Review Article

Biosurfactant Production and Potential Correlation with Esterase Activity

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Abstract

Biosurfactants (microbial surfactants) are surface active compounds produced extracellularly or as part of the cell membrane by several bacterial and fungal species. They have the unique property of reducing the surface and interfacial tension of liquids. Biosurfactants have applications in the field of agriculture, petroleum, microbial enhanced oil recovery, biomedical sciences, cosmetics, food processing and pharmaceuticals. The global biosurfactants market has grown gradually. Regardless of their greater biodegradability and reduced toxicity, cost competitiveness still remains the major concern for biosurfactant production. However, recombinant or metabolically engineered hyper producing strains combined with optimized cultivation conditions have made it possible for many companies to reap the benefits of 'green' biosurfactant technology. Simultaneously, biosurfactants and bioemulsifiers showing esterase activities and having potential applications are reported to form stable oil-water emulsions with hydrophobic substrates such as hexadecane and polyaromatic hydrocarbons. Biosurfactant production and release of esterases by the microbial cells is shown to be synchronized and symbiotically beneficial in some species. Several bacterial biosurfactant and esterase genes have been identified, cloned and expressed for their enhanced production. This review article emphasizes on the present worldwide scenario of biosurfactant production, correlation between biosurfactant production and esterase activity, recent developments in this line of research and future prospects.

Introduction

Biosurfactants are amphiphilic compounds produced by a variety of microorganisms. Biosurfactants have unique properties of lowering the surface and interfacial tensions and Critical Micelle Concentration (CMC) in both aqueous solutions and hydrocarbon mixtures just like chemical surfactants [1,2]. The worldwide biosurfactants market was worth USD 1,735.5 million in 2011 and according to the recent market report published by Transparency Market Research it is expected to reach USD 2,210.5 million by the year 2018, corresponding to an average annual growth rate of 3.5% from 2011 to 2018. With the increased awareness among consumers for environmentally friendly compounds, a number of surfactant manufacturers in the market have budged into the biosurfactant industry. Today there is an enormous increase in the number of biosurfactant companies around the globe. AGAE technologies Ltd, USA have recently introduced R95, an HPLC/MS grade rhamnolipid, while USA based Jeneil Biosurfactants is already a key player in this field. Other major manufacturers include Fraunhofer IGB (Germany), Cognis (Germany and USA) dealing with production of glycolipid surfactants, cellobiose lipids and mannosylerythritol lipids; Saraya (Japan), Ecover Belgium (Belgium), Groupe Soliance (France) and MG Intobio (South Korea) are responsible for manufacturing sophorolipids. Cognis recently announced the production of green surfactant alkyl polyglucoside APG®, which is made from vegetable oil or starch, at its site in China while Jeneil Biosurfactants, USA are currently selling ZONIX, a biofungicide made from rhamnolipids and RECO, a biosurfactant used in cleaning and recovering oil from storage tanks. Paradigm Biomedical Inc (USA), on the other hand, is committed to research of pharmaceutical products derived from rhamnolipids.

Biosurfactants are well known and well documented for their role in enhancement of the emulsification of hydrocarbons, potentially solubilizing the hydrocarbon contaminants and increasing their availability for microbial degradation. Surfactants, on the other hand, are the organic compounds and main ingredients found in washing powders, soaps, toothpastes, shampoos and detergents, which are recalcitrant in nature and persist in the environment for longer period of time. While the scientists are keen on replacing chemical surfactants with biosurfactants, the real bottleneck still lies in the high cost of production of biosurfactants. Recombinant, nonpathogenic, high-yielding strains are being created with the application of latest genome sequencing technologies. With the use of renewable low-cost carbon sources, the yields are being increased, reducing the costs by scaling up the production.

