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Biosurfactant Production by *Bacillus cereus*, B_7 from Lubricant Oil Waste

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Abstract: ***The present study focused on studying the production of a biosurfactant (BS) from microbial origin. For this respect 12 bacteria and 6 fungi were isolated from different polluted sites in Egypt. Bacillus cereus B7 isolated from kitchen drainage tube gave the highest BS production using oil spreading and emulsification assay techniques. The maximum BS productivity by Bacillus cereus B7 was obtained at the following optimal conditions:, 30˚C, pH 5, 30h incubation at static condition, inoculum size of 19.36×10^{6}cfu; 0.5 mllubricant oil waste/25 ml medium(2%, V/V)as the sole C-source, $NaNO_3$as N-source, Dl-α-Amino-n-butyric acid and 200ppm $LiCl_2$,none of the tested C-sources and vitamins exhibited further productivity. The BS extracted with $CHCl_3$:C_2H_5OH in a ratio 2:1. The organic phase was evaporated in a rotary evaporator to give a white powder. The produced BS exhibited antimicrobial activity against Candida albicans, Staphylococcus aureus and Pseudomonas aeruginosa with MIC 5, 5 and 2.5 µl, respectively. The BS gave a good potential application as emulsifier.***

Keywords: biosurfactant, *Bacillus cereus*, lubricant oil, extraction, emulsification

1. Introduction

Bioremediation processes are negatively affected by the low aqueous solubility of some contaminants; therefore their bioavailability may be enhanced by the addition of surfactants. These compounds are organic molecules that can be chemically and biologically produced **(Bustamante *et al.*, 2012)**.

Surfactants are one of the most important organic compounds widely used in almost all industries. As a result, every year millions of tons of surfactants (equivalent to billions of dollars) are commercialized in the world. The major consumers of this market are North America with 35%, followed by Asia–Pacific with 29%; and Western Europe with 23% **(Cirelli*et al.*, 2008).**

Surfactants are amphipathic molecules which reduce the surface tension between water and hydrocarbon interfaces. Most of the surfactants available are petroleum derivatives which are highly toxic and non-degradable **(Seghal*et al.*, 2009)**.

In comparison with synthetic surfactants, biosurfactants have better surface activity, lower toxicity, they can bind heavy metals, have higher biodegradability, selectivity and biological activity, they are produced from renewable resources, can be produced through fermentation and can be reused by regeneration (**Pekdemir*et al.*, 2005; Van Bogaert*et al.*, 2007 &Vatsa*et al.*, 2010).**

The other advantages of microbial surfactants are eco-friendly, high foaming ability and efficiency at extreme temperatures, pH and salt concentrations **(Thavasi*et al.*, 2008 &Sachdev and Cameotra, 2013)**.

Biosurfactants are surfactants derived from several types of microorganisms such as bacteria, yeasts and fungi as membrane components or secondary metabolites **(Maier and Chavez, 2000 & Kitamoto*et al.*, 2002)**.

Biosurfactant produced on microbial cell surface or excreted extracellularly, and contain hydrophobic and hydrophilic moieties **(Fiechter, 1992; Zajic and Stiffens, 1994; Makker and Cameotra, 1998 & Lin *et al.*, 1998).**

Microbial surfactants are complex molecules comprising different structures that include peptides, glycolipids, glycopeptides, and phospholipids **(Mercade and Manresa, 1994)**.

Biosurfactants attracted considerable interest both in theoretical and practical aspects because of their wide usage in oil-producing and mineral resource industry, chemical, pharmaceutical and food-processing industry, agriculture, and for purification of the environment from carbohydrates, heavy metals and other pollutants **(Gogotov*et al.*, 2002 &Shekhara*et al.*, 2014)**.

Biosurfactants have a wide range of potential applications in the medical field. They are useful as antibacterial, antifungal and antiviral agents, and they also have the potential for use as adhesive agents and in vaccines and gene therapy **(Rodrigues *et al.*, 2006a)**.

Biosurfactants can be produced from cheap raw materials which are available in large quantities. The carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used separately or in combination with each other **(Kosaric, 1992**). Oil pollution is an environmental problem of increasing importance. Constantly growing demand for oil and oil products is one of main causes of soil pollution **(Song *et al.*, 1990)**.

The process of oil biodegradation is limited by low solubility of liquids in soil **(Beck and Jones, 1995 &Wurdermann*et al.*, 1995).** Surfactants reduce a surface and interfacial tension and, therefore, increase solubility of

no soluble organic compounds in water and enhance contact of microorganisms with the pollutants **(Lakshmipathy*et al.*, 2010).**

Biosurfactants enhance the emulsification of hydrocarbons, have the potential to solubilize hydrocarbon contaminants and increase their availability for microbial degradation. Biological treatment may efficiently destroy pollutants, while being biodegradable themselves. (**Muyzer*et al.*, 1993** &**Koeuth*et al.*, 1995).**

Aim of the study: In the present investigation a cost-effective production of biosurfactant from lubricant oil waste was carried out. This biosurfactant has the possibility to use in different applications such as antimicrobial activity and emulsification ability of the environmental pollutants.

2. Materials & Methods

2.1 Substrate Used

Lubricant oil waste from Abesco Factory, Industrial District 5, Jeddah, KSA was used as the sole carbon source during the research to study microbial isolates ability for biosurfactant production.

2.2 Samples for isolation of biosurfactant producers

Samples collected from different locations contaminated by different kinds of oils such as, soil samples contaminated with lubricant oil from gas station, samples from wall of drainage tube of kitchen and bathroom, also, waste samples from gas cook tops of kitchen stove. All samples were collected in sterile polythene bags, transported to the laboratory aseptically and refrigerated until isolation procedures.

2.3. Media Used

2.3.1. Nutrient agar medium (Atlas, 2005) was used in purification and maintenance of bacterial isolates.

2.3.2. Czapek'sDox Agar medium (Atlas, 2005) was used in purification and maintenance of fungal isolates.

2.3.3. Modified Czapek'sDox Lubricant Oil Waste (MCZ-LOW) medium
The carbon source (sucrose) in Czapek'sDoxagar medium was replaced by 1% Lubricant Oil Waste as a sole carbon source. This medium can be used in biosurfactant production by means of microbial isolate.

2.3.4. Sabouraud Agar (Atlas, 2005) was used for the growth of *Candida albicans.*

2.3.5. Blood-agar (Cheesbrough, 2000) is a qualitative assay to determine biosurfactant producer. Only those isolates which showed β-hemolysis were considered to be the potential biosurfactant producing microbes

2.3.6. Medium for lipolytic assay activity (Elwan*et al.*, 1977). The medium was composed of (g/l): Tributyrin, 2 ml; Gum Arabic, 4; Agar, 15; Phosphate buffer at pH 4.6, up to 1L**.** Lipolytic activity was detected by clearing zones around the hole in comparison to the turbid background of the assay plates).

2.3.7. Mueller Hinton agar (Atlas, 2005) was used for antimicrobial assay.

2.4 Assay of BS Productivity

2.4.1. Oil spreading assay method (Morikawa*et al.*, 1993)
For this assay 20µl of crude oil (olive oil) is added to 50 ml distilled water in 15 cm Petri-dish. Only10 µl of culture supernatant was gently placed on the center of oil layer. After 30 seconds if biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity.

2.4.2. Emulsification assay method (Cooper and Goldenberg, 1987)
The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples were determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, the cell-free broth obtained after incubation period and centrifuge at 4000 rpm for 4 min, after that vortex the mixture at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm). The percentage of emulsification index calculated by using the following equation:

$$E24 = \frac{\text{Height of emulsion formed} \times 100}{\textit{Total heigh of soltion}}$$

2.5 Studying some parameters optimizing biosurfactant production

The following parameters were carried out to optimize the productivity of the biosurfactant substance. After determining each parameter, the best result was applied in the subsequent parameters. Three flasks were used for each particular parameter.

2.5.1. Effect of different temperatures
Different incubation temperatures 10, 20, 30, 37, and 50˚C, were selected and carried out. After 4 days, flasks were centrifuged, and assay was carried out as previously mentioned.

2.5.2. Effect of static and shaken conditions
Flasks were injected with 1 ml of bacterial suspension and then incubated for 4 days in incubator shaker at 0, 100 and 200 rpm for 4 days at the best temperature. At the end of incubation period, flasks contents were centrifuged, and then assay was carried out.

