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# Marine Biosurfactants: Potent Alternatives of Synthetic Surfactants

***Mahmoud A. AbdelFatah, Ali A. AbdeRahman*, Samar M. Solyman****

*Microbiology and Immunology Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt*

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*Correspondence Authors:

Phone: +201014301403

E-mail address:

dr_ali_abdellah@yahoo.com

Phone: +201023200424

E-mail address:

samar.mansour@su.edu.eg

## Abstract

Marine biosurfactants are surface active agents of marine origin either produced by planktonic microorganisms of marine environment whether sea water or soil or produced by associated microorganisms with marine organisms. In general, biosurfactants are classified according to categories of; Molecular structure, molecular weight and microbial origin. Mineral salts medium "MSM" supplemented with a hydrophobic compounds is mainly used for biosurfactant production enhancement. Then, screening of biosurfactant activity is operated and it is based on qualitative tests which gave a true general indication of biosurfactant type and potency and quantitative test which gave a precise reading of the quantity of biosurfactant and its type. Qualitative tests are mainly; blood hemolysis, oil spreading test , Emulsification index test "$E_{24}$", Drop collapse test and CTAB assay which detects anionic biosurfactants, no single method is suffiecient for detection of biosurfactant or bioemulsifier activity. While, a quantitative test gave us a precise reading of the surface tension, more than one qualitative screening method is required to prove a biosurfactant activity. Biosurfactants have been discussed by many studies on approximately all life aspect of food, agricultural, pharmaceutical, cosmetic, medical, laundry industry, petroleum industry, and environmental applications. They have proved efficiency which is similar or even better than synthetic surfactants. However, they are prefereed over synthetic ones in terms of biodegradability, environmental compatibility and in case of marine produced biosurfactants halophilic resistance which may be beneficial from economic point of view. So, many studies have focused on marine biosurfactants either for isolation of new potent strains, their identification using 16s rRNA or for their potentials' applications utilizing their unique properties.

**Keywords:** Marine biosurfactants, biosurfactant's classification, biosurfactants's screening tests, biosurfactant's applications.

## 1. Introduction

They are surface active agents of biological origin that are produced by microorganisms (**Tabatabaee et al., 2005, Rosen and kunjappu, 2012**) as a primary metabolite during exponential growth phase by bacteria, yeast and fungi (**Shoeb et al., 2012**) extracellularly or intracellularly bound to cell membrane for the role of facilitating diffusion of a hydrophobic matters into the cell (**Ward, 2010**), Cellular differentiation, Amensalism, Complexing of metals, Pathogenicity, Biofilm formation and Motility (**Van-Hamme et al., 2006)**.

Microorganisms responsible for their production can grow and produce biosurfactants from water soluble sources (glucose ,glycerol and ethanol) or majority from water insoluble hydrocarbons or substrates (**Tabatabaee et al., 2005 and Ahmad et al., 2016**).

They are amphiphilic in nature (**Shoeb et al., 2015**) with a hydrophilic head which may be anionic or cationic derivatives of either amino acids or peptides or non ionic derivatives of di or polysaccharides and a hydrophobic tail of fatty acids which may be saturated or unsaturated (**Mehta et al., 2010**), hydroxylated or hydrophobic peptides (**Maier et al., 2003 , Rosen and kunjappu, 2012 and Nordin et al., 2013**). They are preferred over synthetic surfactants for many unique properties that they posses; they have a low molecular weight typically ranges from 500 to 1500 Da (**Maier, 2003 and Ward, 2010**), low critical micelle concentration (CMC) which usually ranges from (1-1200 mg ) (**Ward, 2010 and Pornsunthorntawee et al., 2010**), they are biodegradable, (**Shoeb et al., 2012, Elazzazy et al., 2015, Saravanan and vijayakumar, 2012**), produced from renewable sources (**Abuo Gabble et al., 2011**), they are characterized by low toxicity (**Saravanan and Vijayakumar, 2012**), specificity, low irritancy and compatibility with human skin. Also, They are preferred over synthetic surfactants for their specific activity at extreme salinity & temperature & pH, their diversity, selectivity & large scale production suitability, eco-friendliness (**Shoeb et al., 2012, Elazzazy et al., 2015 and Rodrigues and Teixeira, 2010**). So, They are preferred to be used over synthetic surfacatants in many life aspects (**Mukherjee and Das, 2010**) as agriculture (**Das et al., 2010**), pharmaceutical (**Saravanan and vijayakumar, 2012**), petroleum, bioremediation (**Shoeb et al., 2015**) petrochemical, food and beverage industries, so it may be an alternative to synthetic surfactants (**Shoeb et al., 2012**). Also they are a promising alternatives due to drawbacks of synthetic surfactants used which are produced mainly of petrochemical origin, the increasing awareness of environmental impact and the tightening of regulations in this regard (**Perfumo et al., 2010**).