Biosurfactants, which are selective in nature, act on the surfaces of liquids and at times certain enzymes facilitate them in their action for reducing the surface tensions of liquids and/or improving the solubility of water immiscible substrates. Esterases are among such enzymes, which are shown to be produced in the culture media when the biosurfactants production is at its peak, thus forming a complex with biosurfactants and this interplay between the two greatly helps in the emulsification of the hydrophobic substrates (for example: olive oil). Biosurfactants and esterases thus show some sort of symbiotic relationship which is mutually beneficial to them. Biosurfactants, produced by the recombinant strains, are found to be responsible for the enhanced production and activity of esterases and vice versa [3]. This can further be explained by the fact that biosurfactants are known

to be poteinaceous in nature [4,5] and it is the protein moiety which is responsible for the emulsifying activity or detergent like property of biosurfactants. Esterases and lipases have similar catalytic sites and treatment with a phospholipid can easily convert an esterase into a lipase [6]. Lipases have penetrated into the detergent market like no other enzyme. Today they are being used and have applications in every modern industry. Esterases with their lipase like properties are also known for their role in degradation of natural materials and industrial pollutants [7].

Biosurfactant type	Producing Microbial species
Glycolipids	
Trehalose mycolates	*Rhodococcus erythropolis, Arthrobacter paraffineu, Mycobacterium phlei, Nocardia erythropolis*
Trehalose esters	*Mycobacterium fortium, Micromonospora* sp., *M. smegmatis, M. paraffinicum, Rhodococcus erythropolis*
Trehalose mycolates of mono, di, trisaccharide	*Corynebacterium diptheriae, Mycobacterium smegmati, Arthrobacter* sp.
Rhamnolipids	*Pseudomonas* sp.
Sophorolipids	*Torulopsis bombicola / apicola, Torulopsis petrophilum, Candida* sp.
Rubiwettins R1 and RG1	*Serratia rubidaea*
Diglycosyl digyycerides	*Lactobacillus fermenti*
Schizonellins A and B	*Schizonella melanogramma*
Ustilipids	*Ustilago maydis and Geotrichum candidum*
Amino acid lipids	*Bacillus* sp.
Flocculosin	*Pseudomonas flocculosa*
Phospholipid and Fatty acids	
Phospholipids, Fatty acids	*Candida* sp., *Corynebacterium* sp., *Micrococcus* sp., *Acinetobacter* sp, *Thiobacillus thiooxidans, Asperigillus* sp., *Pseudomonas* sp., *Mycococcus* sp., *Penicillium* sp.
Lipopeptides and lipoproteins	
Gramicidins	*Bacillus brevis*
Peptide lipids	*Bacillus licheniformis*
Polymyxin E1	*Bacillus polymyxa*
Ornithine-lipid	*Pseudomonas rubescens, Thiobacillus thiooxidans*
Viscosin	*Pseudomonas fluorescens*
Serrawettin	*Serratia marcescens*
Cerilipin	*Glucunobacter cerius*
Lysine-lipid	*Agrobacterium tumefaciens*
Surfactin, subtilysin, subsporin	*Bacillus subtilis*
Lichenysin G	*Bacillus licheniformisIM 1307*
Ornithine lipid	*Pseudomonas* sp, *Thiobacillus* sp, *Agrobacterium* sp, *Gluconobacter* sp.
Amphomycin	*Streptomyces canus*
Chlamydocin	*Diheterospora chlamydosporia*
Cyclosporin A	*Tolypocladium inflatum*
Enduracidin A	*Streptomyces fungicidicus*
Globomycin	*Streptomyces globocacience*
Bacillomycin L	*Bacillus subtilis*
Iturin A	*Bacillus subtilis*
Putisolvin I and II	*Pseudomonas putida*
Arthrofactin	*Arthrobacter*
Fengycin	*Bacillus thuringiensis CMB26*
Mycobacillin	*Bacillus subtilis*
Polymeric surfactants	
Lipoheteropolysaccharide (Emulsan)	*Acinetobacter calcoaceticus RAG-1, Arethrobacter calcoaceticus*
Heteropolysaccharide (Biodispersan)	*Acinetobacter calcoaceticus* A_2
Polysaccharide protein	*Acinetobacter calcoaceticus strains*
Manno-protein	*Saccharomyces cerevisiae*
Carbohydrate-protein	*Candida petrophillum, Endomycopsis lipolytica*
Mannan-lipid complex	*Candida tropicalis*
Mannose/ erythrose lipid	*Shizonella melanogramma, Ustiloga maydis*
Carbohydrate-protein-lipid complex	*Pseudomonas fluorescences, Debaryomyces polymorphus*
Liposan	*Candida lipolytica*
Alasan	*Acinetobacter calcoaceticus*
Protein PA	*Pseudomonas aeruginosa*
Particulate biosurfactants	
Membrane vesicles	*Acinetobacter* sp. *H01-N*
Fimbriae, whole cells	*Acinetobacter calcoaceticus*