2.5.3. Effect of different incubation periods
The incubation occurred at different time intervals viz. 0, 2, 4, 8, 12, 16, 20, 24, 30, 36, 42, 48, and 72 hour at 30˚C and static condition. BS productivity was assayed as usual.

2.5.4. Effect of different inoculum sizes
Different inoculum sizes viz. 0.5, 1.0, 2.0, 4.0, and 8.0 ml were introduced into sterilized 100 ml capacity conical flasks containing production medium to make a total volume of 25ml. Haemocytometer was used to calculate the number of inoculated spores/ml. Incubation, filtration and BS assay was carried out.

2.5.5. Effect of different oil concentrations
During the preparation of the medium the oil is add in different conc. viz. 0.5, 1.0, 2.0, 4.0, and 8.0 (ml/25ml, v/v). Then the flasks were sterilized, inoculated and incubated. BS production was assayed.

2.5.6. Effect of different pH's
Initial pH value of the production medium was adjusted to cover a range of 2 to 9 before sterilization by means of 1N HCl and 1N NaOH. Inoculation was carried out followed by incubation under the best parameters determined before. Then BS assay was carried out.

2.6 Extraction of the BS

The filtrate was centrifuged at 4°C and 8500 rpm for 20 min in order to remove the microbial cells. The obtained supernatant was treated by acidification to pH 2.0 using a 6M HCl, and the acidified supernatant was left overnight at 4 °C for the complete precipitation of the biosurfactants **(Yakimov*et al.*, 1996).** After centrifugation, the precipitate was dissolved in a 0.1M NaHCO3 solution, followed by the biosurfactant extraction step with a solvent having a 2:1 CHCl3–C2H5OH ratio at room temperature (25–27 °C) (**Zhang and Miller, 1992; Kim *et al.*, 2000 &Samadi*et al.*, 2007)**. The organic phase was transferred to a round bottom flask connected to a rotary evaporator in order to remove the solvent at 40°C.

2.7. Studying some applications of the produced BS

2.7.1. Antimicrobial activity of the produced BS
The purified BS was tested for its antimicrobial activity against certain resistant pathogenic isolats such as *Candida albicans, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Agar well diffusion method was used in this respect **(Betina, 1957).**

2.7.2. Studying the emulsification ability of the separated biosurfactant
In this respect, 1 ml of oil waste was added into 3 ml of water in test tube, then the biosurfactant obtained from *B. cereus* was introduced into the tube (different concentrations of the biosurfactant were used in this respect separately, 100, 200 & 300 µl). The tubes were vortexed at high speed for 2 min and allowed to stand for 24h. The emulsification ability of the biosurfactant was noticed in comparison to the control which contains the same constituents except the biosurfactant under study **(Cooper and Goldenberg, 1987).**

2.7.3. Oil spreading ability
Concerning oil spreading ability of the biosurfactant, 1000 µl of crude oil is added to only 200µl of purified biosurfactant produced by *B. cereus* , shaked well and then added on 3ml distilled water in 15 cm Petri-dish. After 30 seconds, the displacement or dispersion ability of the biosurfactant was noticed **(Morikawa*et al.*, 1993).**

2.8. Statistical Analysis

All analyses were performed in triplicates and expressed as mean ± standard deviation (SD). Excel was used for the statistical evaluation and graphical representations of the present study. Also, the standard error was calculated. Statistical analysis was performed using ANOVA, one way by Minitab (Version 11) software.

3. Results & Discussion

I. Isolation of some microorganisms capable of biosurfactant production: Oil pollution is an environmental problem because of growing demand for oil and oil products **(Song *et al.*, 1990)**.

The process of oil biodegradation is limited by low solubility of liquids in soil **(Beck and Jones, 1995 &Wurdermann*et al.*, 1995).** To increase the bioavailability, the surfactants are used to reduce a surface and interfacial tension and, therefore, increase solubility of insoluble organic compounds and enhance contact of microorganisms with the pollutants **(Mihelcic *et al.*, 1993 &Lakshmipathy*et al.*, 2010).**

Several microorganisms are known to synthesize surface-active agents; most of them are bacteria and yeasts. These chemicals are apparently synthesized to emulsify the hydrocarbon substrate and facilitate its transport into the cells (**Muyzer*et al.*, 1993; Ochsneret *al.*, 1994& 1996;** &**Koeuthet *al.*, 1995).**

Therefore, this work is aiming at isolation of biosurfactant producing microbes from our local habitats to control the problem of oil waste from one hand and to produce a commercially significant biosurfactant from the other hand. In the present investigation, lubricant oil waste which represent as an environmental pollutant was used as a sole carbon source for biosurfactant production. In this respect twelve bacteria and six fungi were isolated from different localities contaminated by different kinds of oil waste. All microbial isolates were assayed for their biosurfactant "BS" productivity by oil spreading assay technique **(Rodrigues *et al.*, 2006b)**. From all isolates, only 5 bacteria and four fungi were selected as the most potent microbial isolates which exhibited the highest biosurfactant production and subjected for second screening which was carried out by means of both Oil Spreading and Emulsification Assay Technique.

Bacterial isolate B_7 exhibited the highest BS productivity among all the tested isolates under study it gave Fig. (1). so, it was chosen as the most potent microbial isolate for biosufactant production and was subjected for further investigations.

It was subjected for identification on the basis of Analytical Profile index (API) technique (**using API 50-CHB kits (bioMerieux API 50 CHB/E, 07964F-FR-2005/12).**

Also morphological, physiological, and biochemical characteristics were studied according to Microbiological Methods 6th **(Collins *et al.*, 1989),** Bergey's Manual of Determinative Bacteriology **(Holt *et al.*, 1994)** and The Genus *Bacillus* **(Gordon *et al.*, 1973).**The results were compared with reference isolate.

Data represented in table (1) indicated that, isolate B7 is belonging to Gram +ve bacteria, motile, bacilli in shape, and has the ability to form endospores. Moreover, it is facultative anaerobes.

Figure 1: Emusification assay technique of the most potent bacterial isolate B7

Table 1: Showing A Summary Of The Morphological, Physiological And Biochemical Properties Of The Most Potent Bacterial Isolate B7.

Test	*Reference Isolate B. cereus ATCC 11778*	*Isolate B7*
Gram stain	+	+
Shape	Rod	Rod
KOH		-
Spore forming	Central endospore forming	(endospore forming)
Catalase test	+	+
Facultative anaerobic	+	+
Vogesproskour	+	+
Indole		-
Citrate utilization	+	+
Gelatinase enzyme	+	+
Amylase enzyme	+	+
Acid		
D(-) Glucose	+	+
L(+) Arabinose	+	-
Mannitol	-	-
Xylose	-	-
Growth at pH 6.8	+	+
Growth at ph 5.7	+	+
Gas from glucose fermentation	-	-
Growth at 30 ˚C	+	+
Growth at 40˚C	+	+
Growth at 50˚C	-	-
Growth at 55˚C	-	-
Growth at 65˚C	-	-
Nitrate to nitrite	+	+
Motility	+	+
Haemolytic activity	+	β-haemolysis

In view of all the previously mentioned characteristics and according to the international keys this isolate was suggested to be belongs to ***Bacillus cereus*. So,** it can be denoted as ***Bacillus cereus*, B_7.**

The *Bacillus cereus*, B_7 showed a complete hydrolysis of blood, so it is beta hemolytic organism. The β-haemolysis was shown in Fig. (2) this is based on the fact that surfactants interact strongly with cellular membranes and proteins**.** Exotoxins called hemolysins cause lysis of the red blood cells **(Pape and Hoppe, 1988)**.Blood agar lysis was used to screen for biosurfactant production. **Mulligan *et al.* (1984)** had recommended this method as a preliminary screening method. In addition, the hemolytic assay was a simple, fast and low-cost method for the screening of biosurfactant producers on solid medium. Many researchers have used this technique to screen for biosurfactant production by new isolates **(Carrillo *et al.*, 1996; Yonebayashi*et al.*, 2000)**

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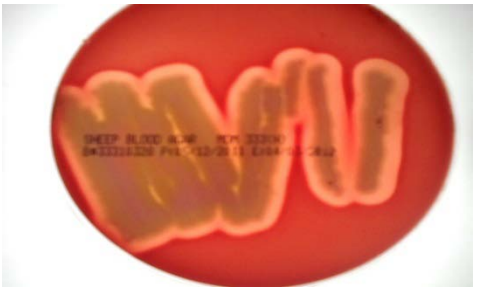


Figure 2: β-haemolysis of *Bacillus cereus*, B7 on blood agar medium

Bacillus cereus, B_7 was investigated its lipolytic activity. Data represented in Fig. (3) indicated that, *Bacillus cereus*, B_7has a lipolytic activity, it gave 14.7 mm of clearing zone.

