positive for oxidase, with variable results for catalase and nitrate reductase, and able to grow at high temperatures between 50-70 ºC, as shown in table (3-1).

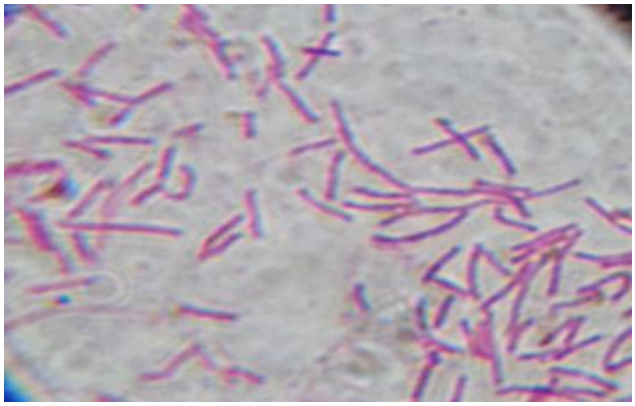


Figure (3-1): Microscopically examination of the G. thermoleovorans bacterial isolates under large objective lens.

Table (3-1): Morphological, physiological and biochemical characteristics of thermophillic bacterial isolates

Thermophillic bacteria	Gram stain	Cell Shape	Catalase	Oxidase	Nitrate reducates	Growth at		
						55 ºC	60 ºC	70 ºC
***Geobacillus thermoleovorans* Ir1 (JQ912239)**	+ve	Long rod	-	+	+	+++	+++	+++
***Anoxybacillus rupiensis* Ir2 (JQ912240)**	+ve	Long rod	+	+	+	+++	+++	+++
***Anoxybacillus rupiensis* Ir3 (JQ912241)**	+ve	Long rod	+	+	+	+++	+++	+++
Anoxybacillus sp.	+ve	Long rod	+	+	+	+++	+++	+++
9SM	+ve	Short rod	-	+	-	+++	++	-
12SM	+ve	Short rod	+	+	+	+++	+++	+++
13SM	+ve	Short rod	-	+	-	+++	++	-
14SM	+ve	Long rod	+	+	_	+++	+	-
21SM	+ve	Short rod	-	+	_	+++	++	++
34SM	+ve	Short rod	+	+	_	+++	++	++

(+): positive result and moderate growth. (-): negative result. (+++): excellent growth (++): good growth (-): no growth.

3.2 Screening the bacteria for biosurfactant production:

In order to screen the previously isolated thermophillic bacteria for their ability to produce biosurfactant, the ten bacterial isolates were grown in mineral salt

medium as in item (2.1.5.2) containing 1% crude oil as a carbon source to induce them to produce biosurfactant. The supernatants of the ten bacterial isolates were subjected to three screening methods:

3.2.1 Emulsification activity (E.A):

Emulsification activity (E.A) for the supernatant of the ten bacterial isolates were determined. The results presented in figure (3-2) show that the E.A ranged between 0.1 to 0.34, with the maximum E.A (0.34) was recorded by *Geobacillus thermoleovorans* Ir1 (JQ912239), followed by E.A (0.32) which recorded by 13SM isolate with lowest E.A (0.1) value attributed to 34SM isolate. The measurement of E.A is an indicator of the activity of biosurfactant production (Jazeh *et al.*, 2012).

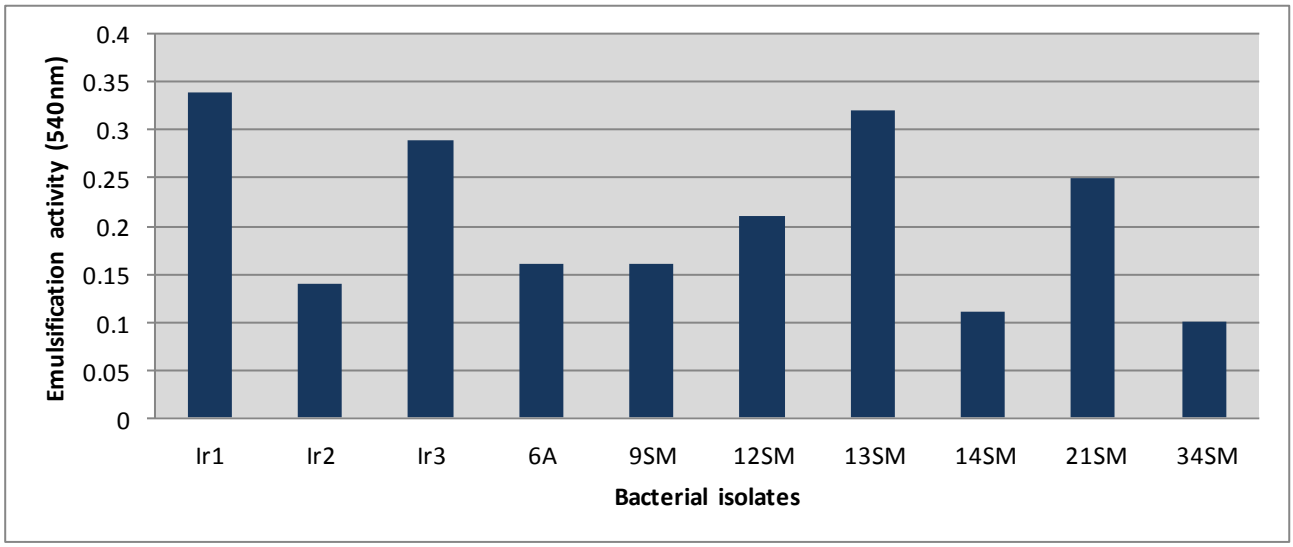


Figure (3-2): Emulsification activities (E.A) of the ten thermophillic bacterial isolates, cultured in mineral salt medium (pH 7) containing 1% crude oil, at 55 ºC in shaker incubator (180 rpm) for 7 days.

Ir1: *G. thermoleovorans* (JQ912239). Ir2: *A. rupiensis* (JQ912240). Ir3: *A. rupiensis* (JQ912241). 6A: *Anoxybacillus* sp.

The emulsification activity of Yansan bioemulsifier of *Y. lipolytica* was 2.63 when using toulen as a carbon source (Amaral *et al*., 2006). On the other hand

Camargo de Morais *et al*. (2006) noticed that the E.A of bioemulsifier produced by *P. citrinum* was 0.20 when using olive oil.

3.2.2 Emulsification index (E24%) and surface tension:

The further detection of biosurfactant production was done by the measurement of emulsification index and reduction of the surface tension.

The results indicated in figure (3-3) show that all isolates were able to produce biosurfactant with a variable emulsification index, and surface tension. The strain (*G. thermoleovorans* Ir1) and13SM isolate showed the highest E24% (68% and 61% respectively) with highest reduction of surface tension (53 and 56 mN/m respectively). While 34SM gave lowest E24%(32%) and highest surface tension (67mN/m).

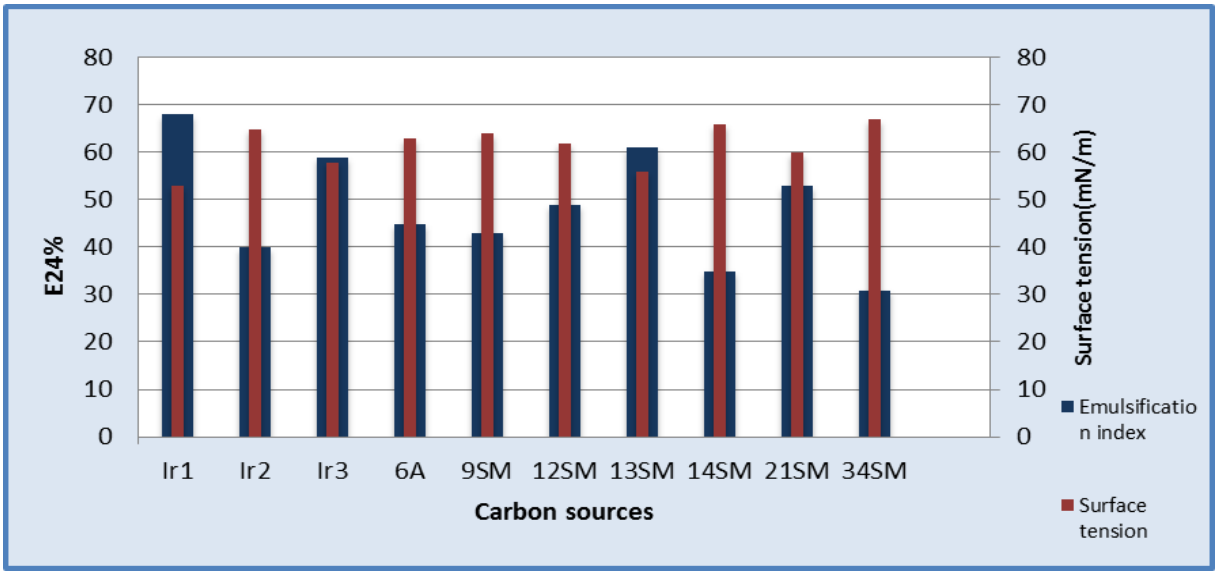


Figure (3-3): Emulsification index (E24%) and surface tension of the ten thermophillic bacterial isolates, cultured in mineral salt medium (pH 7) containing 1% crude oil, at 55 ºC in shaker incubator (180 rpm) for 7 days.

Ir1: *G. thermoleovorans* (JQ912239). Ir2: *A. rupiensis* (JQ 912240). Ir3: *A. rupiensis* (JQ 912241). 6A: *Anoxybacillus* sp.

Bacteria generally prefer to metabolize substrates present in the aqueous phase; they also can take up the substrate if they are in a close contact with the insoluble phase of the hydrocarbons (Abbasnezhad, 2009).

Priya and Usharani (2009) indicated that the highest E24% value (60 %) was observed with *B. subtilis* when using vegetable oil as carbon source. While Kalyani *et al.* (2014) found that the of biosurfactant produced by *Actinomycetes* has an emulsification index of 57.31% when grown in medium containing olive oil as the sole source of carbon.

The ability of the thermophillic bacteria to reduce the surface tension revealed a correlation between the molecular weight of biosurfactant and the reduced surface tension. Zheng *et al.* (2011) reported that *G. pallidus* does not reduce the surface tension below 40 mN/m when growing on media supplemented with different hydrocarbons.

Mulligan (2005) reported that the low molecular weight biosurfactant are able to reduce the surface tension below 40 mN/m, while the high molecular weight bioemulsifiers can form and stabilize emulsions without remarkable surface tension reduction (Batista *et al*., 2006).

The above results showed that all isolates were able to produce biosurfactant compounds and *G. thermoleovorans* (Ir1) was the most efficient one. Thus, this isolate was selected for a subsequent study.

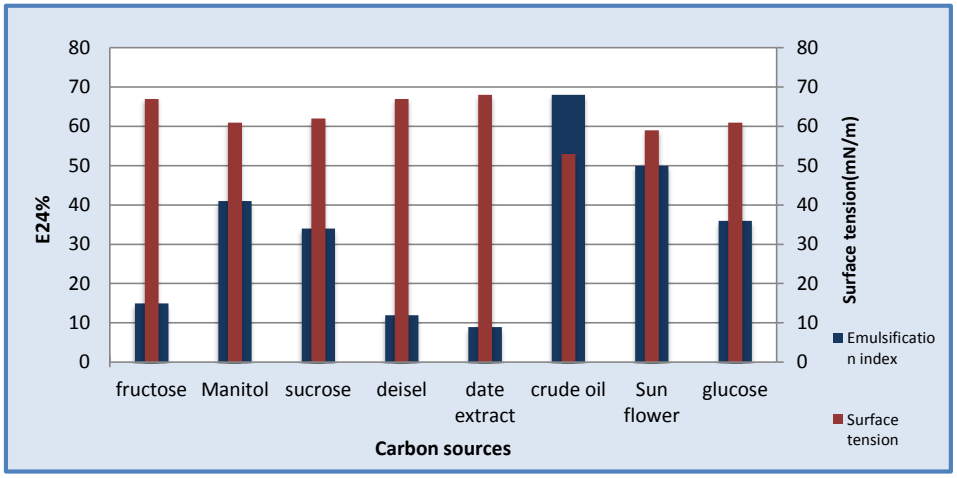
3.3 Optimization of culturel conditions for biosurfactant production:

Several factors were studied to determine the optimal conditions for biosurfactant production by *G. thermoleovorans* (Ir1):

3.3.1 Effect of carbon sources:

There is a correlation between biosurfactant production and growth on hydrocarbons; therefore, several kinds of carbon source were investigated for the biosurfactant production.

Results indicated in figure (3-4) show that the E24% (68%) and surface tension (53mN/m) were achieved when crude oil was used as the sole source of carbon and energy, followed by sunflower with E24% and surface tension 50% and 59 mN/m respectively. While the lowest activity was obtained when date extract (9%and 68 mN/m) was used. These results demonstrated the ability of this bacterium to degrade a wide range of carbon sources and biosurfactant production. Pines and Gutnick (1986) demonstrated that the production of bioemulsifier by *A. calcoaceticus* RAG-1was induced by the addition of hydrocarbons or oils.



Figure(3-4):Effect of different carbon sources on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium (pH7) containing 0.4% ammonium chloride, at 55 ºC in shaker incubator (180 rpm) for 7 days.

The crude motor oil enhanced the biosurfactant production from *P. aeruginosa* PBSC1 with a surface tension 30.98 mN/m and an emulsification index of 74.32±0.52% (Joice and Parthasarathi, 2014).

While Noudeh *et al*. (2007) found that the *B. licheniformis* PTCC produced biosurfactant when growing on almond, castor and olive oil as sole carbon source, but the maximum yield was achieved with olive oil. Also Gudiña *et al.* (2015a) mentioned that the surfactin production by *B. subtilis* 573 was evaluated using the corn steep liquor as an alternative low-cost culture medium.

Also Solaiman *et al*. (2004) on the other hand used soy molasses and oleic acid as co-substrates for the production of sophorolipids by *C. bombicol.* The quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate (Rahman and Gakpe, 2008).

3.3.2 Effect of crude oil concentration:

Different concentrations of the optimal carbon source (crude oil) were used to determine the optimum for the biosurfactant production by *G. thermoleovorans* (Ir1).

Result showed in figure (3-5) indicate that the gradual increase of carbon source concentration was accompanied by an increase in the emulsification index and dropping of surface tension, which was an indicator of biosurfactant production, till the optimum carbon concentration, these dramatic changes in emulsification index and surface tension reached to its better values 68% and 53 mN /m respectively at a concentration of 1%.

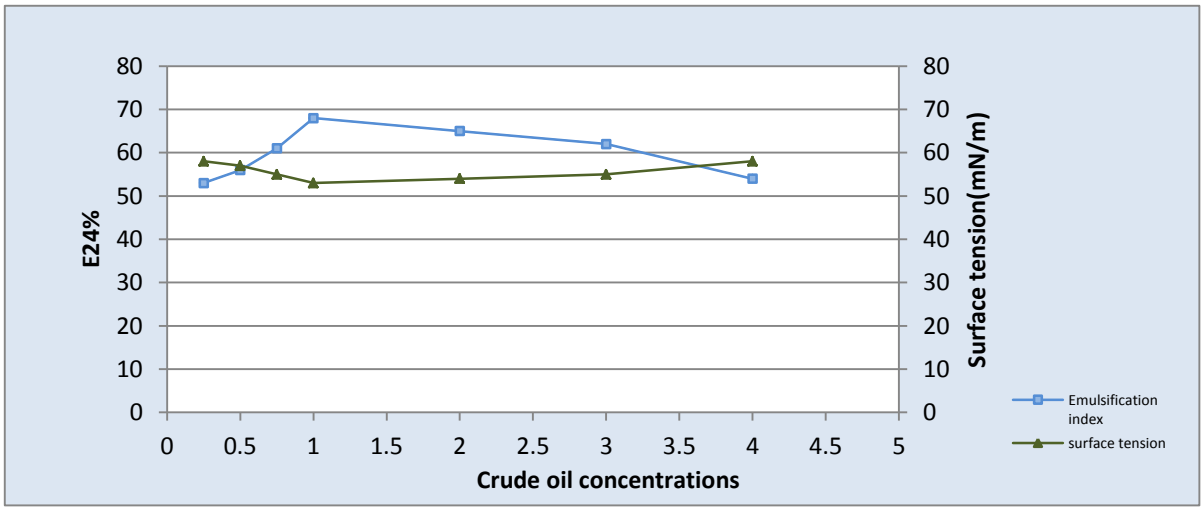


Figure (3-5): Effect of different concentrations of crude oil on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium (pH 7), containing 0.4% ammonium chloride, at 55 ºC in shaker incubator (180 rpm) for 7 days.

The low concentration of crude oil may support the growth of this bacterium and induced it to produce a biosurfactant, while the higher concentration of crude oil may have a toxic effect on the bacterial growth and/or not induce bacterium to produce biosurfactant. The higher concentration of carbon source may reflect the toxic effect to the producing organisms (Johnson *et al.*, 1992).

Abu-Ruwaida *et al*. (1991) mentioned that hydrocarbon concentration plays an important role in synthesizing the biosurfactant by microorganisms.

3.3.3 Effect of temperature:

The temperature is one of the most important parameters affecting the production of biosurfactant, so different incubation temperatures were used.

Result (figure 3-6) showed that the optimal temperature for biosurfactant production was 60 ºC with an emulsification index 70 % and a surface tension 52 mN/ m followed by 55 ºC (the optimal growth temperature for this bacterium). The E24% values were dropped, and surface tension increased with other

temperatures. These results were in accordance with Zheng *et al.* (2011) who demonstrated that the 60 ℃ was the optimum temperature for biosurfactant production by *G. pallidus.*

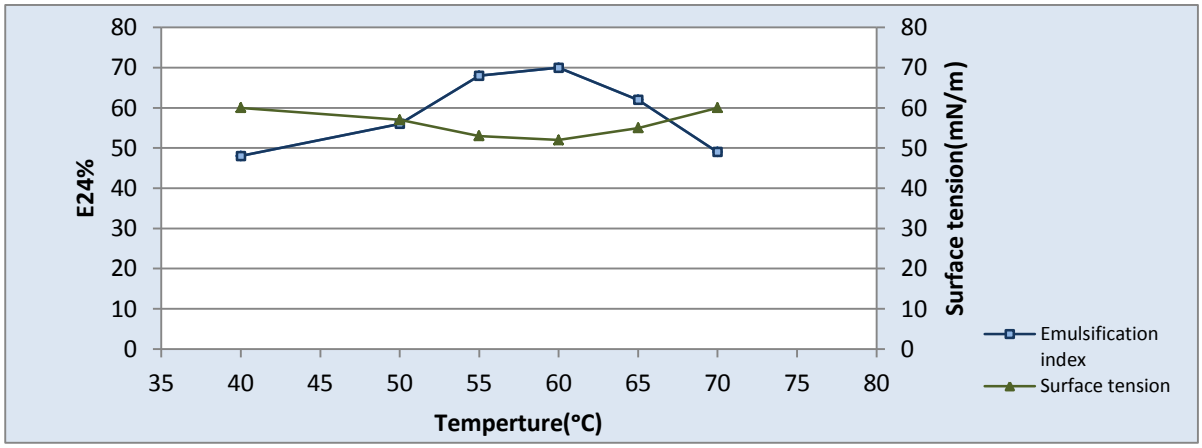


Figure (3-6): Effect of incubation temperature on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium (pH 7) with 1% crude oil and 0.4% ammonium chloride, in shaker incubator (180 rpm) for 7 days.

Maximum enzymatic activation can only be obtained at an optimum temperature (Nakata and Kurane,1999).