Table 1: Major types of biosurfactants produced by microorganisms.

The present review will focus on the recent advances in the field of biosurfactant production. Emphasis will also be laid on the correlation between biosurfactants and esterases. The recombinant strains which show enhanced production of biosurfactants as well as esterases can be harnessed in industrial operations for bulk production. This area of research has very scarce literature available and therefore has many promising research prospects and unexplored edges.

Biosurfactants and their Classification

Biosurfactants have many applications in the field of bioremediation. The term 'biosurfactant' refers to any compound from microorganisms, which has some influence on interfaces i.e. surface active agents, which brings down the interfacial tension between the two liquids. The minimum surface tension value reached and the critical micelle concentration (CMC) needed are the parameters used to measure the efficiency of a surfactant [8]. A successful biosurfactant can reduce the surface tension of water or growth medium from 72 mN/m to around 27 mN/m [9]. Biological surfactants have many advantages over their chemical similitude's as they are easily degraded by the microorganisms, they have low toxicity, they can be produced from very cheap raw materials, they are not easily affected by environmental factors such as temperature, pH, ionic strength and they have the unique property of biocompatibility and digestibility.

Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties present within the same molecule. The hydrophobic moiety of a biosurfactant is either a long-chain fatty acid, hydroxy fatty acid or α-alkyl β-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or an alcohol. While, synthetic surfactants are usually classified according to the nature of their polar groups, microbial biosurfactants are generally classified mainly on the basis of their biochemical composition and microbial origin. The microbial surfactants are complex molecules in the range of peptides, fatty acids, glycolipids, rhamnolipids, lipopeptides and sophorolipids. The low molecular weight biosurfactants are glycolipids whereas the high molecular weight microbial surfactants are generally polyanionic heteropolysaccharides containing both polysaccharides and proteins. The detailed classification of microbial biosurfactants is presented in table 1.

Improvement in Biosurfactant Production and their Applications

Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible substrates. The production of biosurfactants by microorganisms can be during exponential growth or it may be during the stationary phase of growth when the nutrient limiting conditions start prevailing in the growth medium. In case of growth associated biosurfactant production, there exists a parallel relationship between substrate utilization, growth and biosurfactant production. Production of biosurfactants by some microorganisms might be attributable to the presence of certain genes that are turned on in the presence of particular hydrocarbons. These microorganisms are distributed among a wide variety of genera. The hydrocarbon utilizing and biosurfactant producing microbes are mainly from the genera *Bacillus, Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Arthrobacter, Rhodococcus, Mycobacterium, Corynebacterium* and *Candida.*

Studies were undertaken in order to determine how common

culturable surfactant producing bacteria are in undisturbed and contaminated sites [10]. Out of 1305 colonies screened for biosurfactant production in mineral salts medium containing 2% glucose, from the contaminated and undisturbed soils, 45 isolates were positive for biosurfactant production. These 45 isolates were grouped using repetitive extragenic palindromic (REP)-PCR analysis, which yielded 16 unique isolates. 16S rRNA gene sequence of each isolate, revealed one new biosurfactant producing microbe, a *Flavobacterium* sp. Therefore, it was concluded that the biosurfactant producing microbes are present in most soils and can be isolated even by using a relatively limited screening assay.