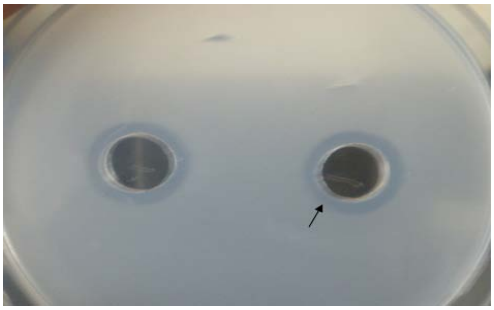


Plate 3: lipolytic activity of *Bacillus cereus*, B_7

Few studies have reported on biosurfactant-producing *B. cereus* strains, for example, **Cooper and Goldenberg (1987)** detected a polysaccharide and a monoglyceridebiosurfactantin *B. cereus* IAF 346. **Hsueh*et al.* (2006)** found that**,** biosurfactant production by *B. cereus* was physiologically related to the formation of a biofilm under nutrient-limiting conditions. In the same accordance, **Vela´zquez-Aradillas (2011)** isolated a Gram-positive bacterium produced a biosurfactant from low-quality coffee beans, this isolate was found to be *Bacillus cereus* QD232.

II. Parameters optimizing biosurfactant production by *Bacillus cereus* B7

Desai and Banat (1997) elucidated that, environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability affect biosurfactant production through their effects on cellular growth or activity.

Several studies have aimed to optimize the biosurfactant production process by changing the variables that influence

the type and amount of biosurfactant produced by a microorganism. Important variables are carbon and nitrogen sources **(Santos *et al.*, 2002)**, potential nutrient limitations and other physical and chemical parameters such as oxygen **(Kronembergeret *al.*, 2008)**, temperature and pH **(Mukherjee *et al.*, 2006).**

Concerning the effect of different incubation temperatures on the biosurfactant production by *B. cereus*, 30 ºC was chosen as the best temperature for biosurfactant productivity (Fig. 4).

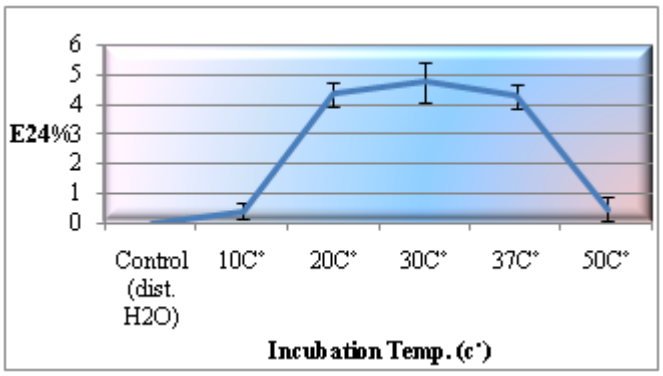


Figure 4: Relation of different incubation temperatures on biosurfactant production by *Bacillus cereus*.

In the same accordance, **Thaniyavarnet *al.* (2008)** reported that, *Pichiaanomala*PY1 biosurfactant produced at an optimum temperature 30°C. Also**, Tabatabaeet *al.* (2005**) indicated that, the best temperature for biosurfactant production by *Bacillus* spp. No4 was between 30- 40°C.

However, **Abu-Ruwaidaet *al.* (1991 b)** found the optimum biosurfactant production of *Rhodococcus*spp. strain ST-5 was at 37ºC.

Concerning the effect of shaking condition at different rpm on biosurfactant productivity by *Bacillus cereus*, B7 agitation was found greatly inhibited the biosurfactant production specifically 200 rpm as shown in Fig.(5). Surface growth showed the maximum biosurfactant productivity comparing to the submerged growth. This may be due to the facultative anaerobic nature of the organism.

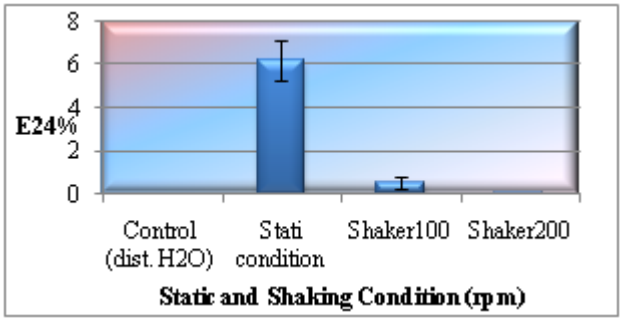


Figure 5: Relation of static and shaking conditions on biosurfactant production by *Bacillus cereus*.

In the same accordance, an increase in agitation speed results in the reduction of biosurfactant yield due to the effect of shear in *Nocardiaerythropolis* **(Margaritiset *al.*, 1979).** On the other hand, **Sarin and Sarin (2008)** indicated that, the production of the biosurfactant by *Bacillus cereus* increase as a result of increasing agitation up to 250 rpm.

In the present investigation, the amount of biosurfactant production increase by increasing incubation period up to 30h, but after that and up to 96h the second phase of biosurfactant production was observed. In conclusion, 30h was the optimum for biosurfactant production by *Bacillus cereus*, B7 as shown in Fig. (6). This result indicated that, the biosurfactant of *B.cereus*, B7 is produced at the end of the exponential phase as a primary metabolite and still produced until 96h.

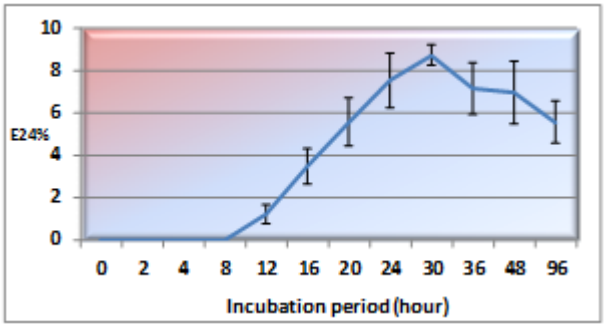


Figure 6: Relation of different incubation periods on biosurfactant production by *Bacillus cereus*.

In the same accordance, **Tabatabaeet *al.* (2005)** demonstrated that, after 14-36 h of growth of *Bacillus* spp. No4, the surfactant concentration started to increase, reaching its maximum after about 36 h. They reported that, the maximum production of biosurfactant at the exponential growth phase, suggesting that the biosurfactant is produced as a primary metabolite accompanying cellular biomass formation. Similar result obtained by **Abu-Ruwaidaet *al.* (1991 a)** for *Rhodococcus*, strain ST-5. But **Cameotra and Makkar (1998)** reported that**,** most of the biosurfactants are secondary metabolites, which are released into the culture medium at the stationary phase. But some of the biosurfactants are also produced throughout the exponential phase.

In addition, many authors indicated that, rhamnolipidbiosurfactant is a secondary metabolite and its production coincides with the stationary growth phase **(Venkata-ramana & Karanth, 1989; Dézielet *al.*, 1999& Santa Anna *et al.*, 2001).**

Data illustrated in Fig. (7) showed the effect of different inocula size to biosurfactant productivity produced by *B.cereus*, B7, it was declared that, by increasing the inoculums size up to 4000 µl (contains 19.36×10^6 CFU/ml) biosurfactant productivity was increased by 14.77%**. Suwansukho, *et al.* (2008)** indicated that, the highest production of the biosurfactant from *B. subtilis*MUV4 with an inoculum of ($4.6x10^6$ CFU/ml) at the concentration of 10% (v/v).

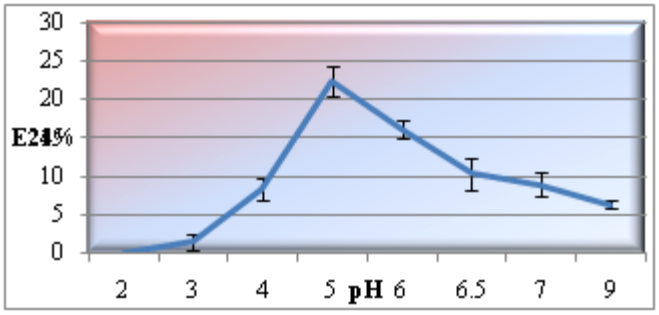


Figure 7: Relation of different inoculum sizes on biosurfactant production by *Bacillus cereus*.