For environmental concern, a good surfactant should begin with the user and end up friendly to the environment (**Mehta et al., 2010**). Microorganisms are proved to produce variety of biosurfactants that besides the previously mentioned desired properties of biodegradability and eco-friendliness, they also have properties of "emulsification, demulsification, wetting, dispersing and "surface and interfacial tension reduction" which are comparable to those of synthetic surfactants or even better. Due to this benefits and especially eco-acceptance properties, their application is promising. (**Rebello et al., 2014 and Perfumo et al, 2010**).

**2. Classification**

Biosurfactants are basically classified according to molecular structure into five main broad groups which are glycolipids as "rhamnolipids, sophorolipids, trehalose lipids, mannosylerythritol lipids and ustilagic acid", lipopeptides&lipoproteins as "Surfactin, iturin, fengycin, lichenysin and viscosin", phospholipids & hydroxylated and cross linked fatty acids as "Spiculisporic acid and phospholipids", polymeric surfactants as "emulsan, apoemulsan, liposan, biodispersan, alasan, mannoprotein, emmulsifiying protein and exopolysaccharide" and particulate surfactants which are extracellular membrane vesicles composed of protein, phospholipid and lipopolysaccharide (**Mukherjee and Das, 2010** and **Sharma, 2016).** They are classified according to molecular weight into low molecular weight as glycolipids, lipoproteins which they decrease surface tension& interfacial tension of solutions and high molecular weights (polymeric and particulate biosurfactants) as lipopolysaccharides and complex biopolymers as emulsan, liposan which they function as bioemulsifier rather than biosurfactants (stabilize o/w emulsions) rather than biosurfactants (**Henkel and Hausmann, 2019)**. Also, they are classified according to microbial origin which they are mainly produced from, for example certain species as *Pseudomonas* mainly produce rhamnolipids and *Acintobacter* mainly produce emulsan (**Mukherjee and Das, 2010**).

**3. Marine biosurfactants:**

Inspite of the promising future of biosurfactant applications in many life aspects, it lacks the economic applicability. So, isolation of high biosurfactant producers that produce potent biosurfactants is of great importance to make their application economically available. So, sampling and isolation of new biosurfactant producing microorganisms is the key factor for this consideration "**Elazzazy et al., 2015, Shoeb et al., 2012, Muller et al., 2012 and Krieger et al., 2010"**. In recent years, many studies have focused on the research of marine microbiome as they are one of the largest and highly diverse microbiomes on the planet. (**Stal and Cretoiu, 2016**) . Marine bacteria is a great source for novel biodiscovery programmes of bioproducts with unique properties (**Joint et al., 2010**). Biosurfactants are bioproducts of such unique properties. Biosurfactant activity is extensively studied by many studies that discussed the potential promising applications of biosurfactants whether in industrial controlled conditions for biosurfactant production or for open in situ application such as bio-remediation (**Elazzazy et al., 2015**). Marine biosurfactants are preferred due to their distinct properties of halophilic tolerance which can be

very beneficial from economic point of view (**Lang *et al.*, 2005**). The issue of discovering marine bioproduct activity was discussed previously by many studies which discussed either an organism associated producing bacteria or even the planktonic marine bacteria and many studies discussed the marine biosurfactants' producing microorganisms discovery while others scoped on the potentials of the isolated marine biosurfactants. (**Wu et al., 2017,Dey et al.,2015 and Li and Liu, 2006**).