Extensive studies in order to examine whether biosurfactant production is growth associated were conducted and it was concluded that in *Acinetobacter calcoaceticus* RAG-1, emulsan or emulsan-like precursors accumulate as capsular material during the exponential growth phase but are released into the medium when the rate of protein synthesis declines i.e. during the stationary phase of growth [11-14]. Similarly, the production of emulsan in *A. calcoaceticus* and the fermentative production of surface-active agents from *Bacillus cereus* IAF-346 and *Bacillus* sp. IAF-343 [15] were found to be growth associated. The production of surfactin in culture broth of *Bacillus subtilis* [16], rhamnolipids by *Pseudomonas aeruginosa* [17], exopolysaccharides in *A. calcoaceticus* BD4 [18] and rhamnolipid AP-6 in *P. fluorescens* 378 [19] were all found to be growth associated. In *Bacillus subtilis* SK320, the production of biosurfactant was also found to be growth associated [3]. Maximum biosurfactant activity was associated with cells at the stationary phase of growth when the nutrient limiting conditions started establishing in the culture medium.

In view of the wide range of industrial applications of biosurfactants as given in table 2, the current biosurfactant industry is targeting the key parameters affecting the production of microbial surfactants in terms of higher yields and lower production costs. The main approach to achieve the target are through screening of the appropriate microorganism, the use of cheap or waste substrates to reduce the cost of production, media optimization and cost-effective downstream processes to maximize recovery of the end product. Surfactin, produced by *Bacillus subtilis*, is a cyclic lipopeptide characterized by a β-hydroxycarbonic acid moiety with strong surface activity as well as antibiotic properties. Production of lipopeptide antibiotic surfactin was carried out using a recombinant *Bacillus subtilis* [20]. Surfactin yield by the recombinant strain was about 1½ times as much as that of *Bacillus subtilis* RB14, the strain in which the surfactin gene originated. Out of the 13 strains of *Bacillus subtilis* tested for the coproduction of the lipopeptide surfactin and the antifungal lipopeptides of the iturin family, only one produced both lipopeptides with a high yield on synthetic medium [21]. Several L-amino acids and various carbon sources were good substrates for this lipopeptide production. The maximum yield of surfactin was about 110 mg/L and that of iturin A about 39 mg/ L /absorbance unit for the best strain, *Bacillus subtilis* S 499.

Various cheap substrates such as vegetable oils and oil wastes, plant-derived oils, lactic whey and distillery wastes, starchy substrates, olive oil mill effluent, animal fat, soap stock and molasses have the potential for enhancing biosurfactant production [22]. Glucose has been the carbon source of choice of various researchers [23-26]. However, *Candida bombicola*, one of the few yeast's to produce biosurfactants can produce sophorolipids from both vegetable oils and sugars [27]. Highly insoluble carbon source such as n-hexadecane, paraffinic oil, glycerol, babassu oil for *P. aeruginosa* PA1 [28], soybean curd residue (okara) for *B. subtilis* YB8 and *B. subtilis* MI113 [29,30],