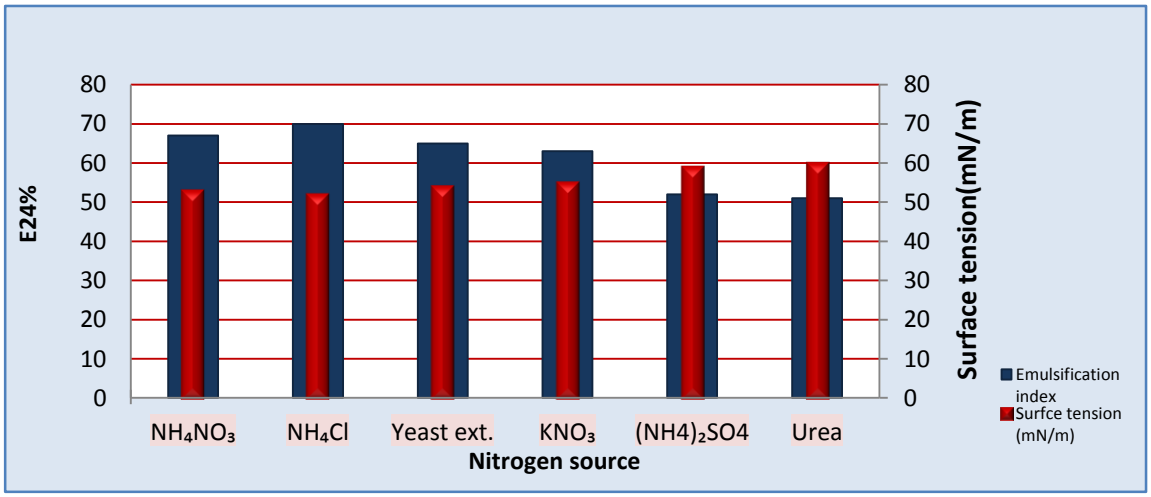
Saharan *et al*. (2011) observed that the growth of *C. bombicola* reached a maximum at a temperature of 30 ºC, while 27 ºC was the best temperature for the production of its biosurfactant. While Kitamoto *et al*. (2001) observed that the highest mannosylerythritol lipid production was at 25 ºC and this temperature was used for both growing and resting cells.

3.3.4 Effect of nitrogen sources:

In order to determine the effect of different types of nitrogen sources on biosurfactant production by *G. thermoleovorans* (Ir1), six nitrogen sources were used.

Results (figure 3-7) declared that the production of biosurfactant varies with

different nitrogen sources ($(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, KNO_3, Yeast extract and urea), the highest E24% (70%) with low surface tension of (52 mN/m) were obtained when an ammonium chloride (NH_4Cl) was used, and this may attributed to the simplicity of NH_4Cl as a nitrogen source and easy to uptake by bacterium.



Figure(3-7):Effect of nitrogen sources (0.4%) on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium (pH7) with 1% crude oil, at 60 ºC in shaker incubator (180 rpm) for 7 days.

Okoliegbe and Agarry (2012) mentioned that the bacteria require nitrogen to complete its metabolic pathways and it is essential for the microbial growth as protein and enzyme syntheses depend on it.

Abu-Rawaida *et al*. (1991) and Guerra-Santos *et al*. (1986) detected that the ammonium salts and urea preferred nitrogen sources for biosurfactant production by *A. paraffineus*, whereas nitrate supported the maximum surfactant production by *P. aeruginosa* and *Rhodococcus* sp. However, Johnson *et al.* (1992) found that the potassium nitrate support the maximum production of biosurfactant by the yeast *R. glutinis* IIP30.

3.3.5 Effect of nitrogen source (NH_4Cl) concentration:

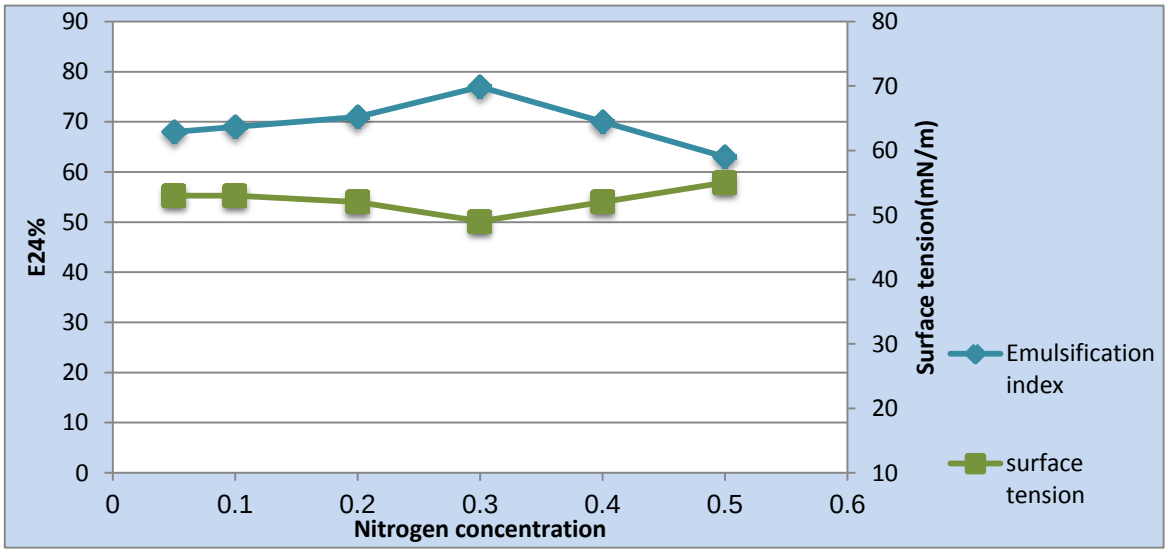
Different concentrations of ammonium chloride were used to determine the optimum concentration for biosurfactant production by *G. thermoleovorans* (Ir1).

Result illustrated in figure (3-8) show that the maximum E24% (77%) and minimum surface tension (49 mN / m), were obtained when NH_4Cl was added in a concentration of 0.3% (w/v). The results also showed a reduction in emulsification index and increased in surface tension when the concentration of NH_4Cl was above or below 0.3%.

Dastgheib *et al.* (2008) referred that the 2% sodium nitrate is the best nitrogen source for emulsifier production by *B. licheniformis.* While Saharan *et al*. (2011) detected that the production of biosurfactant often occurs when the nitrogen source is depleted in the culture medium, during the stationary phase of cell growth, as an example the biosurfactant production increased by *P. aeruginosa*, *C. tropicalis* IIP-4 and *Nocardia* strain SFC-D due to the nitrogen limitation (Kosaric *et al*., 1990; Singh *et al*., 1990).

3.3.6 Effect of pH:

To investigate the effect of initial medium pH on biosurfactant production by *G. thermoleovorans* (Ir1), mineral salt medium was adjusted to different pH values. The obtained results (figure 3-9) indicate that the highest emulsifying index (77%) and lowest surface tension (49 mN/m) occurred with pH value 7. It was also shown that a good activity was recorded with pH values between 6.5 to 9.



Figure(3-8): Effect of ammonium chloride concentration on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium (pH7) with 1% crude oil, at 60 ºC in shaker incubator (180 rpm) for 7 days.

This result pointed that pH 7 was optimum for the bacterial growth (Mahdi, 2013), and for the biosurfactant production.

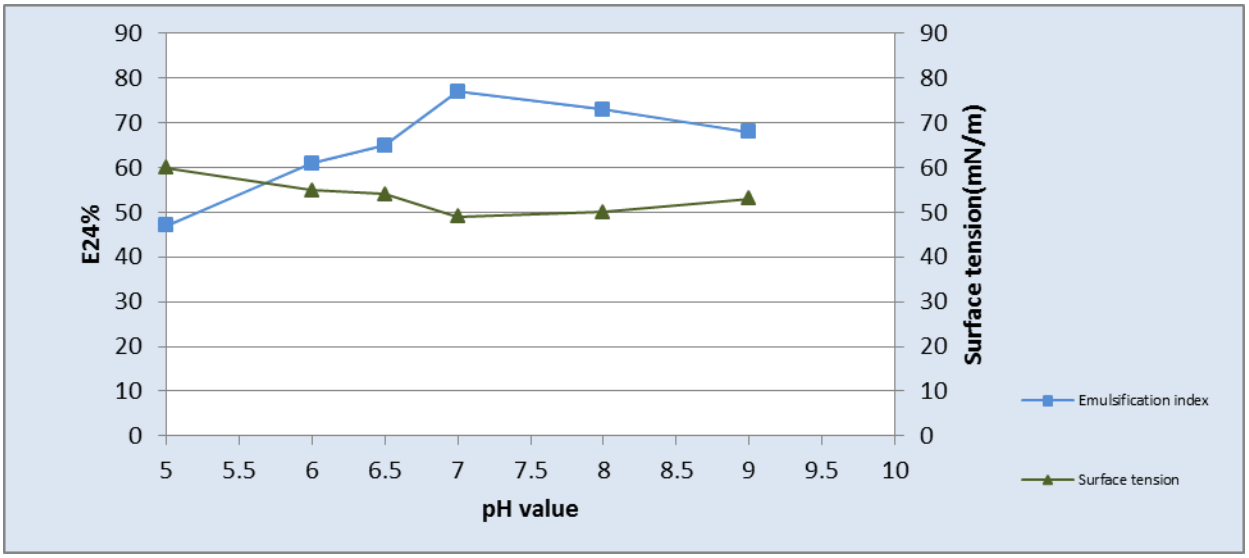
The synthesis of the biosurfactant decreased without the pH control, indicating the importance of maintaining it throughout the fermentation process (Bednarski *et al.*, 2004).

Environmental factors and growth conditions such as pH effect on biosurfactant production through their effects of cellular growth or activity.

Zinjarde and Pant (2002) reported the effect of the initial pH in the production of a biosurfactant by *Y. lipolytica*. They found that the maximum biosurfactant production was obtained at pH 8.0.

Saharan *et al*. (2011) demonstrated that when the pH is maintained at 5.5, the production of glycolipids reaches a maximum by *C. antarctica* and *C. apicola*. Rhamnolipid production by *Pseudomonas* spp. reached its maximum at a pH 7

(Sifour *et al.*, 2007)



Figure(3-9): Effect of pH value on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium with 1% crude oil, and 0.3% ammonium chloride at 60 ºC in shaker incubator (180 rpm) for 7 days.

3.3.7 Effect of aeration:

The aeration represents another important factor influencing the biosurfactant production by *G. thermoleovorans* (Ir1). To evaluate the effect of the aeration, the cultures were incubated at different agitation speed (rpm) values ranging between 120-220 rpm.

The results illustrated in figure (3-10) indicate that maximum E24% (80%) with reduction in surface tension (47mN/m) was obtained at 200 rpm.

This result was in agreement with Zheng *et al*. (2011) who noticed that the optimum agitation speed was 200 rpm when the biosurfactant was produced by thermophillic *G. pallidus*. Priya and Usharani (2009) declared that biosurfactant production by *B. subtilis* and *P. aeruginosa* was optimized in a shaker operating at 120 rpm. Sheppard and Cooper (1990) had concluded that oxygen transfer was one of the key parameters for the process optimization and scale-up of surfactin production in *Bacillus subtilis*. While Ghayyomi *et al*. (2012) detected the

optimum rpm for biosurfactant production by *Bacillus* sp. was150 rpm.

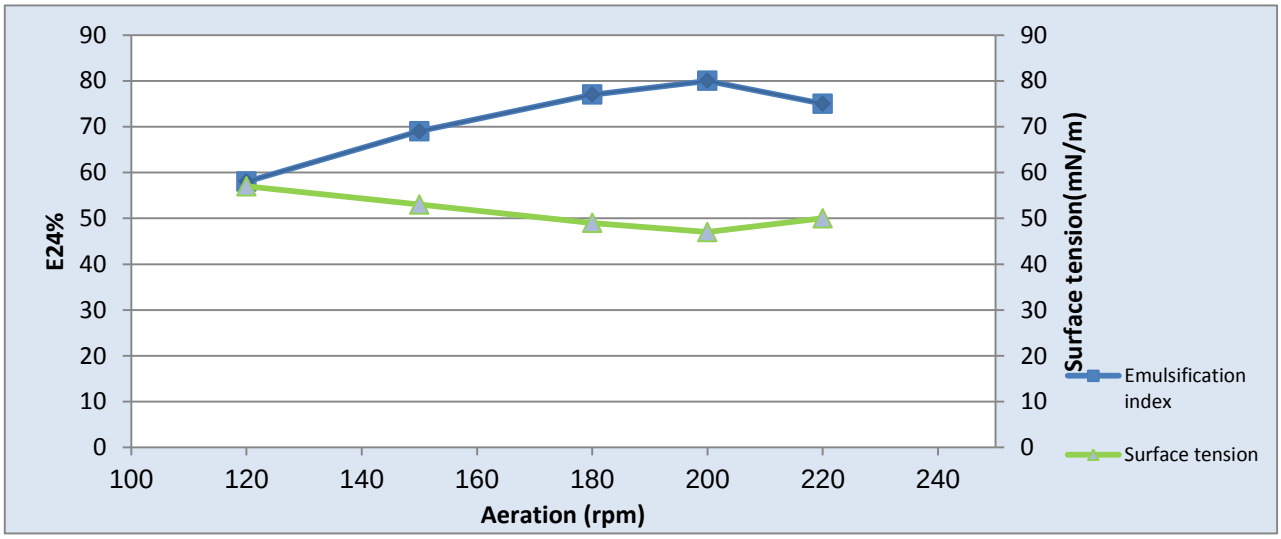


Figure (3-10): Effect of rpm value on biosurfactant production by *G. thermoleovorans*, grown in mineral salt medium (pH 7) with 1% crude oil, and 0.3% ammonium chloride at 60 ºC for 7 days.

3.3.8 Effect of incubation period:

Different incubation periods (2-11 days) were examined in order to determine its effect on biosurfactant production. Result (figure 3-11) showed that the maximum E24% (87%) and the lowest surface tension (43mN/m) were in the tenth day of incubation. E24% was dropped after ten days of incubation and this may attributed to the interference between the metabolites and the formation of emulsion. Bonilla *et al.* (2005) mentioned that biosurfactant biosynthesis stopped, probably due to the production of secondary metabolites which could interfere with emulsion formation and the adsorption of surfactant molecules at the oil–water interface. Rosenberg *et al.* (1979) also reported a maximum emulsan production by *A. calcoaceticus* RAG-1 during the stationary growth phase. While Persson *et al.* (1988) showed that the biosurfactant biosynthesis using olive oil occurred predominantly during the exponential growth phase, suggesting that the

biosurfactant was produced as a primary metabolite accompanying cellular biomass formation (growth-associated kinetics).

Bidlan *et al*. (2007) pointed that biosurfactant produced by *S. marcescens* DT-1P increased with incubation time and the production started at early stationary phase and reached maximum in day 8, beyond both growth and biosurfactant production decrease. On the other hand biosurfactant production by *G. pallidus* needed 14 days (Zheng *et al*., 2011). While *Pseudomonas* sp. strain LP1 produced biosurfactant when growing in medium containing heavy oil with the highest emulsification index (E24%) for the biosurfactant production was 80.33 ± 1.20, on day 8 of incubation, the biosurfactant production is growth-associated (Obayori *et al.*, 2009).

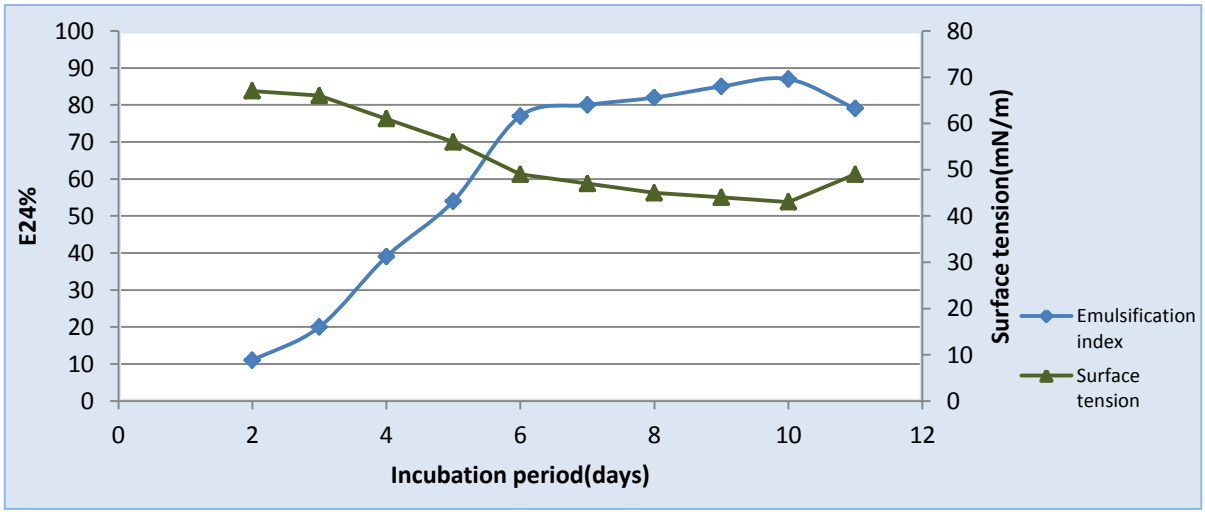
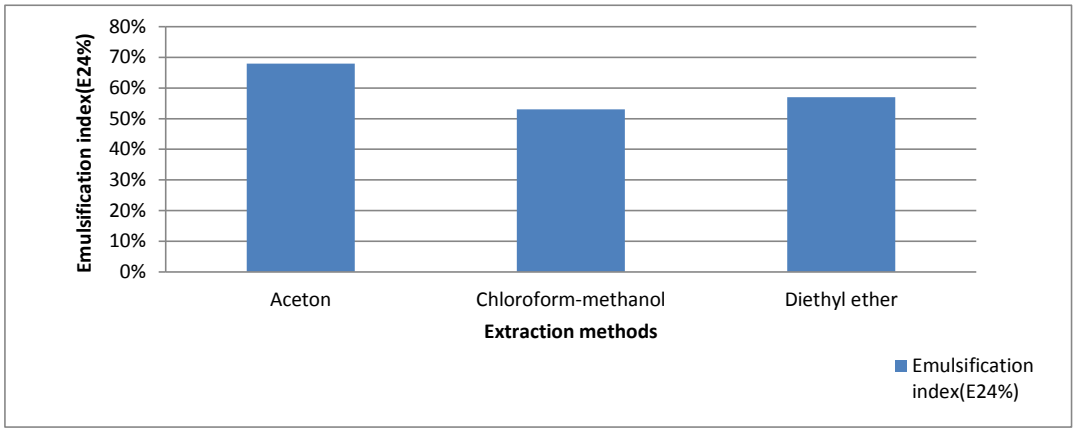


Figure (3-11): Effect of incubation period on biosurfactant production by *G. thermoleovorans* (Ir1), grown in mineral salt medium (pH 7) with 1% crude oil and 0.3% ammonium chloride in shaker incubator (200rpm) at 60 ºC.

3.4 Extraction and purification of biosurfactant:

Geobacillus thermoleovorans (Ir1) was grown in a mineral salt medium (pH7) containing 1% crude oil as a sole carbon source and 0.3% ammonium chloride as a nitrogen source, at 60 ºC, with shaking (200 rpm) for 10 days. After that,

biosurfactant was extracted using three different methods as illustrated in (2.2.6) and the extraction with acetone gave a better biosurfactant activity as in figure(3-12).



Figure(3-12):Methods used for extraction biosurfactant produced by *G. thermoleovorans.*

In order to obtain a purified biosurfactant, silica gel column chromatography was used, by loading the column with partial purified biosurfactant which was dissolved in chloroform. All eluted fractions were collected, then the emulsification activity for each one was measured. By drawing the relation between emulsification activity and fraction number as in figure (3-13), three peaks appeared, two of them appeared when eluted with chloroform, in which the first peak appeared in tubes number (38-45), while the second one at tubes number (46-56) and the third one appeared with ethyl acetate with tubes number (82-94).

Results also indicated that the second peak gave the higher emulsification activity (E.A= 0.48), while the third peak, which eluted with ethyl acetate gave E.A = 0.43.

Silica gel column chromatography was used in several studies to purify biosurfactant compounds, Zhao *et al.* (2013) used silica gel column chromatography to purify rhaminolid produced by *P. aeruginosa*.