### 3.1. Biosurfactants' screening methods:

Screening of biosurfactant producing microbes is starting by the enrichment culture utilizing a hydrophobic compounds as a sole carbon source which are used as an indirect method of screening as the growth on hydrophobic compounds indicates the production of biosurfactants but not always correlates with this traits. Using mineral Salts medium "MSM" supplemented with hydrophobic compounds of either petroleum, PAHs, crude oil, n-alkanes and various vegetable oils have been used by many studies as a sole carbon source for the enrichment medium (**Danyelle et al., 2016, Bentoa et al., 2005 and Safary et al., 2010**). Several screening techniques of biosurfactants have been applied are based on the physical effects of biosurfactants. Also, specific screening methods like the colorimetric CTAB agar assay are suitable only for anionic biosurfactants like rhamnolipids. For biosurfactant detection; qualitative and quantitiative methods are used. Qualitative tests are used for general detection of the biosurfactant effect and the quantitative methods using direct surface tension measurement devices are used which gives a precise, accurate measurements.

Many methods have been proposed by many studies for detection of surface activity and/or emulsification activity, for detection of the hydrophobicity of bacterial cells and the specific test of CTAB agar method which detect the anionic biosurfactants. Detection of surface activity and/or emulsification activity is the basis for detection and it depends on many proposed tests as hemolysis, drop collapse test, microplate assay, penetration assay, oil spreading assay, emulsification capacity assay and for detection of the hydrophobicity of bacterial cells as cell surface hydrophobicity, bacterial adhesion to hydrocarbons assay, hydrophobic interaction chromatography, replica plate assay and salt aggregation assay which are not effective in testing the surface activity (**Walter et al, 2010**)

For general detection of a biosurfactant more than one screening test should be used, as no single method is sufficient for surface activity detection. The following tests are mentioned in many studies for simple, fast, reliable, more accurate results which are correlated to the direct surface tension measurement by accurate devices (**Meenakshisundaram *et al.*, 2016, Nwaguma *et al.*, 2016 and Rehman *et al.*, 2014)**.

#### 3.1.1. Blood Hemolysis assay:

An assay that was developed by mulligan et al (**Walter et al, 2010**). They utilized the principle of "biosurfactants cause lysis of erythrocytes". Pure cultures are streaked on sheep blood agar and incubated at 30º C for 3 days then the results are recorded as α, β and ɣ. This test is considered a preliminary basic test but it has some restrictions that make it non specific. Many microorganisms have pathogenic lytic enzymes which cause hemolysis of blood and some biosurfactants are reported to not causing blood hemolysis. Yet, this technique is used as a preliminary test but it needs other surface activity measurements confirmation (**Tabatabaee et al., 2005, Shoeb *et al.*, 2012, Shoeb *et al.*, 2015 and Safary *et al.*, 2010**).

#### 3.1.2. Oil spreading assay/ oil displacement assay:

An assay which is developed by Morikawa et al (**Walter et al, 2010**). For this assay, 10 µl of crude oil is added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If the 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. For a pure biosurfactant, a linear correlation between quantity of surfactant and the clearing zone diameter is given (**Shoeb et al., 2012)**.

The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. It can be applied when the activity and quantity of biosurfactant is low. It is reliable for biosurfactant production screening from diverse microorganisms (**Shoeb et al., 2015 and Walter *et al.*, 2010)**.

#### 3.1.3. Emulsification activity assay:

Another popular assay for measuring the emulsification property of a biosurfactant. It was developed by Cooper and Goldenberg (**Walter et al., 2010**). For measuring the emulsification capacity, equivalent volumes of a hydrophobic compounds "kerosene, xylene, hexadecane, etc" and the supernatant of the microorganisms culture fluid. Then the mixture is vortexed for 2 minutes and leaved for 24 hr, if the supernatant have an emulsification capacity, then the mixture is still mixed and not separated. Emulsification capacity is another property than the

surface active property. A supernatant may have a surface activity but not have emulsification activity (**Nordin *et al.*, 2013, Nwaguma *et al.*, 2016 and Saravanan *et al.*, 2012**).