In the present investigation, lubricant oil waste was used as the sole carbon source for the biosurfactant production from *Bacillus cereus*, B7. This is in full agreement with **Henkel *et al.* (2012)** who elucidated that, the use of inexpensive substrates, such as crude or waste materials, dramatically affects the production costs of biosurfactants. The choice of inexpensive raw materials is important to the overall economy of the process because they account for a large quote of the final product cost (about 50%) and also reduce, or eliminate, the expenses with wastes treatment (**Makkar and Cameotra, 1999).**

The effect of different lubricant oil concentrations on BS production by *Bacillus cereus*, B7was investigated and illustrated in Fig. (8). By increasing lubricant oil waste concentration up to 0.5 ml oil/25 ml medium (2%, v/v), the biosurfactant productivity increased up to 11.31%. Any further increase in oil concentration resulted in biosurfactant productivity reduction. As a conclusion, by increasing the C/N ratio from 10:3 up to 20:3 the biosurfactant recorded the highest productivity, but any further increase in lubricant oil waste concentration; there is a reduction in biosurfactant productivity.

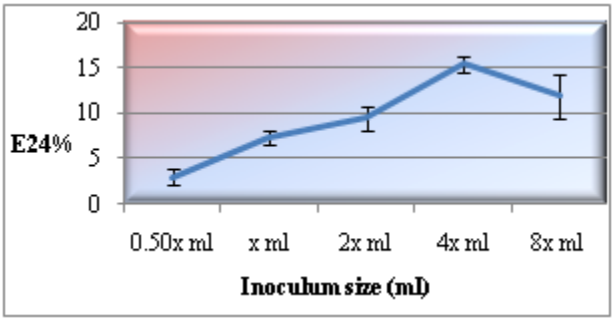


Figure 8: Relation of different lubricant oil concentrations on biosurfactant production by *Bacillus cereus*.

C/N ratio is an important variable to achieve improved productivity of rhamnolipid**(Santos*et al.*, 2002).** However, **Rismani*et al.* (2006)**indicated that, maximal biosurfactant production was obtained when *bacillus licheniformis* was grown on 0.5% crude oil.

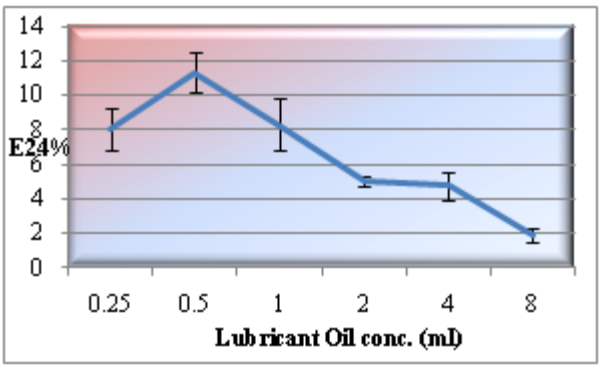


Figure 9: Relation of different pH's on biosurfactant production by *Bacillus cereus***.**

Concerning the relation of hydrogen ion concentrations on biosurfactant productivity by *Bacillus cereus*, pH 5 induced the highest biosurfactant productivity. Above or below this pH, a gradual decrease in the productivity was noticed (Fig. 9). **Gobbert*et al.* (1984)** indicated that, the pH of the medium plays an important role in sophorolipid production by *Torulopsisbombicola*. Rhamnolipid production in *Pseudomonas spp.* was at its maximum at a pH range from 6 to 6.5 and decrease sharply above pH 7**(Guerra-Santos *et al.*, 1984).**

In addition, surface tension and critical micelle concentrations of a biosurfactant product remained stable over a wide range of pH values, whereas emulsification had a narrower pH range **(Abu-Rawaida*et al.*, 1991b).**

Comparable results were obtained by **Kim *et al.* (1997),** who reported that, the surface tension reducing activity of *Bacillus subtillis* C9 was stable to a pH range of 5.0-9.5. Also, **Abu-Ruwida*et a1.* (1991a)** observed the best biosurfactant production at pH 6.5-7.2 by *Rhodococcus*spp. Similarly, **Thaniyavarn*et al.* (2008)** indicated that, *Pichiaanomala* PY1 biosurfactant produced at an optimum pH 5.5. Also, **Guerra-Santos *et al.* (1986)** reported that, biosurfactant production by *Pseudomonas aeruginosa* was higher at pH 6.5. **Huszcza and Burczyk (2003)** found that, *B. coagulans* produced a biosurfactant in a pH range 4–7.5. Data represented graphically in Fig. (10) showed that, all carbon sources used during this study had an inhibitory effect on the biosurfactant productivity by *Bacillus cereus*, B7. The highest production (43.923%) obtained when the medium devoted of any type of additional sugar and only the oil waste was used as the sole carbon source (control).

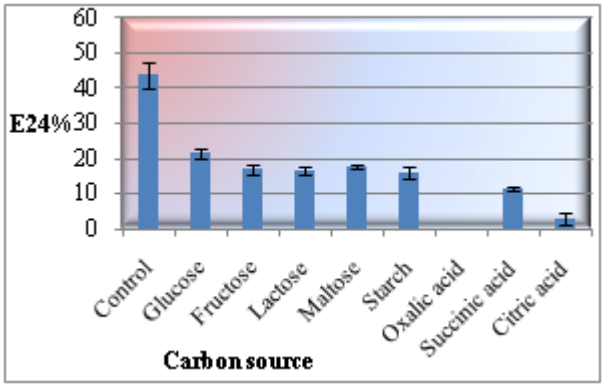


Figure 10: Relation of different carbon sources on biosurfactant production by *Bacillus cereus*, B7.

Bertani*et al.* (2003) indicated that, stress is an important environmental condition which highly influences the biosurfactant production in *P. aeruginosa* and this is related to conditions such as nutrient deprivation and nitrogen exhaustion. Also, **Schuster *et al.* (2004)** reported that, there is a genetic link between rhamnolipid production, nutrient deprivation and environment stress adaptation. Moreover, **Pandy (2004)** indicated that, biosurfactant production is usually induced by hydrocarbons, while water-soluble carbon sources repress their biosynthesis.

On the other hand, the best nitrogen source which stimulated the highest biosurfactant productivity by *Bacillus cereus*, B7 was $NaNO_3$ which originally used in the production medium followed by $NH_4NO_3$and gave 14.88 13.89, respectively. The result was represented graphically in Fig. (11).

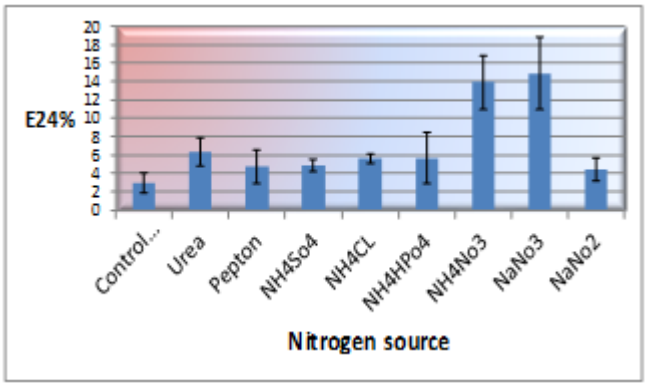


Figure 11: Relation of different nitrogen sources on biosurfactant production by *Bacillus cereus*, B7.

In the same accordance, **Santos*et al.* (2002)** reported that, nitrate has been shown to provide the highest yields of biosurfactant production**.**

In view of the present investigation, Dl-α-Amino-n-butyric acid showed a high production rate 26.79% compared with control, followed by L.Cystine and DL Tryptophan with production rate 17.46 and 9.52 %, respectively. But the rest of the amino acids used during this study had inhibitory effect on biosurfactant production by *Bacillus cereus*, B7. Moreover, aspartic and glutamic result in complete inhibition of biosurfactant production (Fig. 12).