Thanomsub *et al.* (2006) also used silica gel column chromatography to purify the rhamnolipids produced by *P. aeruginosa* B189 with sequential washing by hexane, chloroform, ethyl acetate and methanol.

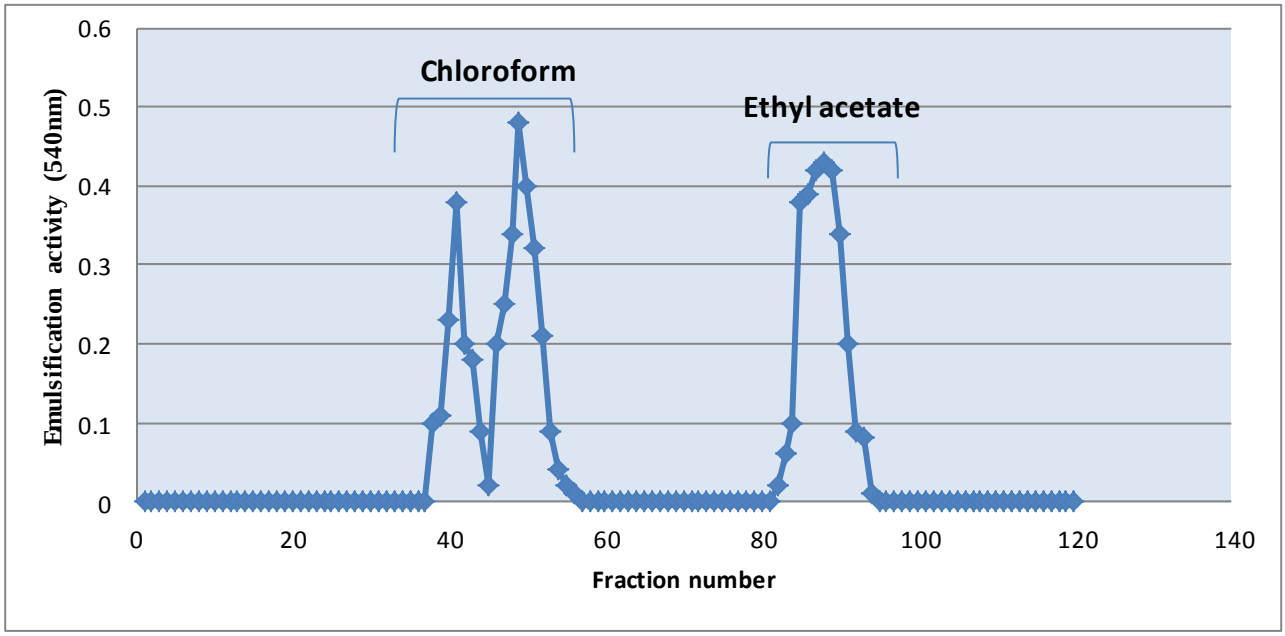


Figure (3-13): Silica gel column chromatography with sequential elution system and flow rate 30ml/hrs., fraction volume 3ml/tube.

3.5 Chemical composition of biosurfactant:

There was no completed idea about the chemical composition of biosurfactant produce by *G. thermoleovorans*, only one study by Feng and Jin (2009) who referred that the bioemulsifier consists of lipid, carbohydrate and protein.

Hence, the partial purified biosurfactant produce by *G. thermoleovorans* (Ir1) was analyzed to determine their composition of lipid, carbohydrate and protein.

The results showed that the lipid content of partially purified biosurfactant was 37.7% according to the lipid standard curve. According to the dobies standard curve the carbohydrate was 26.2%. And analyzed the absorbance of the protein with Bradford standard curve revealed that the concentration of the protein was 10.7%.

The result revealed that the main part of the biosurfactants produced by *G. thermoleovorans* (Ir1) was lipids followed by carbohydrate, and low concentration of the protein.

These values were comparable with Feng and Jin (2009) who reported that the bioemulsifier produced by *G. thermoleovorans* 5366T consisted the highest ratio of lipid (35.8%), then carbohydrate (29.4%), and protein (15.8%).

While Zheng *et al.* (2011) mentioned that the bioemulsifier produced by *G. pallidus* consisted of carbohydrate as the main part (68.6%), then lipid (22.7%), and protein (8.7%).

Luna-Velasco *et al.* (2007) demonstrated that the main part of bioemulsifier produced by *Penicillium* sp. was the lipid part with a ratio of 67%, carbohydrates (11%), and protein (7%). Also the composition of bioemulsifier produced by *A. pallidus* YM-1 was a complex of carbohydrates (41.1 %), lipids (47.6 %) and proteins (11.3 %) (Zheng *et al.*, 2011).

Jagtab *et al*. (2010) found that the bioemulsifier of *B. stearothermophilus* consists of protein (46 %), carbohydrate (16 %), and lipid (10 %).

While Amaral *et al.* (2006) demonstrated that the chemical characterization of Yansan from *Y. lipolytica* IMUFRJ 50682 consists of a polysaccharide–protein complex with a low lipid content and the protein content of this polymer plays an important role in the emulsification activity. And Sarrubbo *et al.* (2001) produced a bioemulsifier, from *Y. lipolytica* in the presence of glucose as carbon source; this biosurfactant consisted of 47 % protein, 45 % carbohydrate and 5 % lipids.

Jagtap *et al*. (2010) revealed that the chemical analysis of bioemulsifier from *Acinetobacter* was proteoglycan with protein (53 %), polysaccharide (43 %), and lipid (2 %). In comparisons, Kokare *et al*. (2007) mentioned that the bioemulsifier produced by marine *Streptomyces* sp. S1 consisted of 82 % protein, 17 % polysaccharide and 1 % reducing sugar.

3.6 Characterization of *G. thermoleovorans* biosurfactant:

In an attempt to complete the chemical characterization of biosurfactant produced by *G. thermoleovorans* (Ir1). The partial and/or purified biosurfactant was subjected to IR, HPLC, GC, HNMR analysis.

3.6.1 Fourier Transform Infrared Spectrometry:

The IR spectrum of partial purified biosurfactant produced by the bacterium (figure 3-14) showed a broad band at 3282 cm^{-1} and another band at 2922 cm^{-1} this may be attributed to the O-H groups of polysaccharide. This result was in accordance with Singh *et al*. (2011) who demonstrated the presence of a broadly stretching intense peak at around 3428 cm^{-1} which is characteristic of hydroxyl groups and a weak C–H stretch band at around 2928 cm^{-1} of hetropolysaccharides of biosurfactant produced by *B. licheniformis*. The results (figure 3-14) also showed a strong band at 1654 cm^{-1}, 1537cm^{-1} and 1432 cm^{-1}, those may be attributed to the C=O, N-H and C-N respectively. In the study of Beech *et al*. (1999) they demonstrated a band at 1655 cm^{-1} which represented C- O stretching of carboxyl group and/or protein related band of amide I, also a band at 1427 cm^{-1}, which belonged to the protein group.

FTIR spectrum (figure 3-14) also showed a band at 1000 cm^{-1} belonging to the polysaccharide. It was revealed that the region from 1200–950 cm^{-1} is associated with (C–O–C) stretching of polysaccharides (Dean *et al*., 2010).

The above IR spectra results of biosurfactant produced by *G. thermoleovorans* (Ir1) was in comparable with IR spectra of bioemulsifier produced by *A. pallidus* YM-1 (Zheng *et al.*, 2011). While Sharma *et al*. (2015) revealed the composition of biosurfactant from *Enterococcus faecium* using FTIR spectrum was lipid and polysaccharide fractions.

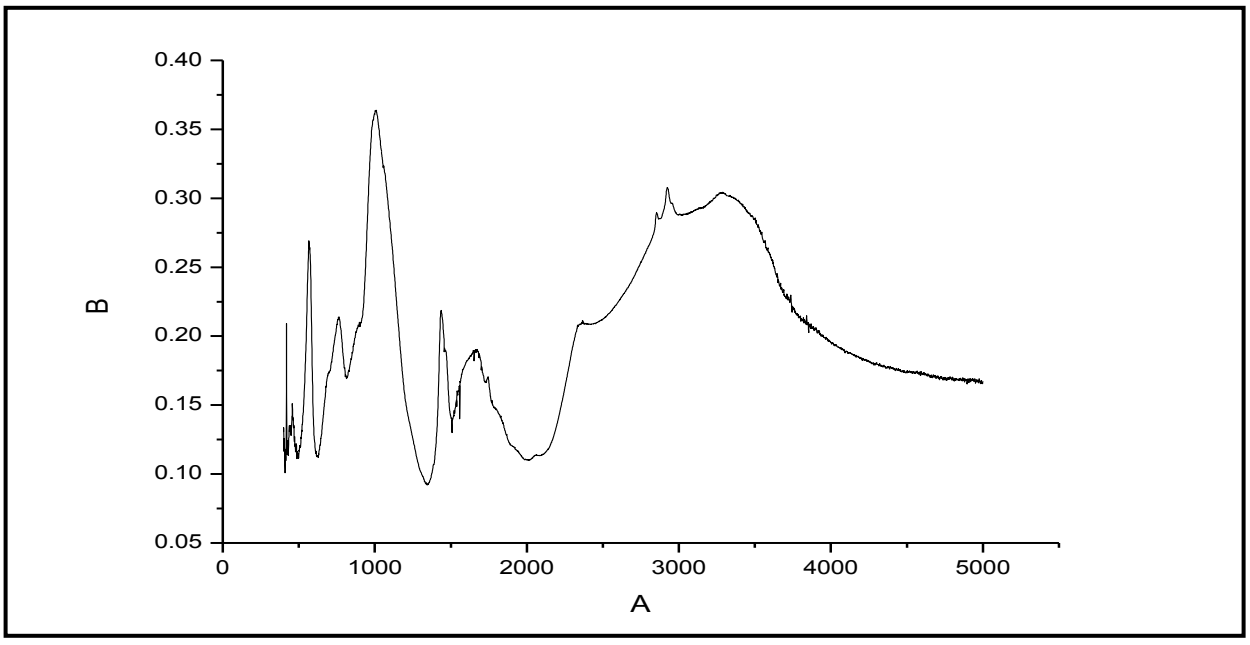


Figure (3-14): FTIR spectrum of partial purified biosurfactant produced by *G. thermoleovorans* (Ir1), 50 scan for each spectrum.

A: 1/cm. B: Absorbance.

3.6.2 High performance liquid chromatography:

For further characteristics of the biosurfactant high performance liquid chromatography (HPLC) was used for detecting the components of fatty acids, carbohydrates and protein found in partial purified biosurfactant produced by *G.*

thermoleovorans (Ir1) depending on standard samples of fatty acids, sugars and proteins.

The (HPLC) result (figure 3-15) revealed that there were many peaks of fatty acid components (lipid part) for biosurfactant. The first peak was palmitic acid (34.1µg/ml), the second peak was stearic acid (35.79 µg/ml), the third peak was oleic acid (34.61µg/ml).

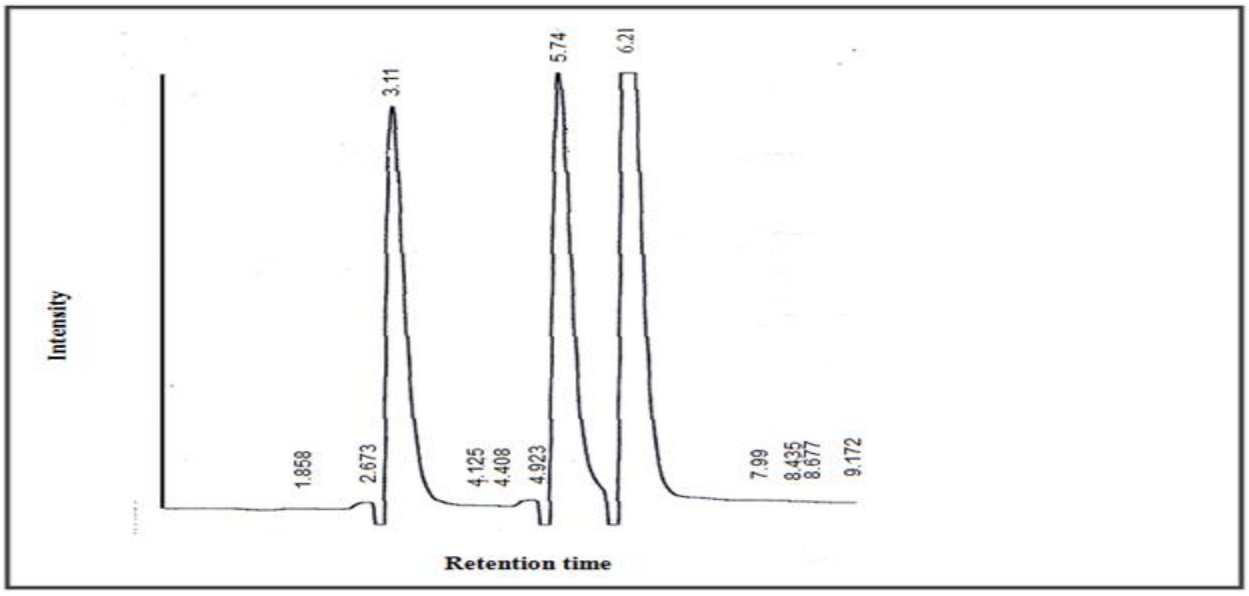


Figure (3-15): HPLC analysis of fatty acids components of partial purified biosurfactant produce by *G. thermoleovorans* (Ir1), equipped with binary delivery pump model LC -10A shimadzu, the eluted peak was monitored by SPD 10A VP detector.

While the results of carbohydrate content showed three peaks. The first peak revealed xylose (20.5 µg/ml), the second peak was mannose (41.7 µg/ml), and the third one was maltose (21.84 µg/ml), when compared to standard carbohydrate (figure 3-16).

The results also showed three peaks for amino acid components of biosurfactant, the first one was aspartic acid with a concentration of 11.55 µg/ml, while the second was glutamic acid (20.60 µg/ml) and glutamine with concentration 15.7 µg/ml as shown in figure (3-17).

Feng and Jin (2009) mentioned that the carbohydrate in the bioemulsifier produced by *G. thermoleovorans* 5366T mainly was D-mannose, and main amino acids were glutamic acid, aspartic acid, alanine. While Adamu *et al.* (2015) revealed that the biosurfactant produced by *B. sphaericus* EN3 was phospholipid and made up of palmitic acid, leucine, alanine, serine, and arginine. Similarly, *B. azotoformans* EN16 produced phospholipid with the following components: glutamine, stearic acid, oleic acid glycine, valine and arginine.

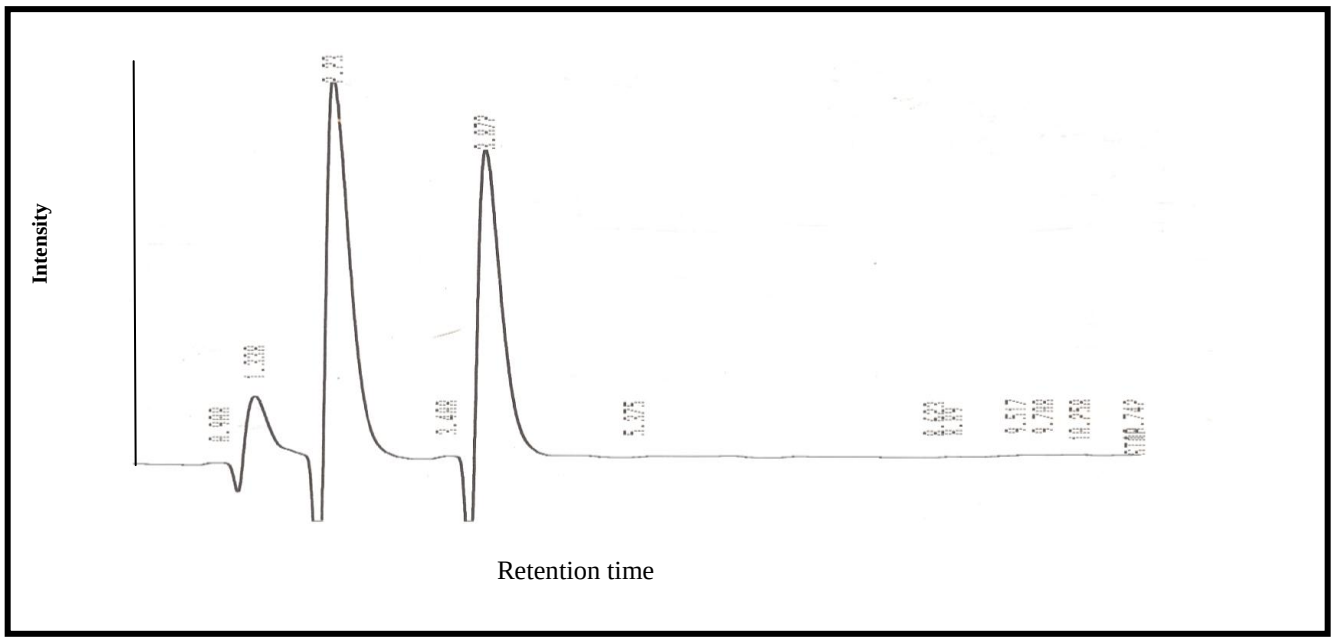


Figure (3-16): HPLC analysis of the carbohydrate components of partial purified biosurfactant produce by *G. thermoleovorans* (Ir1), equipped with binary delivery pump model LC-10A.

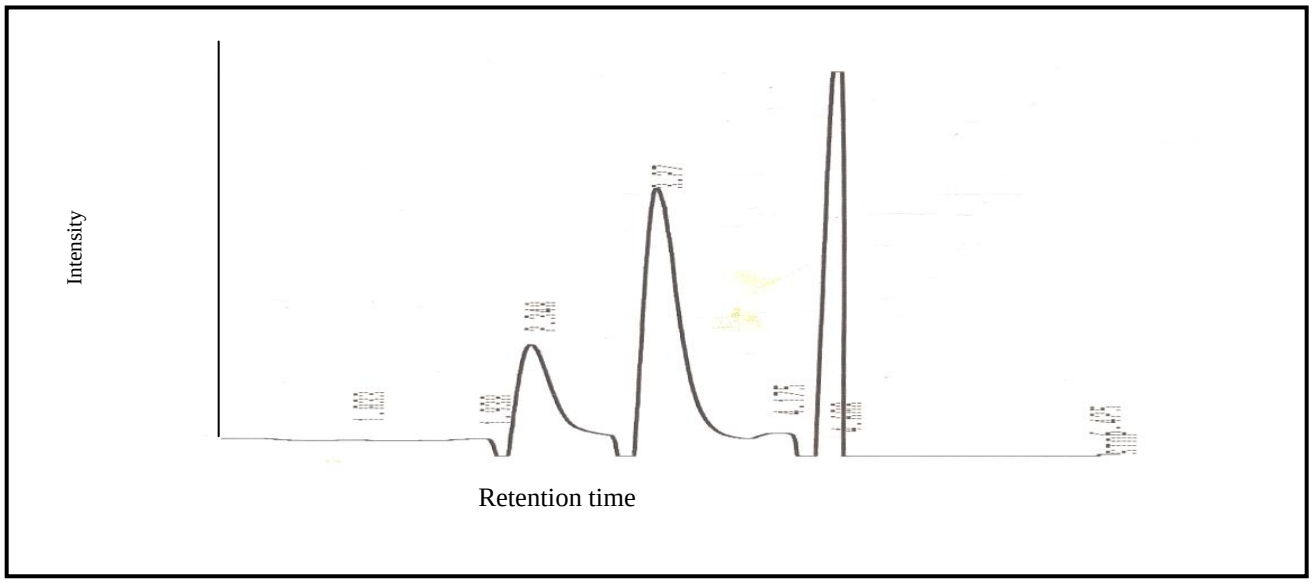


Figure (3-17): HPLC analysis of the amino acid components of partial purified biosurfactant produce by *G. thermoleovorans* (Ir1), with flow rate 1 ml/min.

In addition Satpute *et al.* (2010) used the HPLC for separation of rhamnolipid biosurfactant to its components.