**3.1.4. Drop collapse assay:**

An assay that was developed by jain et al for surface activity detection (Walter et al., 2010). Its idea is based on destabilization of liquid droplets by surfactants. Drops of a culture supernatant are placed on an oil coated, solid surface. If the liquid contains biosurfactants, the drops spread or even collapse because the interfacial tension between the liquid drop and the hydrophobic surface is reduced. and if it doesn't contain a biosurfactant the drops are remained stable. However, this method is not accurate and not precise method even as a qualitative test as it gave many false positive results compared with surface tension or even oil spreading and emulsification activity(**Elazzazy et al., 2015**).

For general detection of biosurface active properties, qualitative tests are used especially hemolysis, oil displacement and emulsification activity assays which they are reported by many studies. Those tests are efficient and sufficient for general detection of a biosufactant and its potency (**Elazzazy et al., 2015** and **Kamal et al., 2017**) . However, measuring of surface tension by direct surface tension measuring devices are the most accurate and precise method as it gives us a definitive idea of the biosurfactant being measured and its degree of potency. Most instruments which are concerned about measuring the surface tension are based on Du-Nouy Ring assay which are based on measuring the force required for detachment of a platinum ring or wire from an interface or surface which are directly proportional to the surface tension. A restriction of this method is that above a certain concentration of the biosurfactant called "CMC" the surface tension remained constant as shown in the graph below. However, we can handle this problem by working by serial dilution until a sharp increase in surface tension is observed. The corresponding dilution of the supernatant is called critical micelle dilution (cmd) and correlates to the concentration of biosurfactant( **Walter et al., 2010, Ahmad et al., 2016 and Thavasi et al., 2011**).