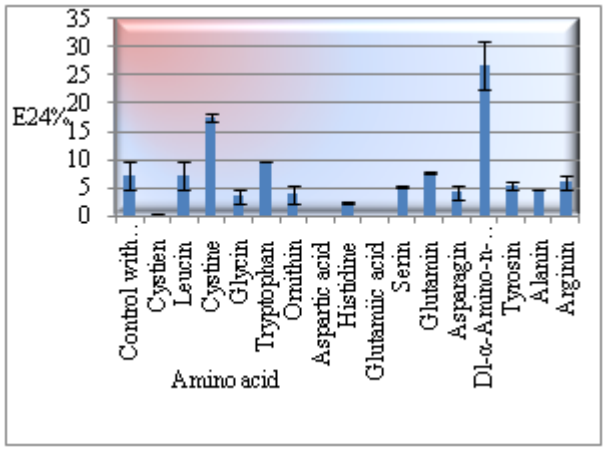


Figure 12: Relation of different amino acids on biosurfactant production by *Bacillus cereus*, B7

In view of the findings of other workers, different nitrogen sources can act as inhibitors (e.g. ammonium, glutamine, asparagine and arginine) or activators (nitrate, glutamate, and aspartate) of rhamnolipid production (**Mulligan and Gibbs, 1989 &Venkata-ramana and Karanth, 1989).**

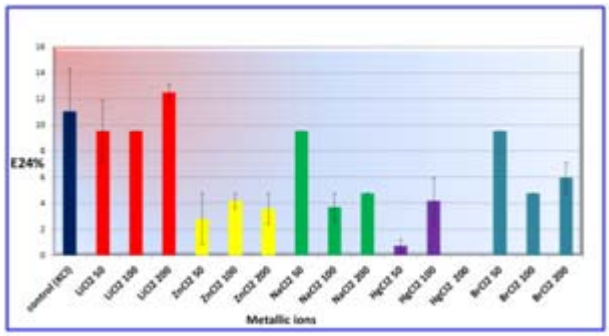


Figure 13: Relation of different metallic ions on biosurfactant production by Bacillus cereus, B7.

In the present investigation a 200 ppm of $LiCl_2$ has the highest biosurfactant productivity by *Bacillus cereus*, B7 among all the tested metallic ions. Moreover, the rest of the metallic ions at all concentrations used had inhibitory effect on biosufactant productivity. Results were represented graphically in Fig. (13).

Mineral salts are important variables to achieve improved productivity of rhamnolipid **(Santos*et al.*, 2002). Guerra-Santos*et al.* (1986)** showed that, limiting concentration of multivalent ions such as Mg, Ca, K, Na, and trace element salts increase rhamnolipid yield.
In the present investigation, none of the tested vitamins or growth promoters at all concentrations used exhibited any further increase in the biosurfactant productivity by *Bacillus cereus*, B7 (Fig14).

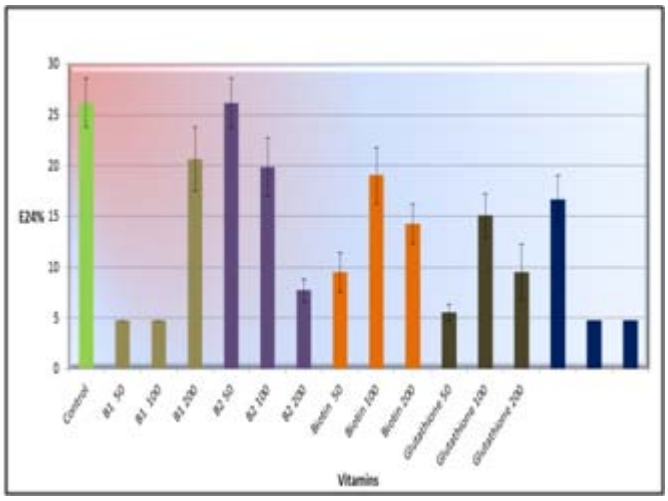


Figure 14: Relation of different vitamins and growth promoters on biosurfactant production by *Bacillus cereus*, B7.

4. Extraction of the BS

In the present investigation, the recovery of biosurfactant produced by *Bacillus cereus* from complex fermentation broth was carried out. Biosurfactant was precipitated and

extracted with a solvent system $CHCl_3$:C_2H_5OH in a ratio 2:1. The organic phase was evaporated in a rotary evaporator to give a white powder. About 500 mg of biosurfactant was extracted per liter of medium (0.5g/l).

Yakimov*et al.* (1996) showed that, the biosurfactant was precipitated according to the method of **Zhang and Miller (1992); Kim *et al.* (2000) &Samadi*et al.* (2007),** with solvent system chloroform: methanol in a ratio 2:1. The organic phase was evaporated in a rotary evaporator to give a white powder.

Sarin and Sarin (2008) indicated that, the bioimulsifier produced by *Enterobacter cloacae* LK5 was white powder product and give a yield of 11.06 g/l. **Desai *et al.* (1994) & Desai and Banat (1997)** reported that, the downstream process including recovery and purification of biosurfactants from fermentation broth is a major problem in the commercialization of biosurfactants. In many cases, it increases the cost of biosurfactant production to as high as 60%.

Zawawi (2005) showed that, improving product yield, low material costs and combining the steps of recovery can reduce the recovery costs. Most biosurfactants are secreted into the medium and they are isolated from either culture filtrate or supernatant obtained after removal of cells.

5. Studying some applications of the biosurfactant produced by Bacillus cereus, B7.

The antimicrobial activity of the produced biosurfactant was evaluated against some resistant pathogenic microorganisms such as *Candida albicans*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Fig. 15). By increasing the concentration of the biosurfactant, the antimicrobial activity against the test organisms under study increased. The MIC of the produced biosurfactant against both *C. albican* and *Staphylococcus aureus* was 5µl. While concerning *Pseudomonas aeruginosa*, the MIC was 2.5 µl.

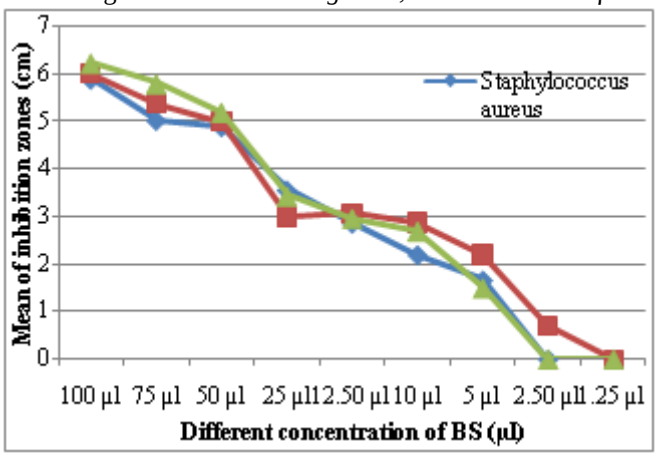
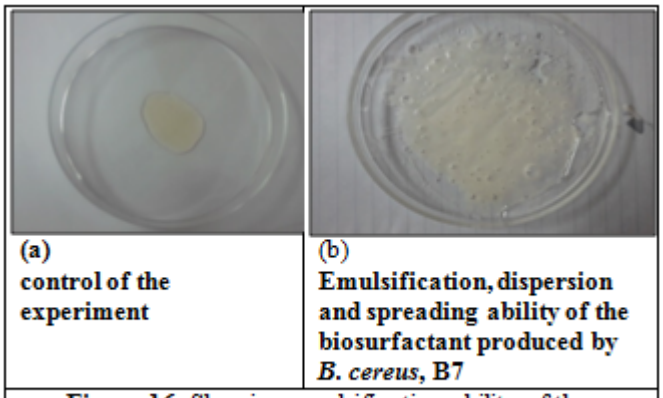


Figure 15: The biological activity of the biosurfactant produced by *Bacillus cereus*, B7 against isolats of *Candida albicans*, *Pseudomonasaeruginosa* and *Staphylococcus aureus*

H-Kittikun*et al.* (1993) concluded that, *Bacillus* MUV4 could produce antibiotic that was macrolactin A compound against the growth of *Bacillus anthracis*, *Salmonella*spp and *Aeromonashydrophila*. During antibiotic production, foam formation was observed and decreased so it was observed that this strain could produce biosurfactant. Several lipopeptidebiosurfactants produced by *B. subtilis* **(Sandrin*et al.*, 1990 &Vollenbroich*et al.*, 1997)** and *B. licheniformis* (**Jenny *et al.*, 1991 &Yakimov*et al.*, 1995**) have been shown to have antimicrobial activity. **Vater (1986)** described the antifungal properties of surfactin. **Hiradate*et al.* (2002), Yu *et al.* (2002)** and **Cho *et al.* (2003)**, reported about another lipopeptide, iturin, produced by *Bacillus* strains to suppress phytopathogenic fungi. . **Raaijmakers*et al.* (2006)** indicated that, *Pseudomonas* strains produce cyclic lipopeptide with growth inhibitory activities against a wide range of plant and human pathogens. **Cao*et al.* (2009)** reported that, the lipopeptidebiosurfactant from *Bacillusnatto* TK-1 exhibited both antibacterial and antifungal activities.