3.6.3 Gas chromatography for lipid part (fatty acid) analysis:

As mentioned before (item 3-5) and according to the above result, it was revealed that the main part of the biosurfactant produce by *G. thermoleovorans* (Ir1) was lipids. So lipid part of the partial and purified biosurfactant were further analyzed by gas chromatography.

Results (figure 3-18) showed that partial purified biosurfactant produced by this bacterium consists of a high percentage (58.9%) of palmitic acid methyl ester(C16:0), Stearic acid (C18:0), Oleic acid (C18:1n9C). Besides there are many other fatty acids with a less percentage.

In comparison, the results of the purified biosurfactant also showed that it mainly consists (high percentage) of palmitic acid mythel ester (C16-0) with

following ratio (23%, 44.4%, 30.3%) for the first, second and third band respectively.

Feng and Jin (2009) analyzed the lipid part of biosurfactant from *G. thermoleovorans* 5366T by gas chromatograph and mentioned that the main component were hexadecanoic acid, octadecenoic acid, octadecanoic acid.

The gas chromatography analysis of the lipid fraction of bioemulsifier synthesized by *Penicillium* sp. revealed that myristic (C-14), stearic (C-18), and oleic (C-18:1) were the major acids that account of 41% of total fatty acids (Luna-Velasco *et al.*, 2007). While Shamra *et al.* (2015) found the gas chromatography and mass spectroscopy data of the fatty acid produced by *E. faecium* was hexadecanoic acid.

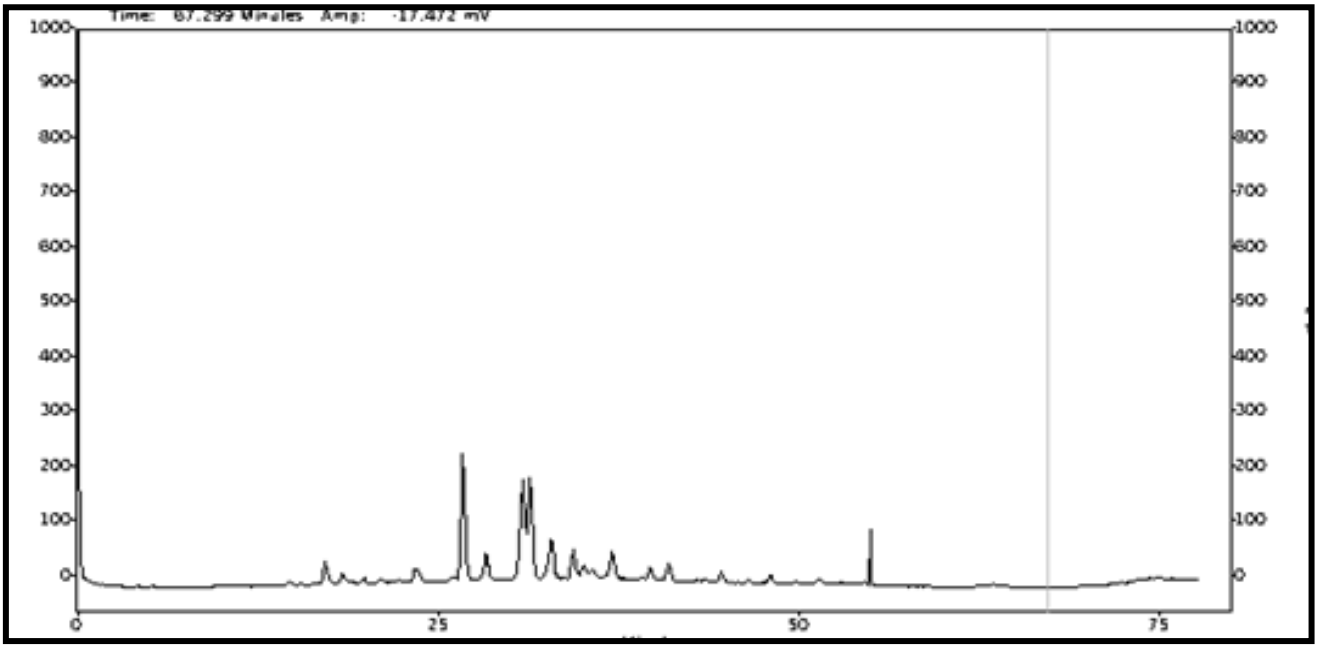


Figure (3-18): GC analysis of fatty acid sample of partial purified biosurfactant produce by *G. thermoleovorans* using helium as a carrier gas on a Shimadzu 17-A GC.

The GC-MS analysis of the lipid fraction of bioemulsifier produced by *A. pallidus* YM-1showed that the hexadecanoic acid and octadecanoic acid were the major fatty acids that account for 94.4 % of the total fatty acids. Other fatty

acids determined at a lower extent were dodecanoic acid and tetradecanoic acid (Zheng *et al.*, 2011).

3.6.4 NMR for lipid part (fatty acid) analysis:

The NMR spectrum (figure 3-19) showed many signals of the partial purified biosurfactant produced by *G. thermoleovorans* (Ir1) and the main signal was attributed to triglycerides.

The partial purified biosurfactant consisted of two compounds, one of them may be one of fatty acid, and the second one was triglycerides.

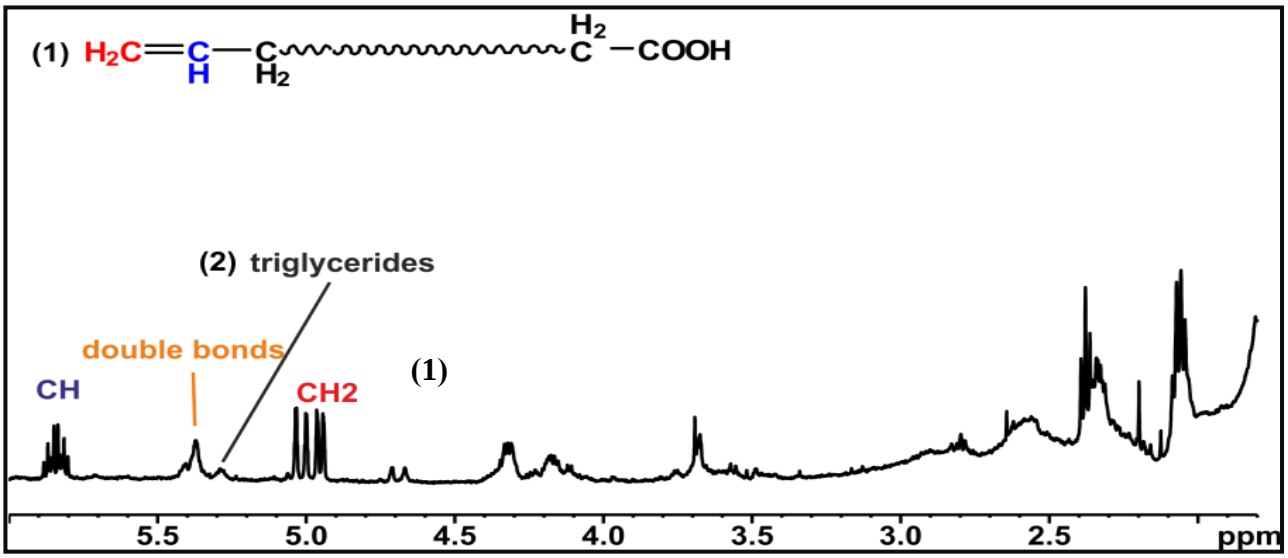


Figure (3-19): Selective region of H-NMR spectra of partial purified biosurfactant produce by *G. thermoleovorans* (Ir1), in $CDCL_3$ at 298K, at 500 MHz.

When comparing the results (NMR spectra) of partial and purified (purified previously with silica gel column chromatography as in item (3-4)) biosurfactant,

the results revealed that all purified bands contain only triglycerides without the other compounds as in figure (3-20).

Singh *et al.* (2011) analyzed exopolysaccharide produced by *B. licheniformis* using H NMR spectrum, they noticed seventeen anomeric signals, depicting complex and heterogeneous nature. While Amaral *et al.* (2006) indicated that the H NMR signals for bioemulsifier produced by *Y. lipolytica* was attributed to polysaccharide at 3.2–4.4 ppm, and the rest of the spectrum referred the presence of low levels of protein.

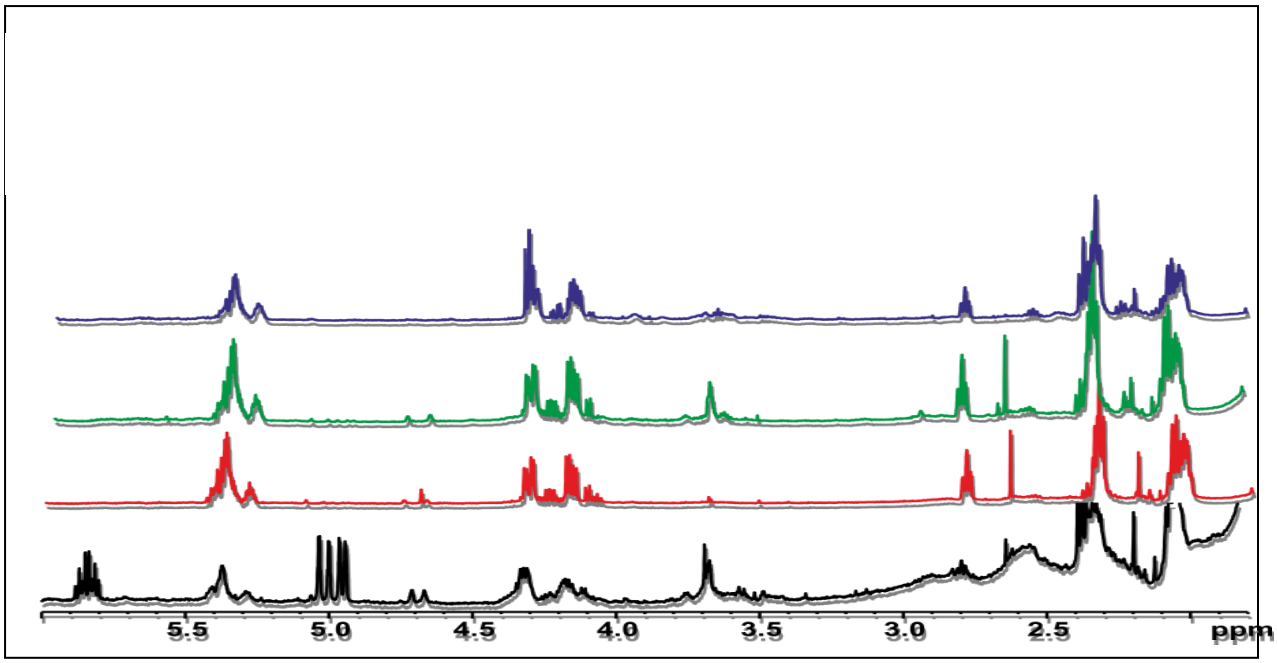


Figure (3-20): Overlap of selective region of H-NMR spectra of biosurfactant produce by *G. thermoleovorans* (Ir1). [black color] partial purified, [red color] purified band 1, [green color] purified band 2 and [blue color] purified band 3, in $CDCL_3$ at 298K, 500 MHz.

Gudiña *et al.* (2015b) used NMR to detected the bioemulsifier produced by *Paenibacillus* sp. as a low molecular weight oligosaccharide-lipid complex in

which the fatty acids and oligosaccharides were structurally associated involving either covalent or non-covalent bonds.

3.7 The biological activity of biosurfactant against tumor cell line:

In order to determine the antitumor activity of the purified biosurfactant produced by *G. thermoleovorans* (Ir1), three different tumor cell lines (MCF7, Jurkate, and Hut78) were exposed to different concentrations of purified biosurfactant for three incubation periods (24, 48, and 72 hrs.).

MTS test was used for determining the number of viable cells in proliferation cell with an absorbance at 490 nm (Duarte *et al*., 2014).

Results showed that the biosurfactant had an inhibitory effect on proliferation of human breast cancer cell line (MCF7) during all periods of exposure as in table (3-2) and appendix (5).

With respect to subjecting cell line to different concentrations with different incubation periods, the inhibitory percent seemed to be increasing with the increasing the concentration. Also the results showed a highly significant effect were accounted among different concentrations in all incubation times except the non – significant effect between 30 µg/ml with 20 µg/ml and 20 µg/ml with 10 µg/ml (table 3-2 and appendix 5).

For the 24 hrs. exposure time there was no pronounced effect on cell viability, especially at the lower biosurfactant concentrations. For the highest exposure times (48 and 72 hrs.), the number of viable cells were found to decrease significantly.

Result indicated in appendix (5) show a high significant inhibition $p<0.01$ effect with all periods of incubation, and exposure to biosurfactant for 72 hrs. with high concentration (100µg/ml) leding to significant inhibition activity. So, the effect

was dose and time dependent manner and this may be attributed to the biosurfactant which have properties of detergent like. Cell proliferation may be affected by different manners as mentioned by Fracchia *et al*. (2010) who demonstrated the effect of surfactin of *B. subtillus* against the MCF7 cell line. They found that at low concentrations, surfactin penetrates rapidly into the cellular membrane forming micelles together with phospholipids, at moderate concentrations, surfactin can induce pore formation on the lipid bilayers, and at high concentrations, the detergent like effect prevails resulting in total membrane loss.

Table (3-2): Inhibitory effect of different concentrations of purified biosurfactant against MCF7cell line with different incubation period.

Concentration (µg/ml)	Inhibitions % MCF7 cells		
	24 hrs.	48 hrs.	72 hrs.
	Mean± S.D.	Mean± S.D.	Mean± S.D.
0	0.000 ±0.00	0.000±0.00	0.000±0.00
100	33.75±15.19	59.00±3.960	66.55±0.919
60	24.60±1.414	48.35±0.495	57.20±13.294
50	13.70±3.960	39.70±4.525	40.90±11.455
40	9.100±2.121	20.15±9.405	28.45±5.303
30	5.200±1.838	8.95±0.495	15.40±9.475
20	0.000±6.25	6.25±3.323	6.45±2.899
10	0.000±0.00	1.45±1.485	2.300±1.273

The results of inhibitory effect illustrated in table (3-3) of purified biosurfactant produced by *G. thermoleovorans* (Ir1) against lymphoblastic leukemia cells (Jurkat cell) showed that the original readings of inhibition percent seemed to be propagated with increasing level of concentrations. The results revealed that the differences obtained for all biosurfactant concentrations, studied were of a statistically highly significant, while a significant effect ($P<0.05$)was found between 60µg/ml with 50µg/ml, 40µg/ml with 20µg/ml, 20µg/ml with 10µg/ml. No significant ($P>0.05$) effect between 50µg/ml with 40µg/ml, 40µg/ml with 30µg/ml, and 30µg/ml with 20µg/ml.

The result illustrated in appendix (6), also indicate that the inhibition percentage of cells treated was significantly increased with the increase of time, so it is the time dependent manner, as increased with prolonged incubation, this may be due to the dead cells release intracellular component, which caused toxicity increase and induced more cells to die.

While the studied inhibitory effect of biosurfactant produced by *G. thermoleovorans* (Ir1) against Human T cell lymphoma (Hut78) (table 3-4 and appendix 7) showed a low inhibitory effect at low concentration, and a highly significant effect ($P<0.01$) with most concentrations. The significant effect ($P<0.05$) also shown in concentration 50µg/ml with 40µg/ml, 40µg/ml with 30µg/ml and no significant effect ($P>0.05$) for 60µg/ml with 50µg/ml and 20µg/ml with 10µg/ml. With respect to the studied periods the results showed significant ($P<0.05$) differences between different incubation periods.
The results (table 3-4) showed a low inhibitory effect at low concentrations even with long incubation periods.

As shown in appendix (7) the inhibitory effect was time and dose dependent manner.

The result showed that the inhibitory effect increased with the prolonged incubation period.

The sensitivity of cell line to biosurfactant varied with type of these cells, since the breast cancer cell line (MCF7 cell line) was more sensitive than Jurkat and Hut78, when treated with purified biosurfactant and this variable in sensitivity may be due to differences in metabolic pathway for different cell lines. Kim *et al.* (2007) found that the metabolic pathways in response to each treatment differed from one line to another.

Table (3-3): Inhibitory effect of different concentrations of purified biosurfactant against Jurkat cell line and different incubation period.

Concentration (µg/ml)	Inhibition % Jurkat		
	24 hrs.	48 hrs.	72 hrs.
	Mean± S.D.	Mean± S.D.	Mean± S.D.
0	0.000±0.000	0.000±0.000	0.000±0.000
100	54.200±0.000	56.350±2.758	60.650±455
60	29.750±11.526	40.750±4.455	51.350±2.192
50	21.700±0.000	30.300±1.414	42.250±3.182
40	19.550±5.728	25.700±1.273	27.000±18.809
30	13.800±6.364	22.450±1.763	23.000±12.445
20	8.500±6.364	19.800±6.505	20.100±1.131
10	0.800±0.566	7.900±6.223	12.700±7.071

In order to find the effect of biosurfactant on normal kidney cell line, the result revealed that no effect on this cell line even with high concentrations this may be

attributed to the metabolism of tumor cell which differ from the metabolism of normal cell. Folkman (2000) mentioned that there was a selective toxicity towards malignant cell lines and this was due to the differences in the malignant cellular physiology such as the presence of some metabolic factors that found in the cancer cell lines but not found in normal cells.

Table (3-4): Inhibitory effect of different concentrations of purified biosurfactant against Hut78 cell line with different incubation period.

Concentration (µg/ml)	Inhibitions % Hut78		
	24 hrs.	48 hrs.	72 hrs.
	Mean± S.D.	Mean± S.D.	Mean± S.D.
0	0.000±0.000	0.000±0.000	0.000±0.000
100	19.400±0.000	28.250±4.879	36.700±3.253
60	11.300±9.758	27.300±4.525	26.800±0.566
50	5.000±0.849	22.500±1.414	24.700±4.525
40	1.210±0.849	13.000±3.253	22.800±0.566
30	0.600±0.849	9.550±10.677	13.350±4.313
20	0.000±0.000	0.120±0.000	2.910±3.946
10	0.000±0.000	0.000±0.000	0.000±0.000

Since the MCF7 cell line showed high inhibitory activity, so it was chosed for subsequent cytotoxicity studies.

3.8 Cytotoxic effect of biosurfactant on cancerous cell lines:

The cytotoxic effect of purified biosurfactant was studied against breast cancer (MCF7) cell line by using the thermo Scientific Cellomics Multi parameter cytotoxicity 3 Kits, which enabled simultaneous measurement of many orthogonal cell- health parameters: cell loss, released cytochrome C from mitochondria and nucleus intensity, cell membrane permeability and mitochondria membrane permeability when compared with control and positive control(Doxorobicin)..

The results illustrated in appendix (8) and table (3-5) indicate that the viability (cell count) of cancer cells (MCF7) treated with different concentrations of purified biosurfactant was highly significant decreased as compared with control and significant effect when compared with positive control. The viablity of MCF7 were (928 ± 33.234 and 688± 9.90) after treatement with 50 and 100 µg/ml of purified biosurfactant respectively.

The result illustrated in appendix (9) and table (3-6) also show that the permeability of MCF7 cells were significantly increased when treated with 50 and 100 (µg/ml) of purified biosurfactant and the values of cell permeability were increased from 31.35 to 36.3 and 56.6 for concentrations 50 µg/ml and 100 µg/ml respectively.

The cytotoxic effect of purified biosurfactant on MCF7 cells line may be attributed to the cell shrinkage membrane blebbing, loss of cell adhesion and interaction with cell membrane lipids. Gudin˜a *et al.* (2013) mentioned that the new therapeutic strategies may be designed, considering that, the use of biosurfactant can alter lipid content to fluidize rigid cancerous tissues and to modulate interfacial properties. While Janek *et al.* (2013) found that the ability of biosurfactant to disrupt cell membranes, leading to a sequence of

events that include lysis, increased membrane permeability, and metabolite leakage, have also been suggested as a probable mechanism of antitumor activity.