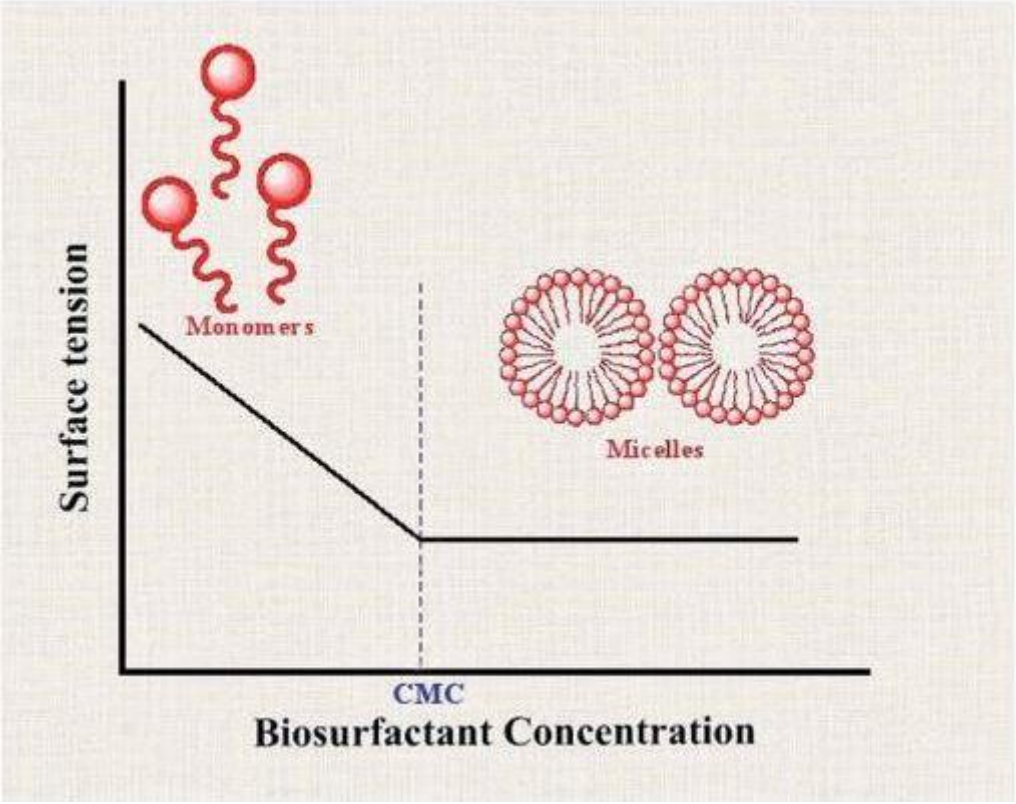


**Fig. 1** Relationship between surface tension and biosurfactant concentration & formation of micelles. (Sourav et al, 2015)

**4. Biosurfactants' applications**:

Biosurfactants have been discussed by many studies for their use as a normal constituent of products or as an alternative to synthetic surfactants.

**4.1 In food industry**: They have been applicated by many studies for enhancing the physicochemical properties of products as the study of "**Fan et al., 2019**" which applicated the MELA in enhancing the physicochemical properties of lactoglobulin by making complexes with lactoglobulin, study of "**Ribeiro et al., 2020**" replaced the egg yolk in cookies formation by addition of a glycolipid biosurfactant produced by *Saccharomyces cervesiae strain URM6670* which lead to physicochemical and physical properties similar to original cookies of egg yolk in concerns of firmness, elasticity and cohesiveness.

**4.2 In agricultural sector**: Restrictions on the use of agrochemicals and fungicides have been present so, alternative biological methods are needed. Biosurfactants have been used in biological control of many crops' diseases and as a plant promoting bacteria as rhizobacteria which increase the plant yields (**Arrebola et al., 2010**). In biological control of plant diseases many studies have applicated the biosurfactant's producing microorganisms in combating the plant diseases. Biocontrol of powdery mildew "phytopathogenic disease" have been studied by (**Clement Mathieu et al., 2008**) which succeeded in combating the disease by *Pseudozyma flocculosa* which have a flocculosin antifungal activity. "pv tomato dc 3000" a serious disease that cause mortality of plants by *Pseudomonas syringae* have been combated by using wild strain of *bacillus subtilis* which produce a lipopeptide surfactin. Pathogenic fungal diseases in citrus have been controlled by application of *B. amyloliquefaciens str PPC (B004) in a study operated by* (**Arrebola et al., 2010**). *They found that the antifungal effect was due to iturin production which lead to disruption of cytoplasmic membrane.* In pest control, (**Ghribi et al., 2012)** succeeded in using *bacillus subtilis SPB1* as a bioinsecticide. They effectively biocontrolled the *Lepidopteran* larvae "third star larvae of *Ephestia kuchniella*".

**4.3 In pharmaceutical and cosmetic sectors**: Biosurfactants have been discussed by many studies in pharmaceutical and cosmetic industry for their advantageous properties of low irritancy, wound healing and skin regeneration. (***Rodrigues-Lopez et al., 2019***) operated a comparative study of two biosurfactants in comparison to SDS and they found that the two biosurfactantants has no irritancy, no hemorrhage and no lysis of CAM vessels "chorioallantoic membrane of hen's egg".