According to **Rosenberg and Ron (1999)**, there are three possible roles of biosurfactant in applications, increasing the surface area of hydrophobic substances, increasing the bioavailability of hydrophobic water-insoluble substrates, and finally regulating the attachment-detachment of microorganisms to and from surfaces.

Emulsification ability: This experiment was design to elucidate the ability of the produced biosurfactant of *Bacillus cereus*, B7 to emulsify the oil under study. Fig. (16& 17) showed the ability of the produced biosurfactant by *Bacillus cereus*, B7 to emulsify the waste oil. While Fig. (18) Illustrate Oil spreading ability of the biosurfactant produced by *Bacillus cereus*, B7.



(a) control of the experiment

(b) Emulsification, dispersion and spreading ability of the biosurfactant produced by *B. cereus*, B7

Figure 16: Showing emulsification ability of the biosurfactant produced by *B. cereus*, B7

Figure 17: Complete emulsification of the lubricant oil waste by the *Bacillus cereus*, B7surfactant

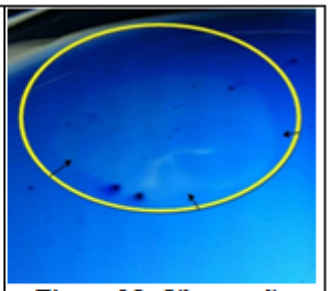


Figure 18: Oil spreading ability of the biosurfactant produced by *Bacillus cereus*, B7.

Various researches have studied the effect of biosurfactant on biodegradation of organic contaminants. Biosurfactants responsible to enhance solubility of the substrate for the microbial cells and interaction with the cell surface, which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily **(Shreve *et al.*, 1995 & Mulligan, 2004).**

In the present investigation, All results were carried out by ANOVA, one way by Minitab (Version 11) software and gave* [p=0.00 ≤ 0.05]

6. Conclusion

Because of the high hazard for human health of synthetic surfactants and increasing consumer demand for natural products, microbial surfactants have become increasingly important. The chemical diversity of biosurfactants offers a wider selection of surface-active agents with properties closely tailored to specific applications. Hence, there could probably be a potential chance of producing biosurfactants from petrochemical wastes. It has been focused here that improving the method of biosurfactant production and studying the possibility of the application of that biosurfactant as an emulsifier.

References

[1] **Abu-Ruwaida, A.S.; Banat, I.M.; Haditirto, S.; Salem, S. and Kadri, M. (1991a).** Isolation of biosurfactant producing bacteria-product characterization and evaluation. *J. of Re. Actabiotechnol.* 11: 315-324.

[2] **Abu-Ruwaida, A.S.; Banat, I.M.; Haditirto, S.; and Khamis, A. (1991b).** Nutritional requirements and growth characteristics of a biosurfactant producing *Rhodococcus*bacterium. *Re. World J. Microbiol. Biotechnol.* 7: 53–61.

[3] **Atlas, R.M.(2005).** Handbook Of Media For Environmental Microbiology. Second Edition. Taylor & Francis Group.USA

[4] **Beck, A.J. and Jones, K.C.(1995).**Limitations to the in situ remediation of soils contaminated with organic chemicals in relation to the potential to achieve clean-up criteria. *J. of Re. Contaminated Soil* 95: 327−336.

[5] **Bertani, I.;Sevo, M.;Kojic, M.&Venturi, V.(2003).**Role of GacA, LasI, RhlI, Ppk, PsrA, Vfr and ClpXP in the regulation of the stationary-phase sigma factor rpoS/RpoS in *Pseudomonas.Arch Microbiol. Epub* 180(4): 264-71.

[6] **Betina, V.(1957).**Nature (Moscow), 44: pp. 378-379.

[7] **Bustamante, M.;Durán, N. and Diez, M.C. (2012) .Biosurfactants is** useful tools for the bioremediation of contaminated soil: a review. *Journal of Soil Science and Plant Nutrition*, 12 (4): 667-687

[8] **Cameotra, S. S. and Makkar, R. S. (1998).** Synthesis of biosurfactants in extreme conditions.*J. of Re. Applied Microbiol. and Biotechnol.*, **50: 520-529**

[9] **Cao, X.H.; Liao, Z.Y.; Wang, C.L.; Yang, W.Y. and Lu, M.F.(2009).**Evaluation of a lipopeptidebiosurfactant from Bacillus natto TK-1 as a potential source of anti-adhesive, antimicrobial and antitumor activities. *J. of Re. Brazilian Journal of Microbiology* 40(2).

[10] **Carrillo, P.G.;Mardaraz, C.; Pitta-Alvarez, S.J. and Giulietti, A.M.** . (1996) "Isolation and Selection of Biosurfactant-producing Bacteria". *World J. Microbiol. Biotechnol*,12: 82-84.

[11] **Cheesbrough, M.(2000).** District laboratoay practice in tropical countries (Part 2), Combridge University Press, England

[12] **Cho, S.J.; Lee, S.K.; Cha, B.J.; Kim, Y.H.; Shin, K.S. (2003).**Detectio and characterization of the Gloeosporiumgloeosporioidesgrowt inhibitory compound iturin A from *Bacillus subtilis* strain KS03. *FEMS Microbiol. Lett.*, 223, 47-51.

[13] **Cirelli, F.A.; Ojeda, C.; Castro, M.J.L. and Salgot, M. (2008).** Surfactants in sludge-amended agricultural soils. *J. of Re. Environmental Chemistry Letters*, 6(3):135-148.

[14] **Collins, H.C.;Lyne, M.P. and Grange, M.J.(1989).**Microbiological Methods: 6th ed. Agency London:200

[15] **Cooper, D. G. and Goldenberg, B. G. (1987).** Surface active agents from two *Bacillus* species. *J. of Re. Appl. Environ. Microbiol.*, (53): 224-229.

[16] **Desai, J.D. and Banat, I.M.(1997).** Microbial production of surfactants and their commercial Reviews, 61: 47- 64.

[17] **Desai, A.J.;Reena M.P. and Desai, J.D.(1994).**Advances in the Production Biosurfactants and Their Commercial Applications. J. of Re. Scientific & Industrial Research. 53: 619-629.

[18] **Déziel, E.;Lépine, F.;Dennie, D.;Boismenu, D.;Mamer, O.A.&Villemu, R.(1999).** Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. *BiochimBiophysActa*

[19] **Elwan, S.H.; EL-Naggar, M.R. and Ammar, M.S.(1977)**. Characteristics of lipase (s) in the growth filtrate dialysate of *Bacillus Stearothermophilus* grown at 55˚C using tributyrin –cup plate assay, *Bull of the Fac. of Sci., Riyadh Univ*., 8: 105- 119.

[20] **Fiechter, A. (1992)**. Biosurfactants: Moving Towards Industrial Application. *J. of Re. Trends in Biotechnol*, 10: 208-217.

[21] **Gobbert, U.; Lang, S. and Wagner, F.(1984).** Sophorose lipid formation by resting cells of *Torulopsisbombicola*. *J. of Re. Biotechnol. Let.*, 6: 225-230.

[22] **Gogotov, I. N.; Belonozhkin, S. V.; Khodakov, R. S. and Shkidchenko, A. N. (2002).** Biosurfactants: producers, properties and their practical usage. Institute of Basic Biological Problems RAS, Mordva State University named by N.P.Ogarev, Institute of biochemistry and physiology of microorganisms named by G.K. Skryabin

[23] **Gordon, R.E.; William, C.; Haynes,Hor-Nay Pang, C.(1973).** The Genus ***Bacillus*:** Agriculture Handbook No. 427. ARS-USDA, Washington, D.C

[24] **Guerra-Santos, L.; Kappeli, O.; and Fiechter, A. (1984).** *Pseudomonas aeruginosa* Biosurfactant Production in Continuous Culture with Glucose as

Carbon Source. *J. of Re. Appl. And Enviro. Microbio.*, 48(2):301-305.

[25] **Guerra-Santos, L.;Kappeli, O.; and Fiechter, A. (1986).** Dependence of *Pseudomonas aeruginosa*continuous culture biosurfactant production on nutritional and environmental factors. *J. of Re. Appl. Microbio. and Biotechno.*, (24): 443-448.