Table (3-5): Effect of different concentrations of purified biosurfactant on cell count of MCF7 cell line:

Treatment (µg / ml)	Cell count (mean ± SE)
Control	1082± 79.195
Doxorubicin 50	76.00± 12.727
100	688± 9.90
50	928± 33.234
P-value	0.000 (HS)

HS= highly significant (p value <0.01).

Table (3-6): Effect of different concentrations of purified biosurfactant on cell permeability of MCF7 cell line:

Treatment (µg / ml)	Cell permeability(mean ± SE)
Control	31.35±5.020
Doxorubicin 50	87.7± 10.748
100	56.6± 8.902
50	36.3± 3.181
P value	0.0006(HS)

HS= highly significant (p value <0.01).

The result elucidated in appendix (10) and table (3-7) declare that the purified biosurfactant caused a highly significant increase in cytochrome C releasing from mitochondria of MCF-7 cells in concentrations 50 and 100 (µg/ml).

Table (3-7): Effect of different concentrations of purified biosurfactant on cytochrome c of MCF7 cell line.

Treatment (µg / ml)	cytochrome C (mean ± SE)
Control	**99.98 ±2.969**
Doxorubicin 50	**132.0 ± 8.343**
100	**123.545± 3.026**
50	**106.68± .975**
P value	**0.007 HS**

HS= highly significant (p value <0.01).

Appendix (11) and table (3-8) show that 50 and 100 (µg/ml) of purified biosurfactant caused significant changes in the mitochondrial membrane potential. This may be attributed to the release of cytochrome C, and that is in accordance with Cao *et al.* (2011) who showed that surfactin induces ROS formation, leading to mitochondrial permeability and membrane potential collapse that ultimately results in an increase of calcium ion concentration in the cytoplasm afterwards, cytochrome C released from mitochondria to the cytoplasm activates caspase-9 eventually inducing apoptosis.

Results (appendix 12 and table 3-9) revealed that the nuclear size of the MCF-7 cell was highly and significantly affected by purified biosurfactant. Different concentrations can cause a nuclear condensation induced at a higher concentration (100µg/ml) from 393.6 to 454.97.

Table (3-8): Effect of different concentrations of purified biosurfactant on mitochondrial membrane potential change of MCF7 cell line.

Treatment (µg / ml)	mitochondrial membrane potential change (MMP)(mean ± SE)
Control	**32.56 ±5.10**
Doxorubicin 50	**21.2 ± 2.262**
100	**15.12± 0.120**
50	**23.31± 2.842**
P value	**0.022 S**

S= significant.

The apoptotic process may be attributed to nuclear condensation and DNA fragmentation. This result was in accordance with Chiewpattanakul *et al*. (2010) who detected the anticancer activity of biosurfactant produced by the dematiaceous fungus *Exophiala dermatitidi*s SK80 against cervical cancer (HeLa) and leukemia (U937) cell lines. This effect is commonly associated with the apoptotic process, in which the DNA is cleaved into fragments of 180 nucleosomal units by the endogenous endonuclease, caspase enzymes and nucleus condensation. Lemasters *et al.* (2009) mentioned that the induction of the permeability transition pore can lead to mitochondrial swelling and cell death through apoptosis or necrosis depending on the particular biological setting a sequence of apoptotic events

was observed, including the condensation of chromatin and DNA fragmentation, thus confirming the apoptosis-inducing potential of MELs in these cells. The activity of protein kinase C (PKC) might be associated with apoptosis induced by MELs. The activation of PKC is one of the first events in the signal transduction that leads to a multiplicity of cellular responses. Indeed, members of the PKC family are key factors in cell differentiation, control of growth, and cell death.

While the activity of Succinoyl trehalose lipid against human monocytoid leukemic cell line (U937) was not caused by a simple detergent-like effect, but was attributed to a specific interaction with the plasma membrane (Isoda *et al*., 1995).

Table (3-9): Effect of different concentrations of purified biosurfactant on nucleus intensity of MCF7 cell line.

Treatment (µg / ml)	Cell count(intensity) (mean ± SE)
Control	**393.6±1.131371**
Doxorubicin 50	**497.5±16.68772**
100	**454.97±14.11**
50	**408.6 ±8.202439**
P value	**0.003 HS**

HS= highly significant (p value <0.01).

The High Content Screening test evaluated the cytotoxic effect of biosurfactant against the MCF7 cell line after 24 hrs. exposure. The evaluation of the HCS

images acquired from figure (3-21) showed that the cytotoxic response to biosurfactant was dose dependent.

Result illustrated a significant cytotoxic effect at 100 µg/ml of purified *G. thermoleovorans* (Ir1) biosurfactant, so at this concentration, many changes were observed. This result was used as a key parameter for the evaluation cytotoxic effect of this biosurfactant.

Cytotoxicity is a complex process affecting multiple parameters and pathways. After toxic insult, cells often undergo either apoptosis or necrosis accompanied by changes in nuclear morphology, cell permeability, and mitochondrial function, resulting in loss of mitochondrial membrane potential and release of cytochrome C from mitochondria (Taylor, 2007).

Mingeot- Leclercq *et al.* (1995) reported that changes in cell membrane permeability are often associated with a toxic or apoptotic response, and the loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity.

While Wakamatsu *et al.* (2001) found that exposure of adrenal gland phaeochromocytoma (PC12) cells to MEL (biosurfactant of *Candida antartica*) enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase, with a resulting outcome of neurites and partial cellular differentiation.

Kim *et al*. (2007) showed that surfactin blocks cell proliferation by inducing proapoptotic activity and arresting the cell cycle. Furthermore, surfactin strongly blocked the PI3K/Akt signaling pathway (PI3K phosphoinositide 3 kinase; Akt also known as protein kinase B (PKB) is a serine/threoninespecific protein kinase); both proteins are involved in multiple cellular processes such as cell proliferation and apoptosis. Furthermore, Du

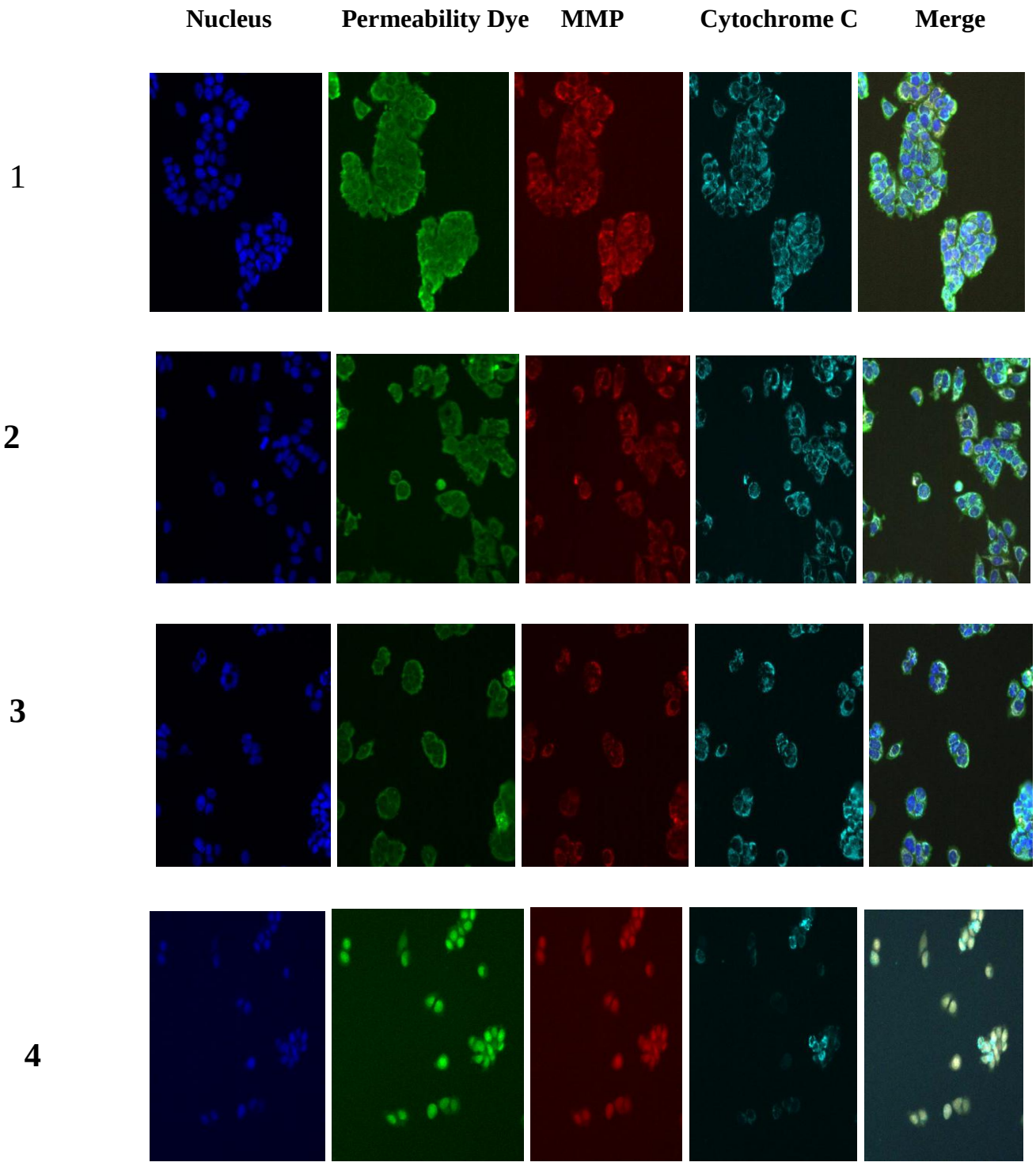


Figure (3-21): The cytotoxic effect of different concentrations of purified biosurfactant on MCF7 cell.

1:Untreated cell 2: Treated cell with (50µg/ml) 3: Treated cell with (100 µg/ml) 4: Treated cell with Doxorubin (50 µg/ml).

et al. (2014) indicated that the disturbance of the cellular fatty acid composition of breast cancer cell lines, by lipopeptide, was related to apoptosis.

STL and MEL glycolipids differentiation-inducing activity was attributed to a specific interaction with the plasma membrane instead of a simple detergent-like effect. Moreover, succinoyl trehalose lipids have been shown to inhibit growth and induce differentiation of HL60 human promyelocytic leukemia cells (Sudo *et al.*, 2000) and human basophilic leukemia cell line KU812 (Isoda *et al.*, 1995).

While Chiewpattanakul *et al.* (2010) showed the biosurfactant monoolein produced by the dematiaceous fungus *Exophiala dermatitidis* SK80, effectively inhibited the proliferation of cervical cancer (HeLa) and leukemia (U937) cell lines in a dose dependent manner, interestingly, no cytotoxicity was found with normal cells, even when high concentrations were used. Cell and DNA morphological changes observed in both cancer cell lines include cell shrinkage, membrane blebbing, and DNA fragmentation.

3.9 Antimicrobial effect of biosurfactant:

The antimicrobial activity of purified biosurfactant was examined against many microorganisms such as gram positive (*S. aureus, Streptococcus* sp.), gram negative bacteria (*E. coli, P. aeruginosa, Proteus* mirabilis) and fungi (*A. niger*, *Penicillium*, *C. albicans*).

The results showed that the biosurfactant had different antibacterial effect on the bacterial growth. As shown in figure (3-22) and when compared with appendix(13), the biosurfactant with concentration 100 mg/ml had an effect on *S. aureus*, *Streptococcus* sp. and *P. aeruginosa* with the inhibition zone 19 mm, 13mm and 10 mm respectively. While no effect of biosurfactant was observed against other remaining bacterial strains.

Result illustrated in figure (3-23) show the antifungal effect of purified biosurfactant against *C. albicans* growth with concentration 100 mg/ml and it caused the inhibition zone (21mm). The concentration 75mg/ml had an effect with inhibition zone 11mm. while there was no any effect of all concentrations of biosurfactant against *A. niger* and *Penicillium*.

This effect may be attributed to the structure of biosurfactant, it is supposed to exert its toxicity on the cell membrane permeability as detergent like effect that emulsified lipid bacterial membranes and/or form a pore-bearing channel inside a lipid membrane.

Deleu *et al*. (2008) found that the effect of lipopeptide biosurfactant of *Bacillus* spp. was attributed to self-associate and form a pore-bearing channel was due to the ability of micellular aggregate inside a lipid membrane.

Landman *et al.* (2008) mentioned that the lichenysin, pumilacidin and polymyxin B are lipopeptides produced by *B. licheniformis, B. pumilusand* and *B. polymyxa*, respectively, has shown antibacterial activities against a wide variety of gram negative pathogens due to the high affinity for the lipid moieties of lipopolysaccharide. Mannosylerythritolcations lipid (MEL), a glycolipid surfactant of *C. antartica*, has shown an antimicrobial activity, particularly against gram-positive bacteria (Kitamoto *et al.*, 1993).

Zhao *et al.* (2010) pointed that the diverse structures of biosurfactant confer them the ability to display versatile performance. While Fernandes *et al*. (2007) who investigated the antimicrobial activity of biosurfactant (lipopeptides) of *B. subtilus* demonstrated that this biosurfactant has a broad spectrum of antimicrobial activity against microorganisms with multidrug-resistant profile.

Yalçin and Ergene (2009) found the antifungal activity of biosurfactant produced by *Pseudomonas* sp. against yeasts (*C. albicans* FMC 17 and *C. krusei* ATCC 6258), with diameters of an inhibition zone ranging between 12 and 17 mm respectively.

Rodrigues *et al.* (2006b) indicated that biosurfactant might also contain signaling factors that interact with the host and/or bacterial cells, leading to the inhibition of infections.

Moreover, they support the assertion of a possible role in preventing microbial adhesion and their potential in developing anti-adhesion biological coatings for implant materials.

Nitschke *et al.* (2010) reported that rhamnolipids produced by *P. aeruginosa* LBI has an antimicrobial activity against several bacteria and fungi, including *B. cereus, S. aureus, M. luteus, M. miehei* and *N. crassa*.

The characteristics of sophorolipid and rhamnolipid were evaluated as antifungal agents against plant pathogenic fungi. Eight percent of mycelial growth of plant pathogen (*Phytophthora* sp. and *Pythium* sp.) was inhibited by 200 mg/L of rhamnolipid or 500 mg/L of sophorolipid, and zoospore motility of

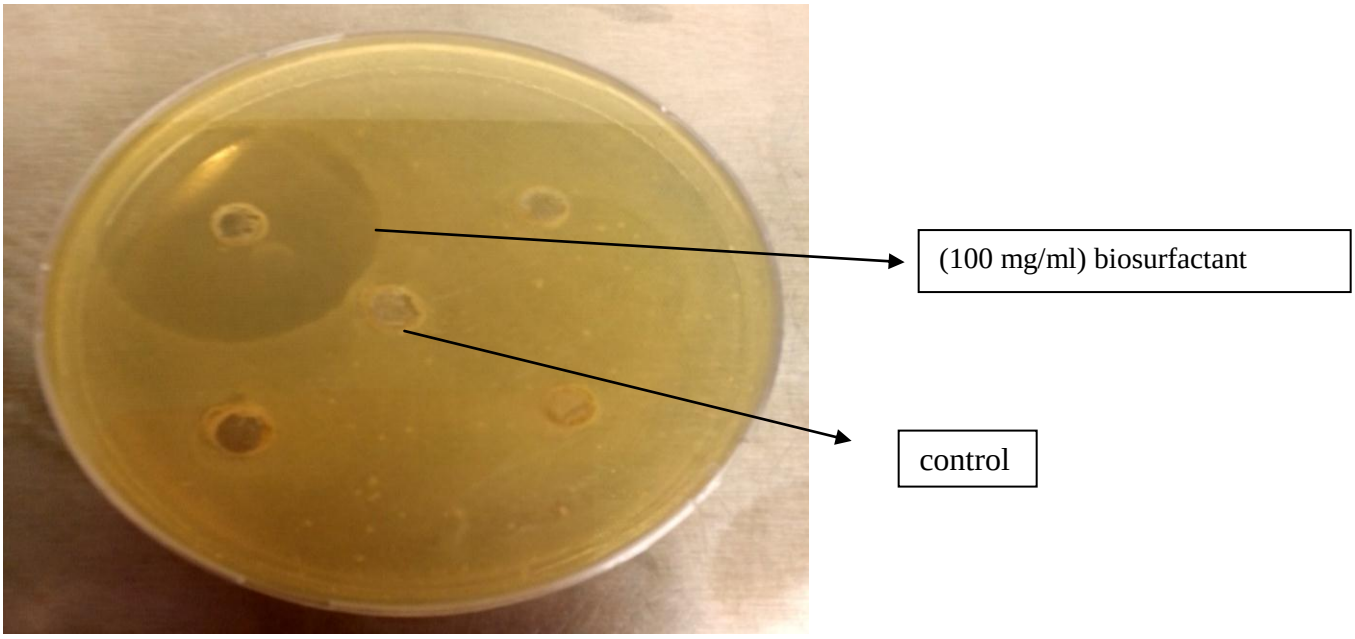


Figure (3-22): Antibacterial activity of purified biosurfactant produced by *G. thermoleovorans*(Ir1) against *S. aureus* grown on Muller Hinton agar incubated at 37 º C for 24hrs.

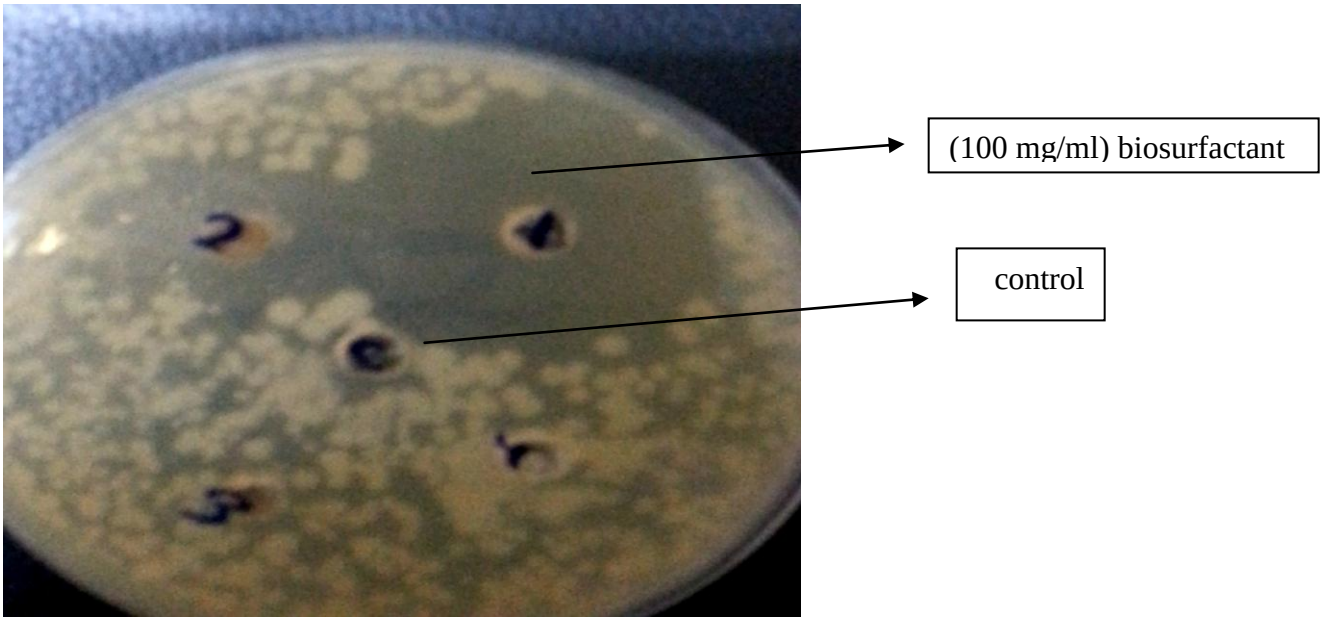


Figure (3-23): Antifungal activity of purified biosurfactant produced by *G. thermoleovorans* (Ir1) against *C. albicans* grown on Muller Hinton agar incubated at 28 º C for 5 day.

Phytophthora sp. decreased by 90 % at 50 mg/L of rhamnolipid and 80% at 100 mg/L of sophorolipid.