**4.4 In medical field**: Many studies have discussed the potential applications of biosurfactant's or biosurfactant producing microorganisms. Trehalose lipid biosurfactant produced by *Rhodococcus fasciens BD8* in a study operated by (**Janek et al., 2018**) proved to have antibacterial and antiadhsive properties against *Proteus mirabilis, Escherichia coli, Enterococcus hirae and Candida albicans* by 70-95% so, may be used in biofilm formation inhibition in medical devices and prostheses. (**Morita et al., 2011)** proved potent anti-inflammatory action of Mannosyl erythritol lipid a,b MEL (A,B) produced by *Pseudozyma Antarctica T34* with anti-inflammatory response similar to cromoglycate. In gene transfection, **Imura et al., 2005** succeeded in forming a thermodynamically stable vesicles with mixture of MELA produced from *Pseudozyma antarctica* and Lα-DLPC (dilauryl phosphatidylcholine), the mixture formed showed stability for 3 months which were better than synthetic surfactants in terms of toxicity. **In drug delivery system, Yi et al., 2018** succeeded in forming a nanoparticle vesicles of emulsan produced by *Acinetobacter calcoaceticus RAG-1* and flax seed oil were used for delivery of photodynamic pheophorbide as a model drug with longer blood circulation and 3.04 fold higher tumour accumulation than pheophorbide alone. **In Immune-enhancing**, **Liu et al., 2011** proved the "immunoenhancing effects" of extracted *Saccharomyces cerevesiae* mannoprotein. Antitumour effect was discussed by many studies; **Saini et al., 2008** proved that viscosin produced and recovered by *Pseudomonas libanensis M9-3* has anticancer effects, **Cao et al., 2010** succeeded in revealing the mechanisms involved in the anticancer activity of the lipopeptide surfactin produced by *B. subtilis*, **Dey et al., 2015** proved the antitumour activity of a lipopeptide iturin A produced by marine bacterium *B. megaterium* "isolated and purified by RP-HPLC" and isoforms with long fatty acid chains are choosed. Iturin A is reported to be safer as anticancer than surfactin as it is less hemolytic and less toxic.

**4.5 In laundry industry**: The cyclic lipopeptide biosurfactant produced by *bacillus subtilis* showed detergency with stability over a pH range of 7-12, good thermal stability up to 80ºC for 60 min without any loss of surface activity(**Mukherjee, 2007)** .

**4.6 In petroleum industry**: Many studies discussed the potential advantages of biosurfactants application in all aspects of petroleum processing. When properly used they are comparable to synthetic surfactants in terms of performance, however it offers advantages with regard to environmental aspects**. Oil clean up of storage tanks:** Microbial cleaning of tanks are first proposed by Gutnick and Rosenberg 1981 in a patent process using alpha and beta emulsans produced by *Acinetobacter venetianus ATCC31012.* Also, another

field trial at the Kuwait oil company was conducted and The oil sludge was treated by rhamnolipid containing culture broth, 91% of hydrocarbon in the sludge was recovered and the value of the recovered crude oil covered the cost of the cleaning operation (**Perfumo et al., 2010)**. **Crude oil transportation in pipelines:** Transportation of Waxy crude oil is a problematic issue since narrowing and blockage of internal diameter of pipes. Solving of these problem by using of emulsifying biosurfactant like emulsan have been reported to be applicated in a field trial for pipeline transportation of a Boscan heavy crude oil of viscosity of about 200000 cP. Another field trial have been reported for transportation of stable emulsion called hydrocarbosol with viscosity to 70 cP was pumped through 380 miles over 64 hr with a surfactant ratio of 1:500 and 70% w/w oil/water stable emulsion formed (**perfumo et al., 2010**). **Microbial enhanced oil recovery "MEOR":** works by using biosurfactants or biosurfactant producing microorganisms in lowering interfacial tension at the oil-rock interface, thus reducing capillary forces that prevent oil from moving through rock pores and so, increasing the recovery of heavy crude oil that was retained at the oil well. Three strategies have been proposed for MEOR: First strategy: injection of biosurfactants produced Ex-situ into the reservoir, Second strategy: Stimulation of indigenous biosurfactant producing microorganism within oil reservoir and third strategy: Injection of microorganism into oil wells (**Joshi et al., 2015 and Mukherjee and Das, 2010**). For the first strategy: Potential application of lichenysin-A which is synthesized by *Bacillus licheniformis R2* in heavy crude oil recovery was reported by **Joshi et al., 2015** and 37.1% Recovery of oil from Berea sand stone cores at 80ºC was recorded and in the study operated by **Liu et al., 2015** reported that Surfactin produced by mutated high yield *B. subtilis BS-37* have low CMC of 20 mg/l which lowers surface tension to 27.7mN/m and are produced by high titers of 585mg/l. Surfactin solution(30mg/l) showed 88.5% of oil washing effiency and 13.48% of crude oil displacement efficiency. Another study used a halotolerant and thermotolerant *Bacillus licheniformis JF-2* had been exploited through various processes for oil recovery through injection into oil bearing formations alone or as a part of microbial consortium, increase of 14% of oil production was observed after flooding with *B. licheniformis JF-2* and presence of living cells in the production fluids were detected 6 weeks after injection.(**Perfumo et al., 2010**).