[26] **H-Kittikun, A.; Abe, N.;Kamio, Y. and Iszaki, K. (1993)**. Isolation,purification and identification of antibiotics production by *Bacillus subtilis* MUV4. *Annual Reports of IC Biotech*. (16):379-390.

[27] **Henkel, M.; Müller, M.M.;Kügler, J.H.Lovaglio, R.B.;Contiero, J.;Syldatk, C.*et al.*(2012).**Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. *Process Biochemistry.*, 47(8):1207-19.

[28] **Hiradate, S.; Yoshida, S.;Sugie, H.;Yada, H. and Fujii, Y.(2002)**. Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. *Phytochemi.*, 61:693-698.

[29] **Holt,J.k.; Krieg, N.R.;Sneath, P.H.; Staley, J.T. and Williams, S.T.(1994).**Bergey's Manual of Determinative Bacteriology 9th. Baltimore.

[30] **Hsueh, Y.H.; Somers, E.B.;Lereclus, D. and Lee-Wong, A.C. (2006)**. Biofilm formation by *Bacillus cereus* is influenced by PlcR, a pleiotropic regulator. *Appl Environ Microbiol* 72:5089–5092

[31] **Huszcza, E. and Burczyk, B.(2003).** Biosurfactant production by Bacillus coagulans. J. of Re. *Surfactants Deterg*, 6:61–64.

[32] **Jenny, K.;Kappeli, O. and Fiechter, A. (1991).**Biosurfactants from *Bacillus licheniformis*, structural analysis and characterization . ApplMicrobiolBiotechnol 36:5–13.

[33] **Kim, S.H.; Lim, E.J.; Lee, S.O.; Lee, J.D. and Lee, T.H.(2000).** Purification and characterization of biosurfactants from *Nocardia*sp. L-417. *J. of Re. Biotechno. and Appl. Biochem.* 31:249-253.

[34] **Kim, S.Y.; Oh, D.K.; Lee, K.H. and Kim, J.H.(1997)** Effect of soybean oil and glucose on sophorose lipid fermentation by *Torulopsisbombicola* in continuous culture. *J. of Re. Appl.Microbio. and Biotechno.* 48(1):23-26.

[35] **Kitamoto, D.;Isoda, H. and Nakahara, T.(2002).** Functions and potential applications of glycolipid biosurfactants: from energy saving materials to gene delivery carriers. *J. of Re. Journal of Biosci. and Bioeng.*, 94: 187–201.

[36] **Koeuth, T.;Versalovic, J. and Lupski, J.R. (1995)** Differential subsequence conservation of interspersed repetitive Streptococcus pneumoniae BOX elements in diverse bacteria. *J. of Re. Genome Res*, 5(4):408-418.

[37] **Kosaric, N.(1992).**Biosurfactants in industry. *J. of Re. Pure & Appl. Chem.*, 64(11):1731-1737.

[38] **Kronemberger, F.D.A.; Santa Anna, L.M.M.;Fernandes, A.C.L.B.;Menezes, R.R.D.; Borges, C.P. and Freire, D.M.G.(2008).** Oxygen-controlled biosurfactant production in a bench scale bioreactor. *ApplBiochemBiotechnol.* 147(Issue 1-3) : 33-45

[39] **Lakshmipathy, D.T.; Prasad, A.S.A. and Kannabiran, K. (2010).** Production of Biosurfactant and Heavy Metal Resistance Activity of *Streptomyces* Sp.VITDDK3-a Novel Halo Tolerant Actinomycetes Isolated from Saltpan Soil. *J. of Re. Advances in* . 4(2):108-115

[40] **Lin, S.C.; Lin, K.G.; Lo, C.C. and Lin, Y.M.(1998).**Enhanced biosurfactant production by *Bacillus licheniformis* mutant. *J. of Re. Enzyme and micro. Techn.*, 23:267-273.

[41] **Maier, R.M. and Soberon-Chavez, G.S.(2000).***Pseudomonas aeruginosa*rhamnolipids: biosynthesis and potential applications. *J. of Re. ApplMicrobiolBiotechnol*,(54): 625-633.

[42] **Makkar, R.S. and Cameotra, S.S.(1998).** Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*. *J. Ind. Microbiol. Biotechnol.* 20: 48–52.

[43] **Makkar, R.S. and Cameotra, S.S.(1999).**Biosurfactant production by microorganisms on unconventional carbon sources. *J. Surf. Det.*, 2: 237-241.

[44] **Margaritis, A.;Zajic, J.E. and Gerson, D.F.(1979).**Production and surface active properties of microbial surfactants. *J. of Re. Biotechnol. Bioeng.*, 21:1151–1162.

[45] **Mercade, M.E. and Manresa, M.A.(1994).**The use of agro-industrial by products for biosurfactant production. *J. of Re. AM. Oil chem. Soc.*, 71: 61-64.

[46] **Mihelcic, J.R.; Lueking, D.R.; Mitzell, R.J. and Stapleton, J.M. (1993).** Bioavailability of sorbed- and separate-phase chemicals. *J. of Re. Biodegradation*,(4): 141-153.

[47] **Morikawa, M.; Daido, H.; Takao, T.;Marato, S.;Shimonishi, Y. and Imanaka, T.(1993).** A new lipopeptidebiosurfactant produced by *Arthrobacter sp.* strain MIS 38. *J. of Re. Bacteriol.*,(175): 6459–6466.

[48] **Mukherjee, S.; Das, P. and Sen, R.(2006).**Towards commercial production of microbial surfactants. *Trends Biotechnol*. 24(11):509-515.

[49] **Mulligan, C. N. (2004).** Environmental Applications for Biosurfactants. *J. of Environmental Pollution.*, 133, 183-198

[50] **Mulligan, C.N. and Gibbs, B.F. (1989).** Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa.Appl Environ Microbiol.*, 55(11):3016-3019

[51] **Mulligan, C.N.; Cooper, D.G. and Neufeld, R.J.(1984).**Selection of Microbes Producing Biosurfactants in Media without Hydrocarbons. *Journal Fermentation Technology*. 62 (4). 311-314.

[52] **Muyzer, G.; de Waal, E.C. and Uitterlinden, A.G. (1993).** Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *J. of Re. Appl. Environ. Microbiol.*, 59(3):695-700

[53] **Ochsner, U.A.; Fiechter, A. and Reiser, J. (1994).** Isolation, characterization, and expression in Escherichia coli of the *Pseudomonas aeruginosa*rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipidbiosurfactant synthesis. *J.BiolChem*, 269(31): 19787-19795.

[54] **Ochsner, U.A.; Hembach, T. and Fiechter, A. (1996).** Production of rhamnolipidbiosurfactants.

AdvBiochemEngBiotechnol, 53: 89-118.

[55] **Pandey, A. (2004).** Concise Encyclopedia of Bioresource Technology. In "Biosurfactant Production and Application, Kourkoutas, Y. and Banat, I.M., eds" Food Products Press®, USA.

[56] **Pape, W. and Hoppe, U. (1988).** "Evaluation of Acute Irritation Potentials of Tensides Using the In Vitro Alternative Red Blood Cell Test System". Second World Surfactants Congress, Paris. Proceedings, IV. 414-428.

[57] **Pekdemir, T.;Copur, M. and Urum, K. (2005).**"Emulsification of crude oil-water system using biosurfactant," Process Safety and Environmental Protection83: 38-46.

[58] **Raaijmakers, J.M.; de Bruijn, I. and de Kock, M. J.D. (2006)** Cyclic lipopeptide production by plant associated Pseudomonas spp.: diversity, activity, biosynthesis, and regulation. *J. of Mol. Plant. Microbe. Interact*. (19): 699-710.

[59] **Rismani, E.; Fooladi, J. and Ebrahimi, G.H. (2006).** Biosurfactant production in batch culture by a *bacillus licheniformis* isolated from the Persian Gulf. *Pakistan J. of Re. Biological Science*, 9(13): 2498-2502.

[60] **Rodrigues, L.; Banat, I.M.; Teixeira, J. and Oliveira, R.(2006a).**Biosurfactants: potential applications in medicine. *J. of Re. Antimicrobial Chemotherapy*.,57 :609–618.