The effective concentrations of zoospore lysis were two times higher than those of zoospore motility inhibition. The highest zoospore lysis was observed with *Phytophthora capsici*; 80 % lysis at 100 mg/L of di-rhamnolipid or lactonic sophorolipid, showing the dependency of structure on the lysis. These results showed the potential of microbial glycolipid biosurfactants as an effective antifungal agent against damping-off plant pathogens (Dal-Soo *et al*., 2005).

Another lipopeptide with an antimicrobial activity and other interesting biological properties is viscosin, a cyclic lipopeptide from *Pseudomonas* (Saini *et al.*, 2008).

3.10 Effect of some physical and chemical factors on partial purified biosurfactant activity:

3.10.1 The physical properties of biosurfactant:

The physical properties of the partial purified biosurfactant produced by *G. thermoleovorans*(Ir1) showed that the biosurfactant has a white to brown color, and soluble in a slightly warm Tris solution, chloroform and water but partially soluble in butanol and methanol. And the melting point was >250 ºC.

Singh *et al.* (2011) showed that the exopolysaccharide bioemulsifier produced by *B. licheniformis* was dissolved in deionized water heating at 80 ºC.

3.10.2 Effect of different concentrations of biosurfactant on emulsification activity:

Different concentrations of partial purified biosurfactant produced by *G. thermoleovorans* (Ir1) were utilized for emulsification of sunflower oil. The result showed that the optimum concentration of biosurfactant was 7 mg/ml and E24%value 86%. The E24% was reduced at a concentration below this value, with no detectable E24% at concentration 2 to 3 mg/ml (figure 3-24).

Liu *et al.* (2010) demonstrated that the biosurfactant synthesized by *B. subtilis* CCTCC AB93108 one of the most powerful biosurfactant, has good activity at concentration 2.96 g/L.

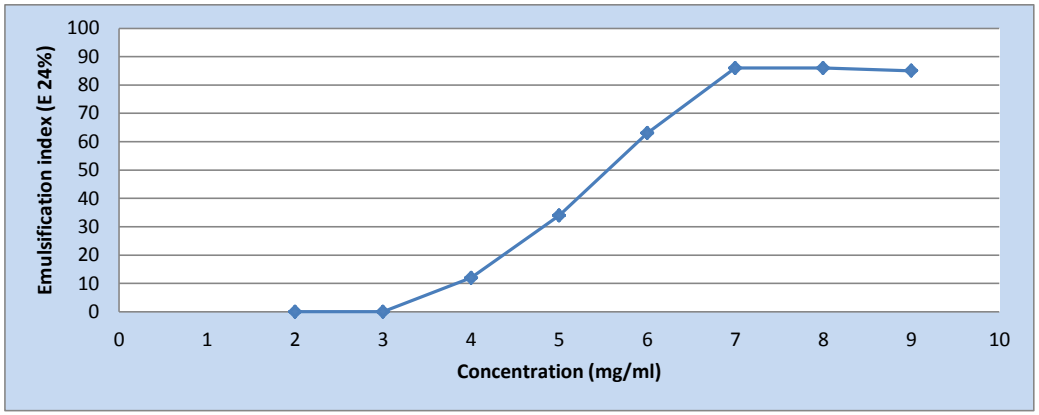


Figure (3-24): The effect of different concentrations of *G. thermoleovorans* (Ir1) biosurfactant on emulsification index (E24%) of sunflower oil.

3.10.3 Effect of temperature on biosurfactant activity:

The results showed that the biosurfactant of *G. thermoleovorans* (Ir1) has good stability with a wide range of temperatures (20-120 ºC), the maximum activity at 60 ºC was E24% = 87% (figure 3-25).

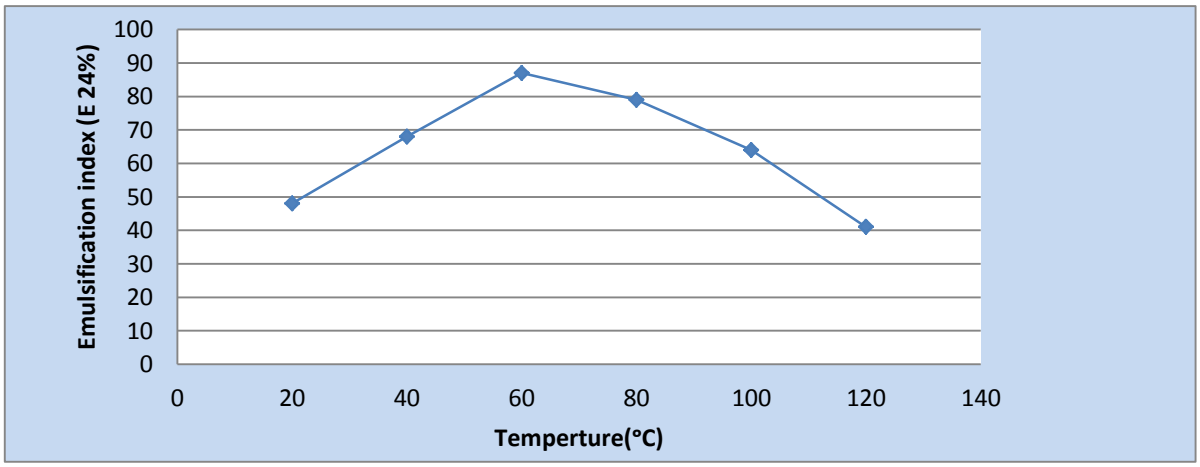


Figure (3-25): The effect of temperature on stability of biosurfactant produce by *G. thermoleovorans* for 30 min.

Also the results showed that the biosurfactant has an activity at 100 ºC, and this result revealed that this biosurfactant was heat stable, this stability may relate to its structure (lipids, carbohydrates and protein).

The emulsification activity of extracellular bioemulsifier produced by *B. stearothermophilus* VR-8 grown in a medium containing 4% crude oil was stable over a broad range of temperatures (50–80 ºC), the emulsification activity was 100% at 80 ºC for 30 min, and 60% at 90 ºC and 100 ºC (Gurjar *et al*., 1995).

Result illustrated in figure (3-25) also indicate low activity of biosurfactant with a high temperature above 100 ºC and this may be attributed to denature of its structure (especially the protein part) at high temperature.

Sarubbo *et al*. (2006) mentioned that the loss of emulsifying activity during heating at 100 ºC can be explained by the denaturation of the protein fraction of bioemulsfier of *Microbacterium* sp. MC3B-10.

Several bioemulsifiers are effective at high temperatures, including the protein complex from *M. thermoautotrophium* (De Acevedo and Mclnerney, 1996) and the protein-polysaccharide-lipid complex of *B. stearothermophilus* ATCC 12980 (Gurjar *et al.,* 1995). While Maneerat and Dikit (2007) observed that the cell-associated bioemulsifier crude extract produced by *Myroides* sp. SM1 was stable in a broad temperatures ranging from 30 to 121ºC and this range did not affect the emulsification activity.

3.10.4 Effect of some salts on biosurfactant activity:

In order to determine the stability of biosurfactant with salts, three kinds of mineral salts (NaCl, KCl and $CaCl_2$) at different concentrations were mixed with biosurfactant to detect their effect on the emulsification index.

Results presented in figure (3-26) show that the emulsification index was stable at concentration 2%, 2% and 8% of NaCl, $CaCl_2$ and KCl respectively.

Besides the results showed that the best salt, which maintain the E24% was $CaCL_2$ then NaCl. It was also noticed that the low (2%) and high (20%) concentration of KCl gave the lowest E24%.

From the above results, it is clear that the biosurfactant activity was stable over a wide range of salt concentrations and the activity was stable even at a high concentration of salt. This may be attributed to the salt, which plays a major role in the physiological cellular activity and metabolism.

Abu-Rawaida *et al*. (1991) mentioned that the salt plays an important role in biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10 % (w/v), although little reduction in the critical micelle concentrations was demonstrated.

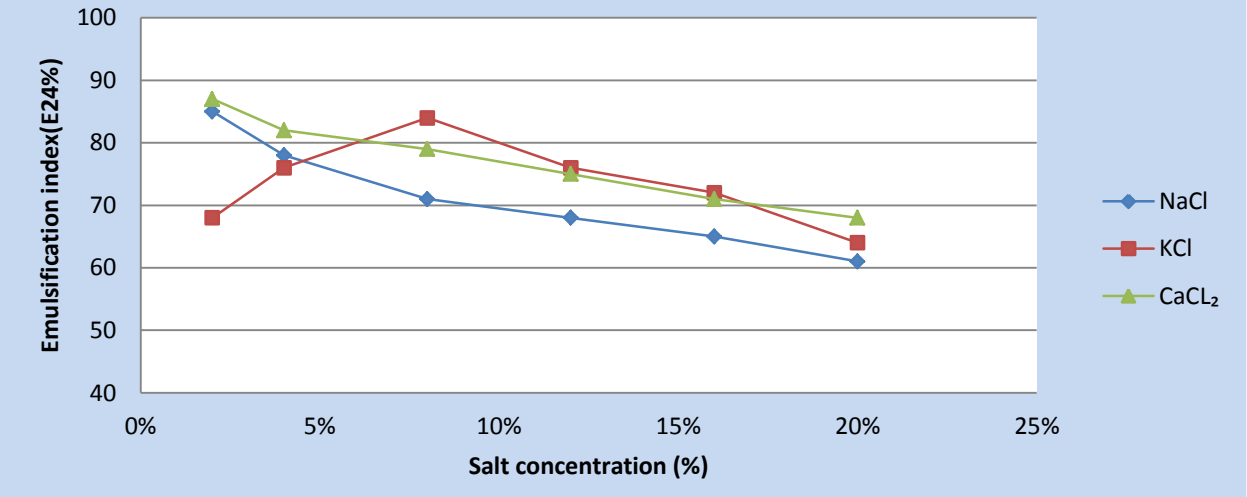


Figure (3-26): The effect of different salts (NaCl, $CaCl_2$ and KCl)) concentrations on stability of biosurfactant produced by *G. thermoleovorans* (Ir1).

Bioemulsifier extracted from *Myroides* sp. SM1was able to emulsify weathered crude oil in the presence of NaCl from 0.51 M up to 1.54 M, but a loss in activity was found when NaCl concentration was above 1.54M. The highest emulsification activity was observed in the presence of 1.02 to 1.54 M of NaCl. However, the activity was found in the absence of NaCl, while 3M $CaCl_2$ inhibited the emulsification of weathered crude oil by crude extract. The activity was not inhibited in the presence of $MgCl_2$ up to 0.1 M, but was enhanced at 0.02 M $MgCl_2$ (Maneerat and Dikit, 2007).

Camacho-Chab *et al.* (2013) studied emulsifying activity and stability of a bioemulsifier synthesized by *Microbacterium* sp. MC3B-10 and mentioned that the highest levels of activity were observed at 3.5% NaCl. While the emulsification activity of biosurfactant produced by *Aeromonas* spp., was maintained with concentration of NaCl up to 5% (Ilori *et al.*, 2005).

3.10.5 Effect of pH on biosurfactant activity:

The result declared in figure (3-27) indicate that biosurfactant of *G. thermoleovorans* (Ir1) remain active over a wide range of pH (2-10). The highest (E24%= 87%) was observed at pH 7, while the minimum E24%=58% was recorded with pH 2.

This result was in agreement with Kokare *et al.* (2007) who mentioned that the maximum activity of biosurfactant of *Streptomyces* sp. at pH value 7, and reduced in acidic as well as in basic pH.

However, slightly higher levels of emulsifying activity that recorded at acid and alkaline pH values, suggesting the ionization of functional groups that resulted in the activation of less surface-active species within the bioemulsifier matrix (Sarubbo *et al.*, 2007).

In comparison, biodispersan of *A. calcoaceticus* A2 had an optimum functional pH range of 9 to 12 for limestone-dispersing activity (Rosenberg *et al*., 1988). While Adamu *et al.* (2015) found that the phospholipids produced by *Bacillus sphaericus* EN3 and *Bacillus azotoformans* EN16 were more effective at pH of 8 to 10. While a precipitation was observed at pH below 6 in the crude extracted bioemulsifier of *Acinetobacter*, but no changes in activity were observed in the pH (6-12).

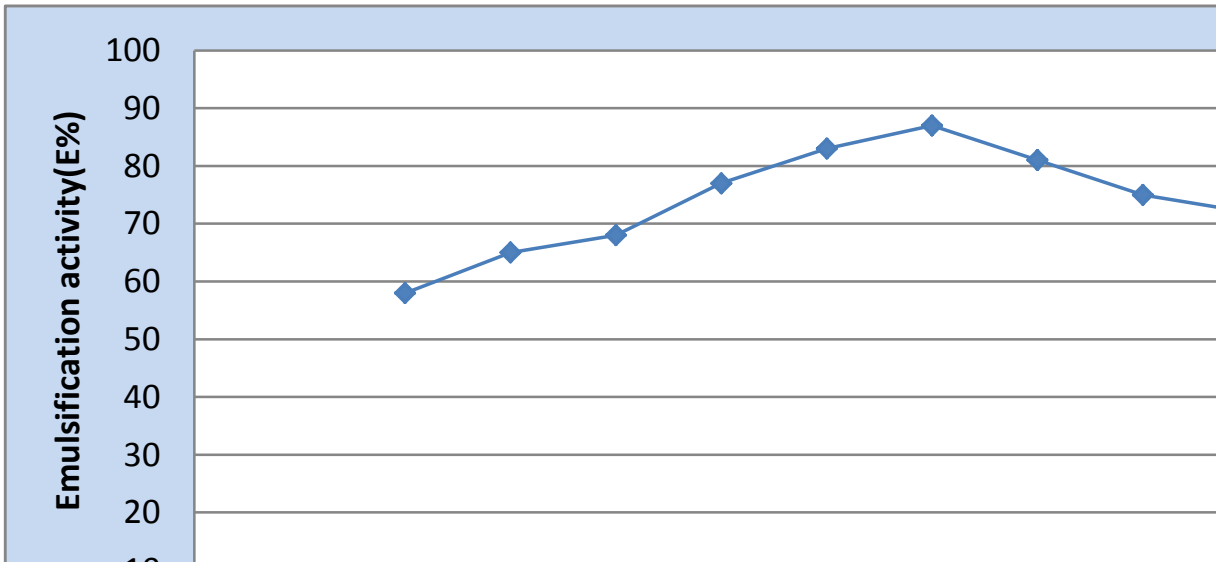


Figure (3-27): The effect of different pH values on activity of biosurfactant produced by *G. thermoleovorans* (Ir1).

The stability of crude extract bioemulsifier from *A. calcoaceticus* sub sp. *anitratus* SM7 in alkaline pH indicated that ester linkages were not required for its emulsifying activity, and at a pH close to isoelectric point, there was no electrostatic repulsion between neighboring molecules, and the compounds tend to coalesce and precipitate (Phetrong *et al*., 2008).

According to the above results, it can be concluded that the biosurfactant is active with a wide range of temperature, different concentration of following salt (NaCl, KCl and $CaCl_2$) and at different pH values, so biosurfactant produced by *G. thermoleovorans* (Ir1) can be used in many applications that require using extreme conditions.

3.11 Detection of gene coded for biosurfactant production:

In an attempt to amplify and characterize the gene responsible for biosurfactanteeeeee of *G. thermoleovorans* (Ir1), the primers for *sfp* gene coded for biosurfactant produced by *Bacillus* sp. was used, because there was no data found in NCBI about the gene(s) or DNA sequence coded for biosurfactant in *Geobacillus* spp.

3.11.1 Isolation of genomic DNA:

Genomic DNA of *G. thermoleovorans* (Ir1) and *B. subtilis* (positive control) was extracted using (2.1.6) kit, in order to amplify the gene coded for biosurfactant using PCR. The results showed that the recorded range of DNA concentration was 75-180 ng/µl and the DNA purity was 1.7-1.9, which indicated a high purity. A pure DNA preparation expected A260/A280 ratio of 1.8 which was based on the extinction coefficients of nucleic acids at 260 nm and 280 nm (Green and Sambrook , 2012). Such results were also observed when the DNA samples were analyzed by gel electrophoresis, in which sharp DNA bands were detected, indicating purified DNA samples as shown in figure (3-28).

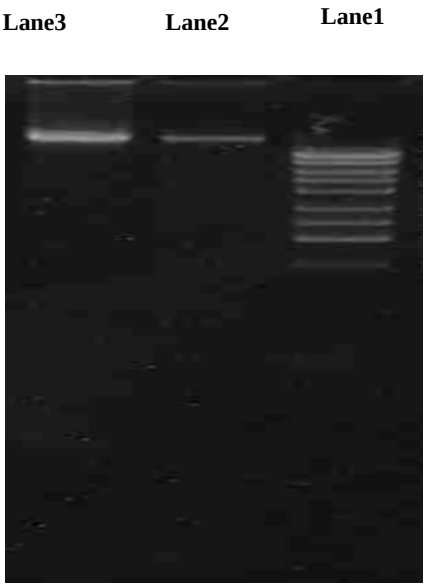


Figure (3-28): Gel electrophoresis for genomic DNA of *G. thermoleovorans* (Ir1), gel electrophoresis was performed on 1% agarose gel and run with 5V/cm for 1.5 hrs. Lane (1) is 10000 bp ladder, Lane: (2) genomic DNA of *G. thermoleovorans*, Lane: (3) genomic DNA of *B. subtilis.*

3.11.2 Amplification of gene coded for biosurfactant:

Genomic DNA was used to amplify the gene coded for the biosurfactant production using a specific primer (table 2.1.4).

The results illustrated in figure (3-29) show that *B. subtilis* gave the positive result, the amplified fragment was about 675 bp in size, which was the same size for biosurfactant gene phosphopantetheinyl transferase (*sfp)*. In comparison result showed no amplification product was seen, when using the same primers and

similar conditions, with G. *thermoleovorans* (Ir1). This means that the *(sfp)* gene not found within genomic of this bacterium.

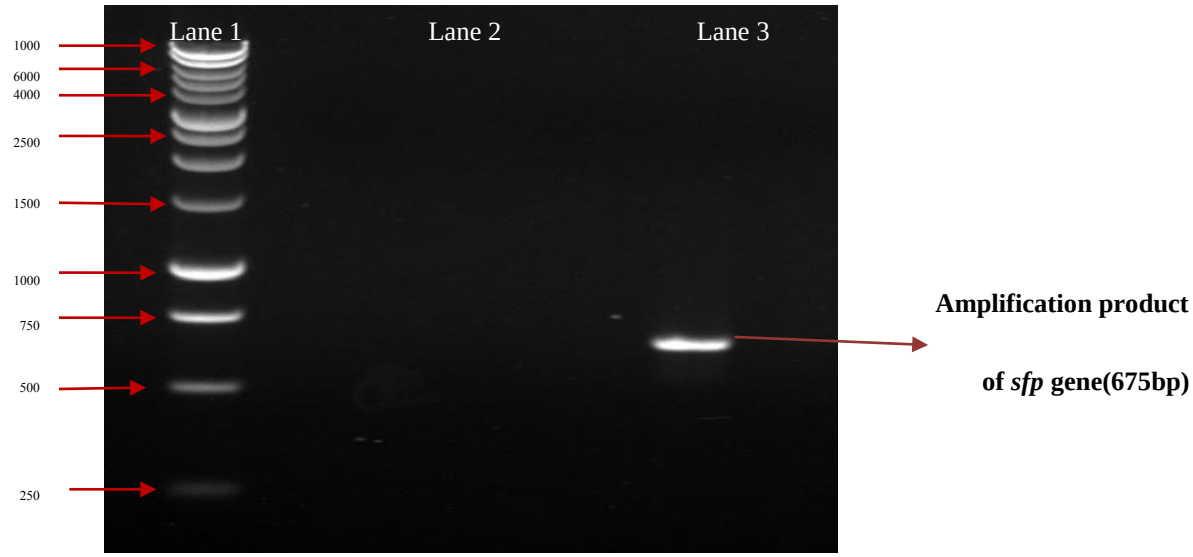


Figure (3-29): Gel electrophoresis for amplification of *sfp* gene using specific primers of *sfp*. Electrophoresis was performed on 1.5% agarose gel and run with 5V/cm for 1hr. Lane (1) DNA ladder (1kb), Lane (2): Amplification of *G. thermoleovorans*, Lane (3): Amplification of *Bacillus subtilis*.