**4.7 Bioremediation**: Bioremediation of hydrocarbon pollutants in the environement is a natural and continous biological process to clean the nature from pollutants which are leaked into the environment such as petroleum derivatives, aliphatic& aromatic hydrocarbons, industrial solvents, pesticides and metals. It had been proposed as an effective, economic and environmental friendly technology (**Whang et al., 2008**). **Kang et al., 2010** studied the potential application of sophorolipid microbial biosurfactant produced by *Candida bombicola ATCC 22214* in washing and biodegradation enhancement at 10 g/l. Sophorolipid effectively showed higher soil flushing efficiency than any other tested nonionic surfactants(Tween 60, 20 and span 20/80/85) except for tween 80, it showed 30% washing of 2-methylnaphthalene. **Whang et al., 2008** proved the potential application of two biosurfactants (rhamnolipids produced by *P.aerugenosa J4* and surfactin produced by *B. subtilis ATCC21332* in enhancing of biodegradation of diesel in enriched diesel degrading consortia in two batch systems (diesel/water system and diesel/soil system).

**5. Conclusion:**

Future applications of biosurfactants or biosurfactant producing microorganisms at large market scale is based on the economic matter as economy is considered the bottleneck for biosurfactants production. Many methods have been proposed to overcome this issue, one of the most promising methods to make their production of economic importance is screening new biosurfactant's producing microorganisms of marine origin as marine microbiome is one of the largest microbiome on earth so, a great source of new novel bioproducts. Discovery of new potent biosurfactant producers from marine source is important for industrial bioprocesses because of their halophilic resistance and environmental biocompatibility. Many attempts have been done on exploring marine biosurfactant producing microorganisms, marine biosurfactants and their applications. **Elazzazy et al., 2015** isolated a good biosurfactant producing isolate from sea water and soil samples in the Jeddah region, Saudi Arabia. It was identified as *virgibacillus salaries* with good halophilic activity, thermoresistance and alkaline pH tolerance, **Safary et al., 2010** isolated two strains of good biosurfactant activity of the Caspian Sea, Mazandaran province, Iran. They were capable of utilizing crude oil as a sole carbon source, **Mounira and Abdelhadi, 2015** isolated two strains of good biosurfactant activity from five saline soil samples collected of Chott El Hodna-M'sila (Algeria), **Kamal et al., 2017** isolated a potent biosurfactant producer which was identified as *Aeromonas salmonicida* from Marchika lagoon, located in the north-west Mediterranean coast of Morocco, **Shoeb et al., 2015** isolated eighty nine distinct bacterial isolate from

fifteen seawater samples collected from Arabian Sea coast of Karachi of which thirty nine isolate showed surface activity and forty eight isolate showed emulsifying activity, in the study of **Dey et al., 2015** isolated a marine lipopeptide iturin A by RP-HPLC", iturin from a marine bacterium B.megaterium, and isoforms with long fatty acid chains are choosed. The anticancer activity for iturin was shown in vitro for breast cancer cell line MCF-7 and MDA-MB-231 and in vivo for cell line MDA-MB-231 breast cancer. Iturin A treated cancer cells showed apoptosis in vitro and in vivo through molecular mechanisms, **Sivapathasekaran et al., 2010** isolated a marine surfactin and fengycin like lipopeptide from Bacillus circulans DMS-2 using glucose mineral Salts medium (GMSM), the biosurfactant produced has a CMC of the crude and purified products to be 90 and 40 mg/l respectively with a surface tension reduction down to 27 mN m/l. The crude biosurfactant production of 1.64 ±0.1 g/l. They proved a significant antiproliferative activity was displayed of the purified marine lipopeptides against the human colon cancer cell lines HCT-15 ($IC_{50}$ 80 µg/ml) and HT-29 ($IC_{50}$ 120 µg/ml).

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