[61] **Rodrigues, L.R.; Teixeira, J.A.; Mei, H.C. and Oliveira, R. (2006b)**. Physicochemical and Functional Characterization of a Biosurfactant Produced by *Lactococcuslactis* 53, Colloids and Surfaces B: Biointerfaces, 49, 79-86

[62] **Rosenberg, E., and Ron, E.Z. (1999).** High- and low-molecular-mass microbial surfactants. *J. of Re. Applied Microbiology and Biotechnology*, 52: 154-162.

[63] **Sachdev, D.P. and Cameotra, S.S. (2013).**"Biosurfactants in agriculture," *Applied Microbiology and Biotechnology*, 97:1005-1016

[64] **Samadi, N.N.;Abadian, A.;Akhavan, M.R.;Fazeli, A.;Tahzibi, and Jamalifar, H.(2007).** Biosurfactant production by the strain isolation from contamination soil. *J. of Re. bio. Sci.* 7 (7):1266-1269.

[65] **Sandrin, C.; Peypoux, F.; Michel, G. (1990).** Coproduction of surfactin and iturin A lipopeptides with surfactant and antifungal properties by *Bacillus subtilis*. *Biotechnol. Appl. Biochem.*, 12: 370-375.

[66] **Santa Anna, L.M.; Sebastian, G.V.; Pereira Jr., N.; Alves, T.L.M.; Menezes, E.P. and Freire, D.M.G. (2001)**. Production of biosurfactant from a new and promising strain of Pseudomonas aeruginosa PA1. Applied Biochemistry and Biotechnology- Part A Enzyme *Engineering and Biotechnology.*91-93 : 459-467

[67] **Santos, A. S.; Sampaio, A.P.W.; Vasquez, G.S.; Santa Anna, L.M.; Pereira, Jr. N. and Freire, D.M.G. (2002).** Evaluation of different carbon and nitrogen sources in production of rhamnolipids by a strain of Pseudomonas aeruginosa. Applied Biochemistry and Biotechnology J. 98-100, (1-9): 1025-1035

[68] **Sarin, S. and Sarin, C. (2008).** Production, isolation and application of biosurfactant/emulsifier by oil contaminated soil isolate Enterobacter cloacae LK5. *J. of Naresuan University*, 16(2): 113-125.

[69] **Schuster, M; Hawkins, A.C.; Harwood, C.S. & Greenberg, E.P. (2004)**. The *Pseudomonas aeruginosa*RpoSregulon and its relationship to quorum sensing. MolMicrobiol. Epub 51(4), 973-85.

[70] **Seghal, G.K.;Hema, T.A.;Gandhimathi, R.; Joseph, S.;Anto, T.T.;Rajeetha, R.T. and Natarajaseenivasana, K.(2009)**. Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillususstus* MSF3. *J. of Re. Colloids and Surfaces B*: Biointerfaces, 73: 250-256.

[71] **Shekhara, S.; Sundaramanickama, A. & Balasubramaniana, T. (2014).** Biosurfactant producing microbes and its potential applications: A Review. Critical Reviews in Environmental Science and Technology. Publisher: Taylor & Francis

[72] **Shreve, G.S.; Inguva, S. and Gunnan, S. (1995).**RhamnolipidBiosurfactant Enhancement of Hexadecane Biodegradation by Pseudomonas aeruginosa. *J. of Re. Molecular Marine Bio. Biotech.*, 4: 331-337.

[73] **Song, H.G.; Wang, X. and Bartha, R. (1990).** Bioremediation potential of Terrestrial fuels spills. *J. of Re. Appl. Environ. Microbiol.*, 56(3): 652−656.

[74] **Suwansukho, P.; Rukachisirikul, V.; Kawai, F. and H-Kittikun, A. (2008).** Production and applications of biosurfactant from *Bacillus subtilis* MUV4. SongklanakarinJ. *of Re. Sci. Technol.*, 30(1):87-93.

[75] **Tabatabaee, A.; Assadi, M.M.; Noohi, A.A. and Sajadian, V.A. (2005).** Isolation of Biosurfactant Producing Bacteria from Oil Reservoirs. *J. of Re. Iranian J Env Health Sci Eng.*, 2(2): 6-12.

[76] **Thaniyavarn, J.T.; Chianguthai, T.; Sangvanich, P.; Wang, N.R.; Washio, K.; Morikawa, M. and Thaniyavarn, S. (2008).** Production of Sphorolipidbiosurfactant by *Pichiaanomala*. *J. of Re. Biosci. Biotechnol. Biochem.*,72(8):2061-2068

[77] **Thavasi, R.;Subramanyam, V.R.M.N.;Jayalakshmi, S.;Balasubramanian, T. and Ibrahim, M.B.(2008).** Biosurfactant Production by *Azotobacterchroococcum* isolated from the Marine Environment. *J. of Re. Marine Biotechnology*, DOI 10.1007/s10126-008-9162-1.

[78] **Van Bogaert, I.N.A.; Saerens, K.; Muynck, C.D.; Develter, D.; Soetaert, W. and Vandamme, E.J. (2007).** "Microbial production and application of sophorolipids," *Applied Microbiology and Biotechnology*, 76: 23-34

[79] **Vater, P.J. (1986).**Lipopeptides, an attractive class of microbial surfactants. Prog. Colloid. *Polym. Sci.*, 72, 12-18.

[80] **Vatsa, P.; Sanchez, L.; Clement, C.;Baillieul, F. and Dorey, S.(2010).**"Rhamnolipidbiosurfactants as new players in animal and plant defense against microbes," *International Journal of Molecular Sciences*, 11: 5095-5108.

[81] **Vela´zquez-Aradillas, J.C.; Jime´nez, J.T.; A ´ ngelesGonza´lez-Cha´vez, M.; Bautista, F.; Cebria´n, M.E. and Esparza-Garcı´a, F.J. (2011).**Characterisation of a biosurfactant produced by a *Bacillus cereus* strain tolerant to cadmium and isolated from green coffee grain. *World J MicrobiolBiotechnol* 27:907–913

[82] **Venkata-ramana, K. &Karanth, N.G. (1989).** Factors affecting biosurfactant production using *Pseudomonas aeruginosa* CFTR-6 under submerged conditions. *ChemTechnolBiotechnol.* , 45(1), 249-57.

[83] **Vollenbroich, D.; Pauli, G.; Ozel, M.; Vater, J. (1997).**Antimycoplasma properties and application on cell cultures of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Appl. Environ. Microbiol.*, 63, 44-49.

[84] **Wurdemann, H.; Lund, N.C. and Gudehus, G.(1995).** Assessment of a biological in situ remediation . In: "In situ aeration: air sparging, bioventing, and related remediation processes. Eds. Hinchee R. E., Miller R. N., Johnson P. C." Columbos, USA, Battee Press, : 237−247.

[85] **Yakimov, M.M.; Fredrickson, H.L. and Timmis, K.N.(1996).** Effect of heterogeneity of hydrophobic moieties on surface activity of lichenysin A, a lipopeptidebiosurfactant from *Bacillus licheniformis* BAS50. *J. of Re. Biotechnol. Appl. Biochem.*, 23:13–18. .

[86] **Yakimov, M.M.; Kenneth, Timmis, K.N.; Wray, V. and Fredrickson, L. (1995)**. Characterization of a new lipopeptide surfactant produced by Thermotolerant and Halotolerant Subsurface *Bacillus licheniformis* BAS50. *Applied Environmental Microbiology*: 1706-1713.

[87] **Yonebayashi, H.; Yoshida, S.; Ono, K. and Enomoto, H. (2000).** "Screening of Microorganisms for Microbial Enhanced Oil Recovery Process". Sekiyu Gakkaishi. 43 (1). 59-69.

[88] **Yu, G.Y.; Sinclair, J.B.; Hartman, G.L.; Bertagnolli, B.L. (2002).** Production of iturin A by *Bacillus amyloliquefaciens* suppressing Rhizoctoniasolani. *Soil Biol. Biochemie*., 34: 955-963.

[89] **Zajic, J.E. and Stiffens, W. (1994).**Biosurfactants. *J. of Re. CRC Re Biotechnol*, 1: 87-106.

[90] **Zawawi, R. M. (2005).** Production of biosurfactant by locally isolated bacteria from petrochemical waste. M.Sc. Faculty of Science UniversitiTeknologi Malaysia.

[91] **Zhang, Y. and Miller, R.M.(1992).** Enhanced octadecane dispersion and biodegradation by a *Pseudomonas*rhamnolipid surfactant (biosurfactant). *J. of Re. Appl. Environ. Microbiol.* 58: 3276-3282.