Roongsawang *et al*. (2002) mentioned that the *sfp* plays an important role in the production of biosurfactant, and the species were identified as positive for the *sfp* gene in the PCR experiment were close relatives to the species *B. subtilis*. While Cosmina *et al*. (1993) showed that the *sfp* gene is an important member of the *srfA* operon, which codes for a nonribosomal peptide synthetase complex also known as surfactin synthetase. The *sfp* gene is an essential component of peptide synthesis

systems and it also plays a role in the regulation of surfactin biosynthesis gene expression (CLSI, 2007).

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

Conclusions and Recommendations

Conclusions:

- All thermophillic bacteria used in this study were able to produce biosurfactant, and *G. thermoleovorans* Ir1 (JQ 912239) was the most efficient one.
- The activity and productivity of biosurfactant was increased by optimized cultural condition for *G. thermoleovurance* Ir1(JQ912239).
- The chemical composition of biosurfactant revealed that it consists of 37.7% lipids, 26.2% carbohydrate and 10.7% protein. The chemical characterization revealed that the lipid part consists of palmitic acid, stearic acid and oleic acid. The carbohydrate consist of xylose, mannose and maltose. The protein part consists of aspartic acid, glutamic acid and glutamine.
- Biosurfactant showed an inhibitory effect against three different tumor cell lines (MCF7, Jurkate, and Hut78). The effect was dependent on the type of tumor cell line, biosurfactant concentration, and exposure time, with no cytotoxic effect on the normal kidney cell line.
-Biosurfactant had a cytotoxic effect against MCF7 cell line by decrease of cell count, with increase in cell permeability, cytochrome C released from mitochondria, and nucleus intensity and a significant reduction in the potential of the mitochondrial membrane.

- The purified biosurfactant had different antibacterial effect against gram negative and gram positive bacteria, it also had an antifungal activity.
- The biosurfactant is active with a wide range of temperature, different concentration of salts and at different pH values, therefor, it can be used in many applications required using extreme conditions.

- The *(sfp)* gene is not found within genomic DNA of *G. thermoleovorans* Ir1(JQ912239).

Recommendations:

1-More analytical techniques are required to clarify the chemical composition of biosurfactant.

2- Studing the antiviral activity, antiadhesive and immunmodulatory effect of this biosurfactant.

3- In vivo study of the antimicrobial and antitumor activity of biosurfactant.

4-Producting of biosurfactant and using it in different industrial applications that require extreme conditions.

5- Genetic study of *G. thermoleovorans* Ir1 to detect the gene(s) responsible for the biosurfactant production.

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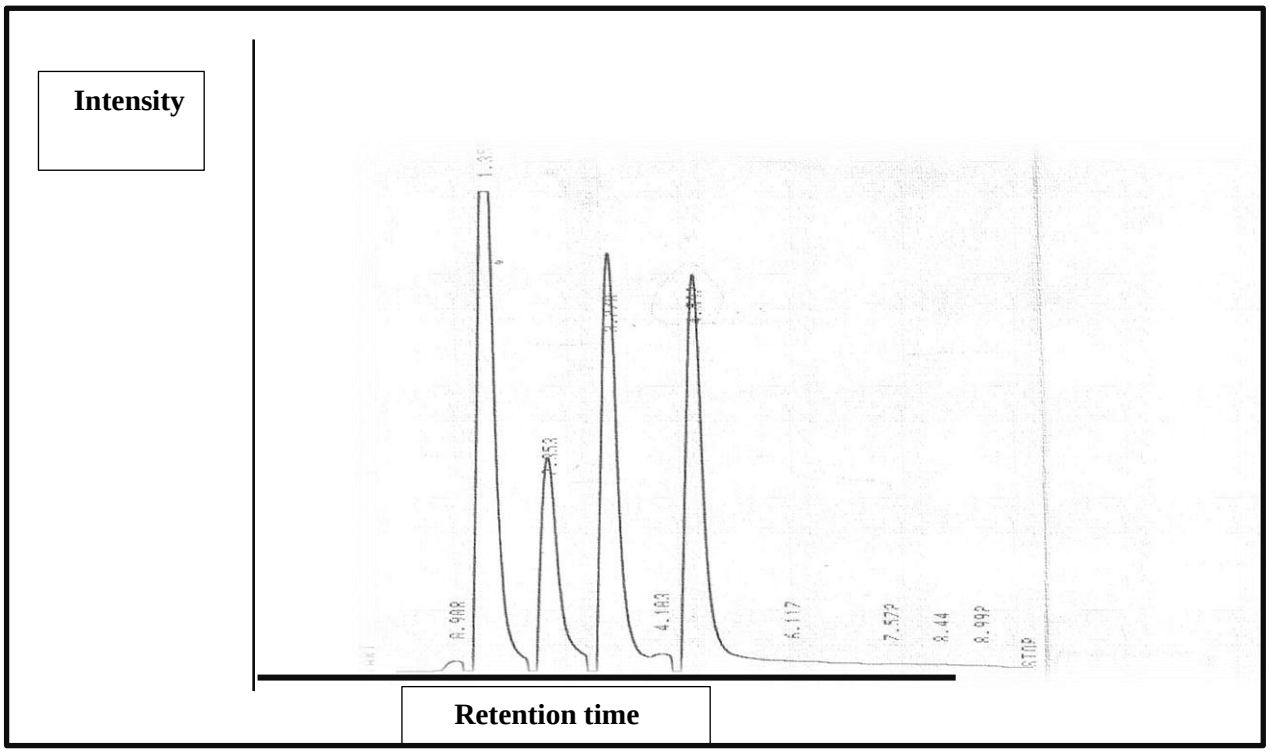
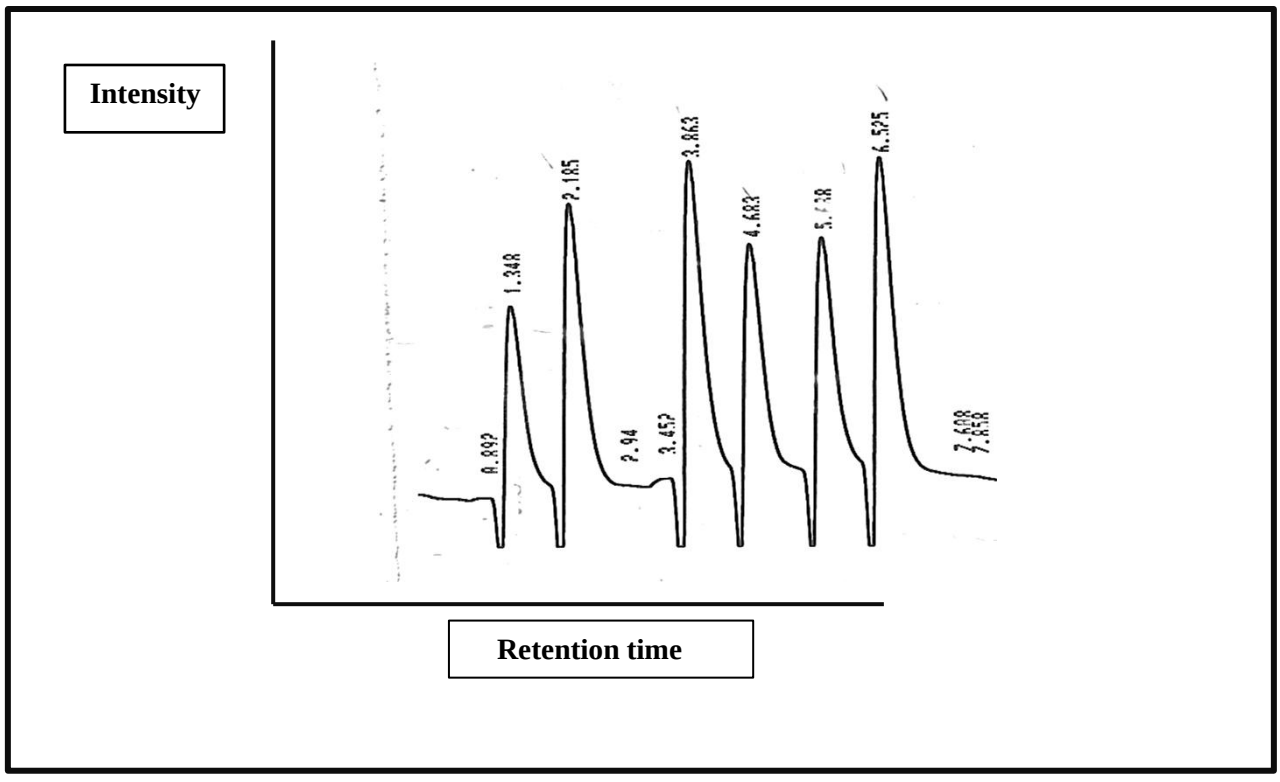
APPENDICES

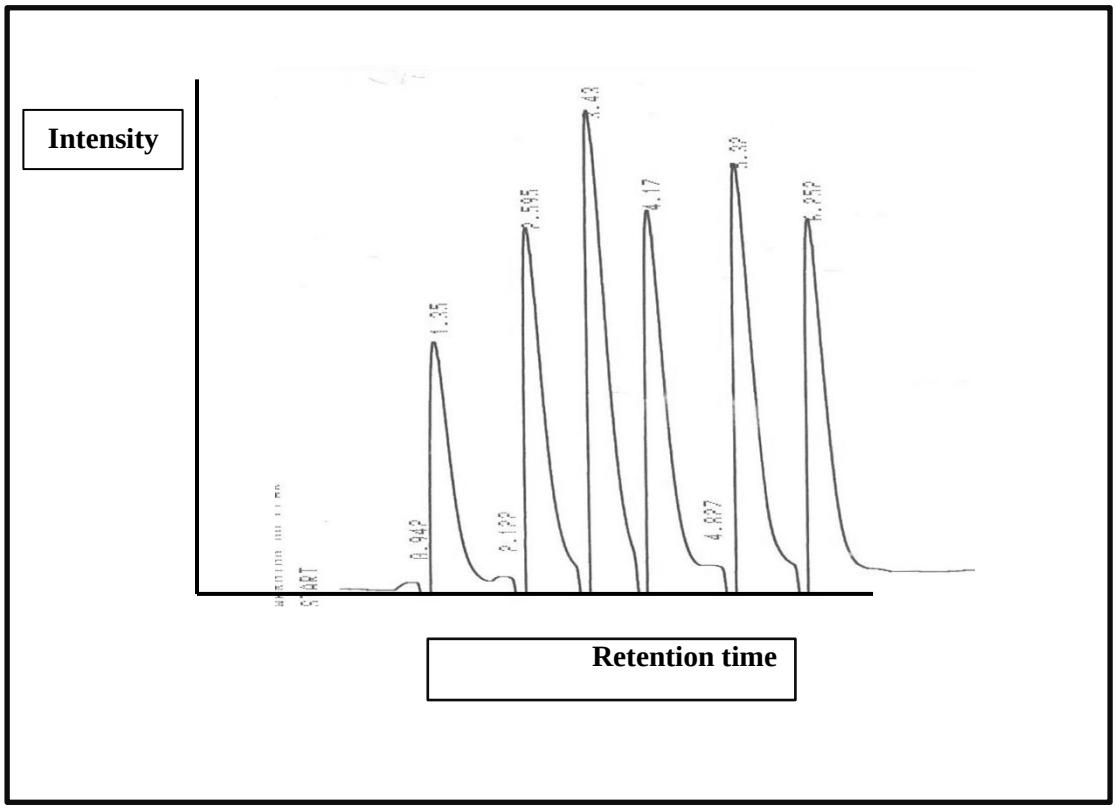
Appendix (1) HPLC analysis of the standard amino acid components, with flow rate 1 ml/min.



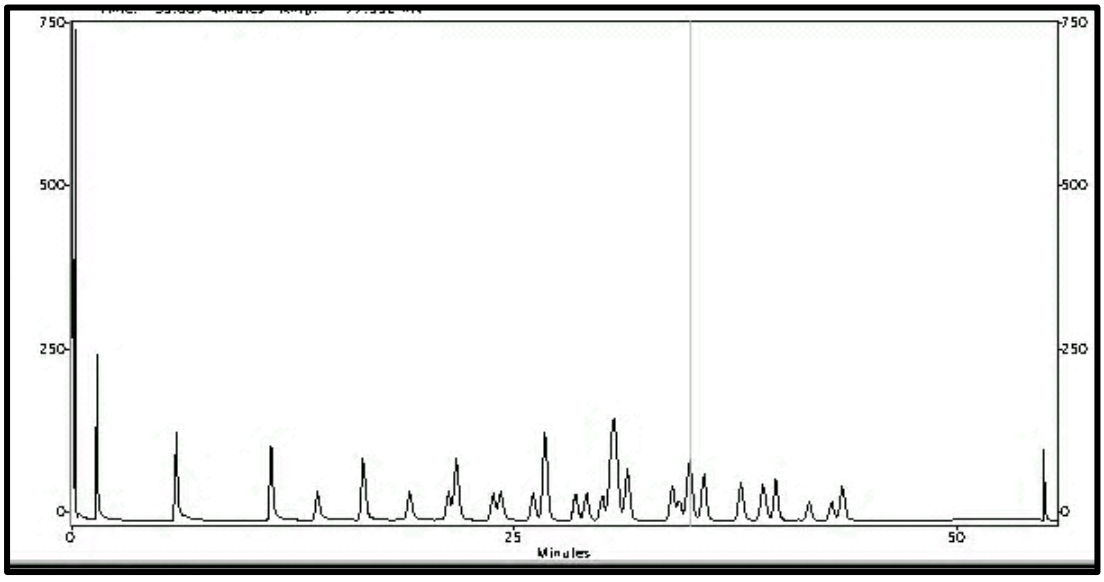
Appendix (2) HPLC analysis of the standard carbohydrate components, equipped with binary delivery pump model LC -10A.



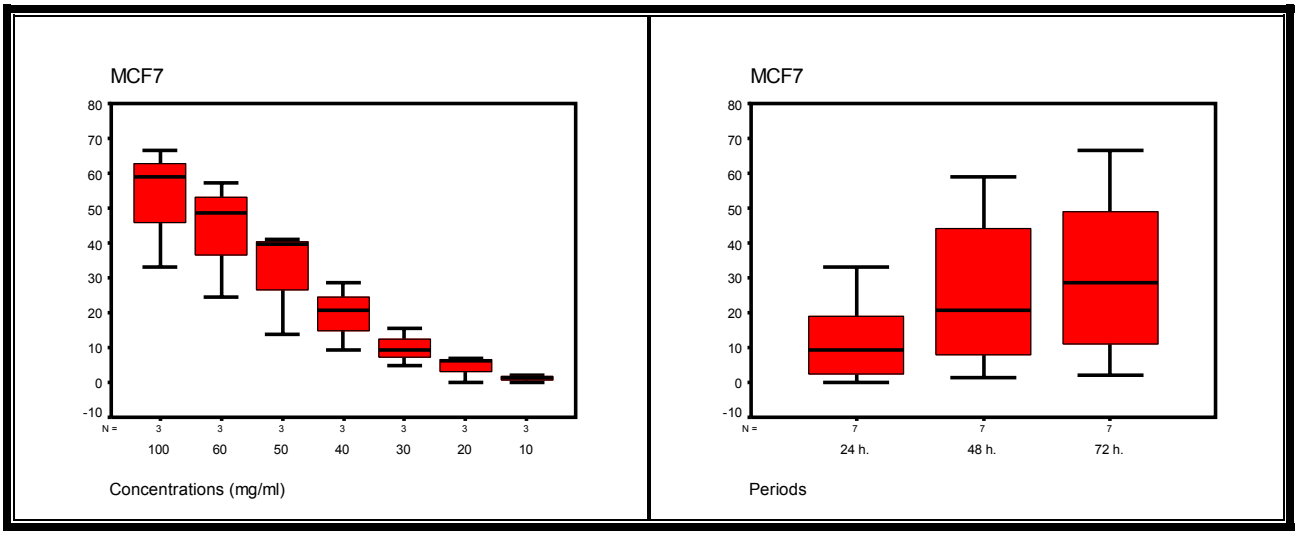
Appendix (3) HPLC analysis of standard fatty acid components, equipped with binary delivery pump model LC -10A shimadzu, the eluted peak was monitored by SPD 10A VP detector.



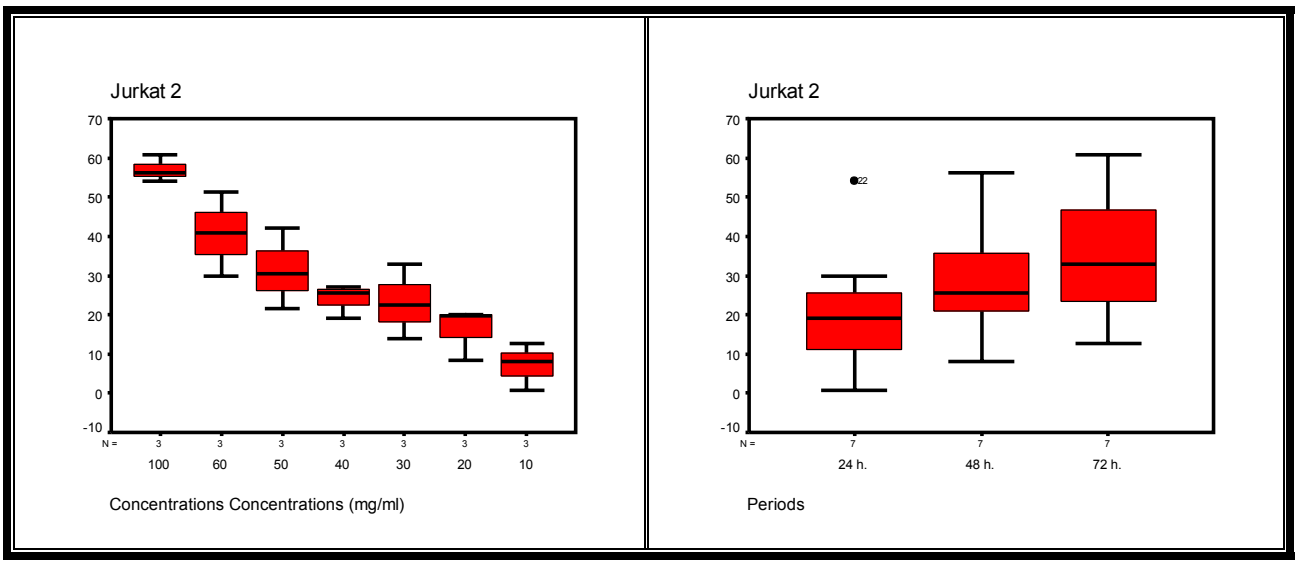
Appendix (4) Gas chromatography analysis of standard fatty acid methyl ester.



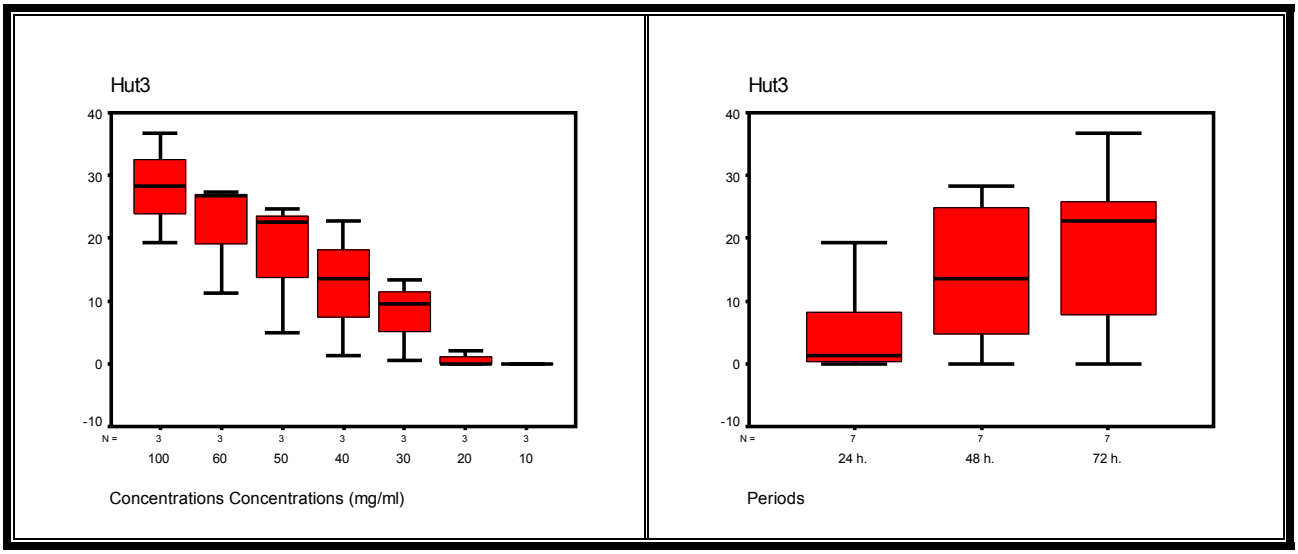
Appendix (5):Inhibition percent associated with MCF7 cells line in different concentrations and time periods.



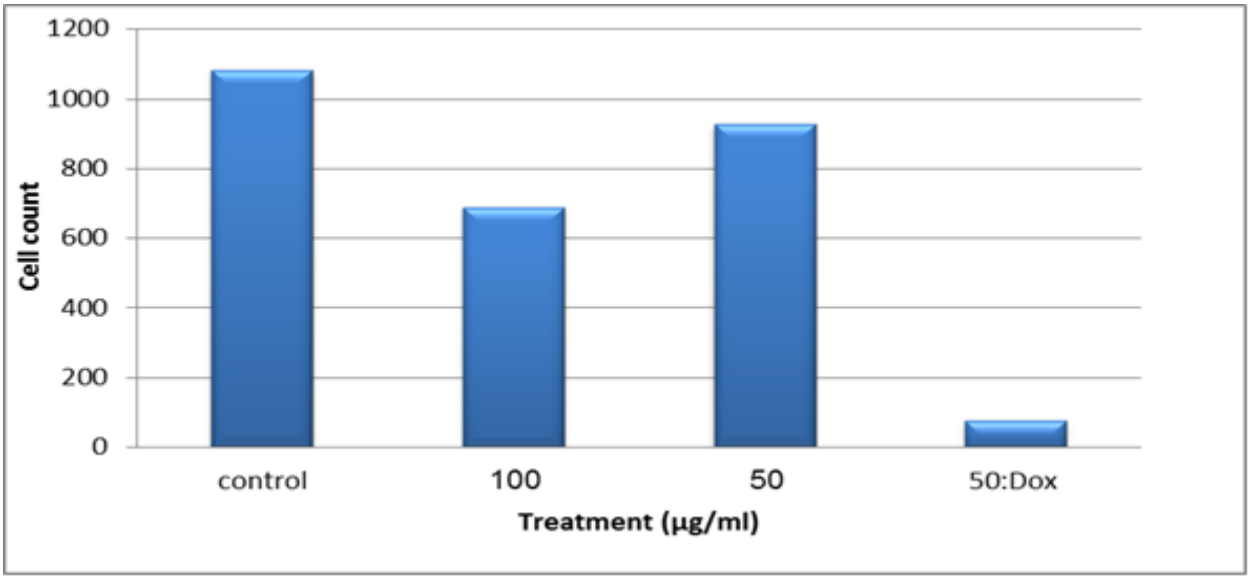
Appendix (6): Inhibition percent associated with Jurkat cells line in different concentrations and time periods**.**



Appendix (7): Inhibition percent associated with Hut78 cells line in different concentrations and time periods**.**

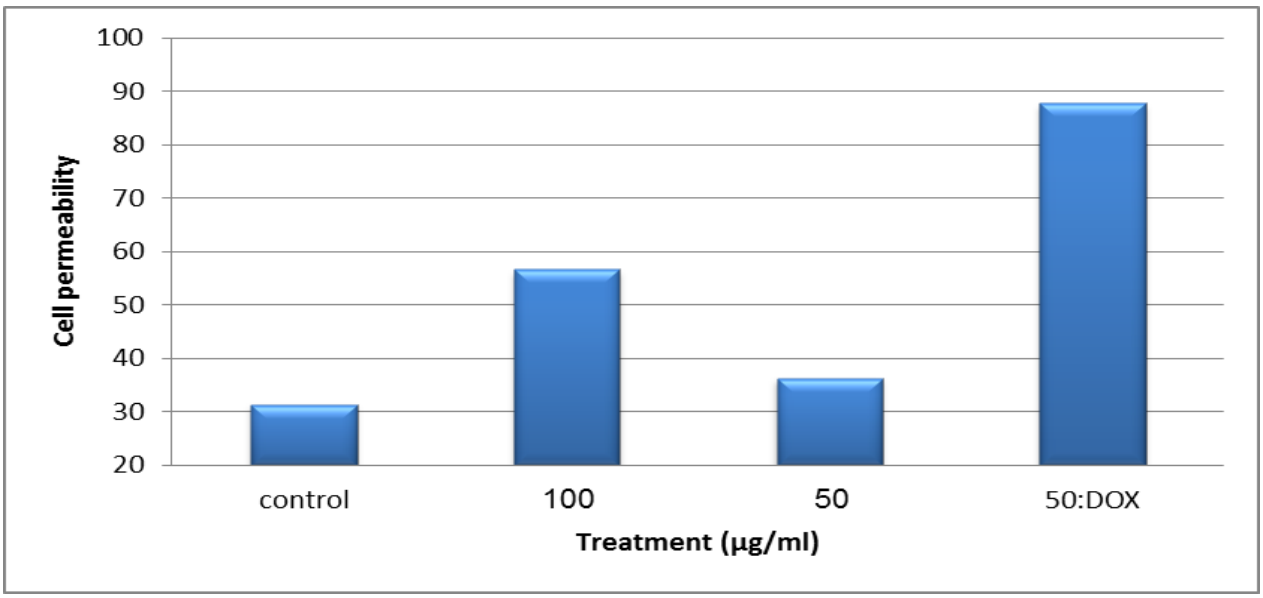


Appendix(8): Effect of different concentrations of purified biosurfactant on cell count of MCF7 cell line.



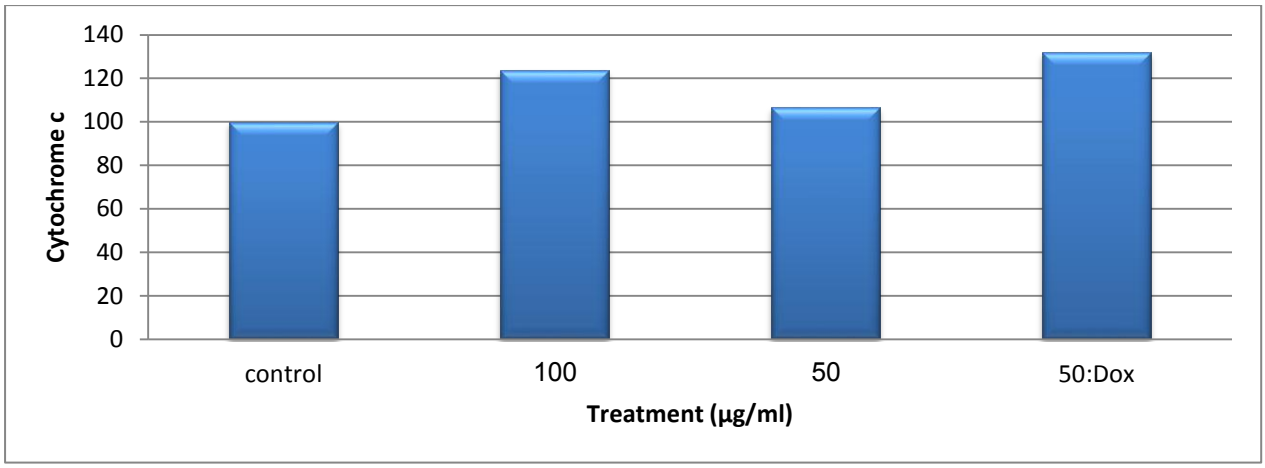
Dox:positive control.

Appendix(9): Effect of different concentrations of purified biosurfactant on cell permeability of MCF7 cell line.



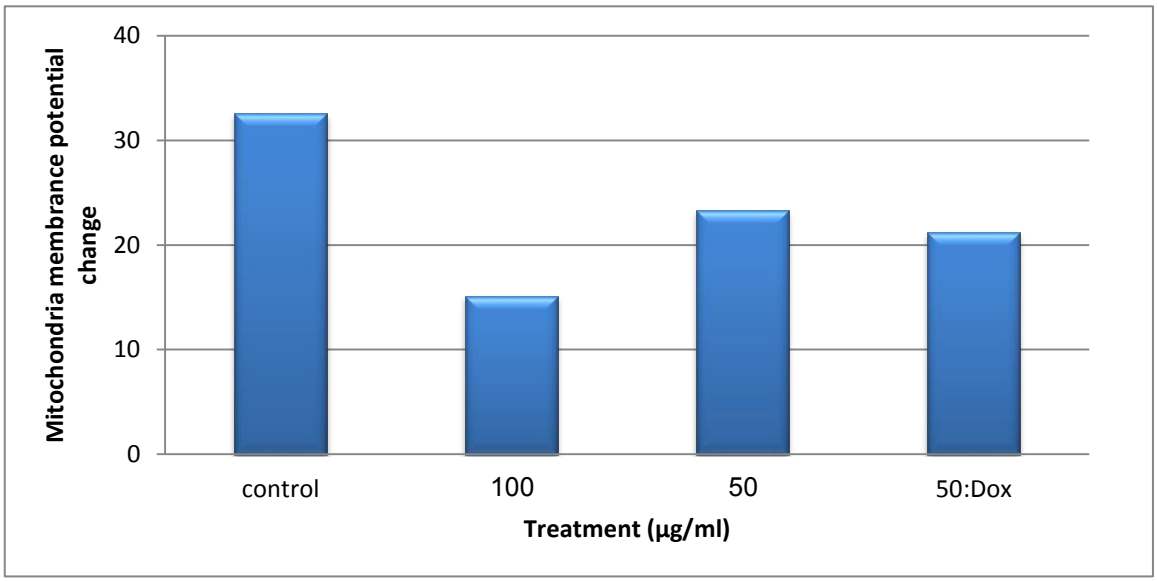
Dox:positive control.

Appendix(10): Effect of different concentrations of purified biosurfactant on cytochrome c of MCF7 cell line.



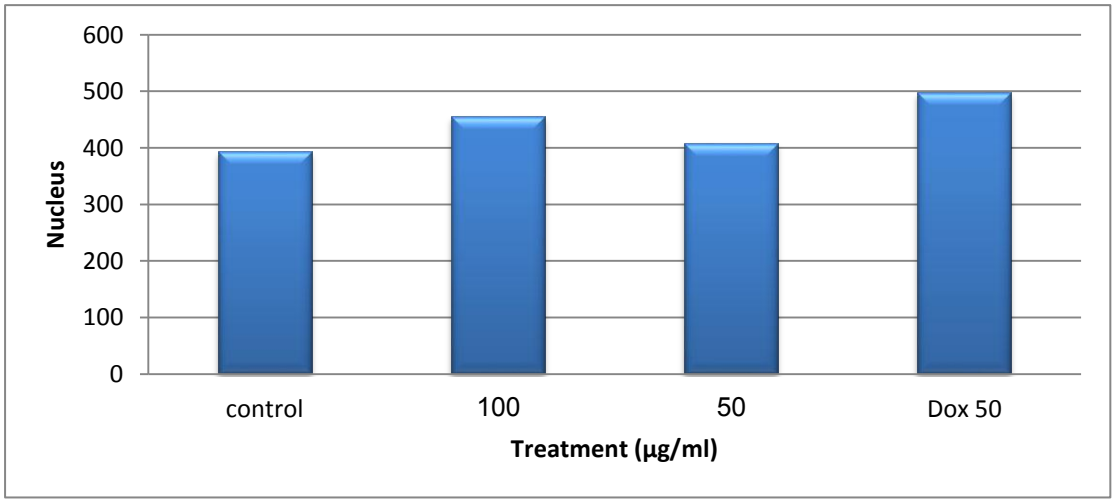
Dox:positive control.

Appendix(11):Effect of different concentrations of purified biosurfactant on mitochondrial membrane potential change of MCF7 cell line.



Dox:positive control.

Appedix(12): Effect of different concentrations of purified biosurfactant on nucleus intensity of MCF7 cell line.



Dox:positive control.

Appendix(13):Antibiotic sensitivity of the test bacterial isolates:

Bacteria	Antibiotic	Zone of inhibition
Staphylococcus aureus	Oxacillin-	22
	Cefoxitin(30µg)	21
	Ciprofloxacin (5µg)	21
	Clindamycin(2µg)	21
Streptococcus SPP.	Tetracycline(30µg)	23
	Erythromycin(15mg)	22
Enterobactericeae	Ceftazidime(30mg)	21
	Aztreonam(15µg)	21
Pseudomonas aeruginosa	Meropenem(10µg)	20
	Ceftazidime (30µg)	21

الخلاصه

العزلات البكتيرية العشرة التي استخدمت في هذه الدراسة لديها القدرة على الاستفادة من النفط الخام والمركبات الاروماتيه تم عزلها في دراسه سابقه واعتبرت كمجموعة جديدة من البكتيريا المحبه للحرارة المتطرفه و المحلله للهيدروكربونات الاروماتيه.

غربلت هذه العزلات للكشف عن قابليتها لانتاج المستحلبات اعتمادا على مؤشر الاستحلاب (E24٪), قياس الشد السطحي (ملي نيوتن / م) وفعاليه استحلاب (EA) باستخدام النفط الخام كمصدر الكربون والطاقه. أظهرت النتائج أن جميع العزلات كانت قادرة على انتاج المستحلب الحيوي وكان *Geobacillus thermoleovorans* Ir1(JQ912239) الأكثر كفاءة.

تم تحديد الظروف المثلى لإنتاج المستحلب الحيوي بفعل السلاله *G.thermoleovorans* Ir1 وقد اوضحت النتائج أن هذه الظروف هي: بتنمية هذه البكتيريا في وسط الأملاح المعدنية (pH7) الحاوي على النفط الخام (1٪) كمصدر وحيد للكربون وكلوريد الأمونيوم (0.3٪) كمصدر للنيتروجين،والحضن بدرجة حرارة 60 مئوية، مع التحريك (200 دورة في الدقيقة) لمدة 10أيام.

أستخلص المستحلب الحيوي باستخدام ثلاثة طرق. وأظهرت النتائج أن الاستخلاص بالأسيتون أعطى أفضل فعاليه للمستحلب الحيوي . تم تنقية المستحلب الحيوي باستخدام عمود هلام السيليكا ، وتم الحصول على ثلاثة قمم وأعطت نشاط استحلابي.

كشف التركيب الكيميائي للمستحلب الحيوي أنه يتكون من 37,7٪ دهون, 26,2٪ من الكربوهيدرات و 10,7٪ من البروتينات.

أخضع المستحلب الحيوي المنقى و/ أو المنقى جزئيا لFTIR، HPLC، GC وNMR لاستكمال الخواص الكيميائية.

وكشفت نتيجة FTIR أن المستحلب الحيوي يحتوي على الدهون, الكربوهيدرات والبروتينات. في حين أشارت نتائج ال HPLC أن مكونات الأحماض الدهنية للمستحلب الحيوي كانت حمض البالمتيك وحمض استريك وحمض الاوليك، أما الكربوهيدرات فكانت هي الزايلوز والمانوز والمالتوز، في حين أن الأحماض الأمينية هي حمض الأسبارتيك ,حمض الجلوتاميك و الكلوتامين.

وأظهر تحليل GC ان الجزء الرئيسي (الدهون) من المستحلب الحيوي يتكون من نسبة عالية من حامض البالمتيك ميثيل استر (C16:0)، حمض استريك (C18:0) وحمض الأوليك (C18:1n9C)، ونسبة أقل من الأحماض الدهنية الاخرى.

أظهر طيف HNMR أن المستحلب الحيوي المنقى جزئيا يتكون من مركبين: المركب الرئيسي هو الدهون الثلاثية والثاني ربما يعزى لحمض دهني . أما نتائج المستحلب الحيوي المنقى، القمم الثلاث التي تم الحصول عليها من عمود هلام السيليكا، فقد تبين ان جميعها تحتوي على الدهون الثلاثية فقط. قد تكون هذه الدراسة تعد الأولى من نوعها التي يتم تنفيذها في العراق والتي تلقي الضوء على قدرة البكتريا الالفه للحرارة لانتاج المستحلب الحيوي.

لأجل تحديد الفعالية المضادة للأورام للمستحلب الحيوي المنقى، تم معاملة ثلاثة خطوط خلايا سرطانية (MCF7, Hut78 وJurkat) بتراكيز مختلفة من المستحلب الحيوي. وأظهرت النتائج أن التأثير كان يعتمد على نوع خط الخلايا السرطانية، و تركيز المستحلب الحيوي ومدة التعرض. كان اعلى تأثير على خط الخلايا السرطانية MCF7 (الأكثر حساسية) اذ ادى التركيز 100 ميكروغرام / مل الى تثبيط 66.55٪ خلال 72ساعه (فترة الحضانة).

أخضع خط الخلايا السرطانية MCF7 لدراسة السمية الخلوية. وأظهرت النتائج أن 50 و100مايكروكرام / مل من المستحلب الحيوي المنقى سببت انخفاض كبير في عدد الخلايا، مع زيادة كبيرة في نفاذية الخلية، وخروج السيتوكروم C من الميتوكوندريا، وكثافة النواة واختزال كبير في كفاءة غشاء الميتوكوندريا.

أختبرت الفعالية الضد ميكروبية للمستحلب الحيوي المنقى تجاه بعض الأحياء المجهرية، وتبين أن المستحلب الحيوي قد ثبط نمو البكتيريا (*Staphylococcus aureus, Streptococcus sp., Pseudomonas aeruginosa*) والفطريات (*Candida albicans*).

درست بعض الخصائص الفيزيائية والكيميائية للمستحلب الحيوي. ووجد أن المستحلب الحيوي كان فعالا في مدى واسع من قيم الحموضة، متحمل لدرجات الحرارة العالية وثابت في مدى واسع من تراكيز الأملاح NaCl , $CaCl_2$ و KCL.

وفي محاولة لتحديد الجين (الجينات) المسؤولة عن انتاج المستحلب الحيوي في بكتريا *G. thermoleovorans* Ir1, استخلص الدنا الجيني من هذه البكتيريا ومن بكتريا

B. subtilis وتم تضخيمه باستخدام البادئ الخاص لجين *sfp* (المشفر لأنتاج المستحلب الحيوي في *Bacillus* spp.). وأظهرت النتائج الحصول على ناتج تضخيم (675 زوج قاعدي) مع بكتريا *B.subtilis* بينما لم يتم الحصول عليه مع بكتريا *G. thermoleovorans* Ir1

وهذا يعني ان هذا الجين ليس مسؤول عن إنتاج المستحلب الحيوي في *G.thermoleovorans* .

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جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعه النهرين

كلية العلوم

قسم التقانه الاحيائيه

انتاج، تنقيه وتوصيف المستحلب الحيوي من بكتريا *Geobacillus thermoleovorans* ودراسه فعاليته المضادة للاحياء المجهريه والاورام

أطروحة

مقدمة الى مجلس كليه العلوم/ جامعة النهرين كجزء من من متطلبات نيل درجة الدكتوراه

فلسفه في علوم التقانة الأحيائية

من قبل

هبه منصور ناصر

بكالوريوس علوم, تقانه أحيائيه, جامعه النهرين 2002

ماجستير علوم , تقانه أحيائيه, جامعه النهرين 2005

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