Applications	Role of biosurfactants
Environmental	
Soil remediation and flushing	Emulsification through adherence to hydrocarbons, dispersion, foaming agent, detergent, soil flushing.
Bioremediation	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration.
Agriculture	
Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypo-virulence.
Phosphate fertilizers	Prevent caking during storage
Spray application	Wetting, dispersing, suspending of powered pesticides and emulsification of pesticide solutions, promote wetting, spreading and penetration of toxicant
Petroleum production /products	
Enhanced oil recovery	Improving oil drainage into well bore, stimulating release of oil entrapped by capillaries, wetting of solid surfaces, lowering of interfacial tension, dissolving of oil.
De-emulsification	De-emulsification of oil emulsions, oil solubilization viscosity reduction, wetting agent.
Drilling fluids	Emulsify oil, disperse solids, and modify rheological properties of drilling fluids for oil and gas wells.
Biological	
Microbiological	Physiological behavior such as cell mobility, cell communication, nutrient accession, cell-cell competition, plant and animal patho-genesis.
Pharmaceuticals and therapeutics	Antibacterial, antifungal, antiviral agents, adhesive agents, immuno modulatory molecules, vaccines, gene therapy
Cosmetic	
Health and beauty products	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action.
Food and beverages	
Emulsification and	Emulsifier, solubilizer, demulsifier, suspension, wetting, foaming, defoaming, thickener, lubricating agent.
de-emulsification	Interaction with lipids, proteins and carbohydrates, protecting agent.
Functional ingredient	For cleaning sanitizing
Food processing plants	Improve removal of pesticides and in wax coating
Fruits and vegetables	Solubilize flavor oils, control consistency, retard staling
Bakery and ice cream	Improve washing, reduce processing time
Crystallization of sugar	Prevent spattering due to super heat and water
Elastomers / plastics	
Emulsion polymerization	Solubilization, emulsification of monomers
Foamed polymers	Introduction of air, control of cell size
Latex adhesive	Promote wetting, improve bond strength
Plastic articles	Antistatic agents
Plastic coating/laminating	Wetting agents
Industrial cleaning	
Janitorial supplies	Detergents and sanitizers
De-scaling	Wetting agents and corrosion inhibitors in acid cleaning of boiler tubes and heat exchangers
Soft goods	Detergents for laundry and dry cleaning
Leather	
Skins	Detergent and emulsifier in degreasing
Tanning	Promote wetting and penetration
Hides	Emulsifiers in fat liquoring
Dyeing	Promote wetting and penetration

Table 2: Some major applications of biosurfactants.

peat hydrolysate for *B. subtilis* [31], soybean oil, safflower oil, glycerol for *P. aeruginosa* GS9-119 and DS10-129 [32] and sludge palm oil for *Klebsiella pneumoniae* WMF02 [33] have been reported to induce high biosurfactant production. Olive oil was an unconventional carbon source used for the production of lipopeptide biosurfactant by *Bacillus subtilis* SK320 [3] and biosurfactant produced by marine *Nocardiopsis* B4 [34]. The important finding was the temperature stability of both the biosurfactants increasing their scope of application at higher temperatures like in microbial enhanced oil recovery. Use of numerous inexpensive raw materials for the production of biosurfactants by various microbial species has been summed up in table 3.

Historical record is rich with data where researchers have worked with the culture parameters and used cheap substrates in order to achieve maximum yield of biosurfactants. Total production yield of the lipopeptides from *Bacillus subtilis* BBK-1 was about 480 mg/L at 30°C for 24 h [35], whereas *Bacillus* sp. strain IAF 343 gave the yield of 1 g/L on medium containing only water soluble substrates [15]. *P. aeruginosa* DS10-129 produced 4.31, 2.98 and 1.77 g/L rhamnolipid biosurfactant using soybean oil, safflower oil and glycerol, respectively, as substrates [32]. *Bacillus cereus* IAF 346 produced a monoglyceride biosurfactant

that lowered the surface tension of water to 28 mN/m with a yield of 1.6 g/L (pH 6.5) and 1.7 g/L (pH 7.0) [15]. *B. subtilis* grown on medium containing 4% glucose gave the yield of 1-2 g/L of biosurfactant with minimum surface tension of 27 mN/m [23]. *B. licheniformis* JF-2 anaerobically produced biosurfactants when grown in glucose rich medium and reduced surface tension of water to 28 mN/m [25]. Purified lichenysin A from *B. licheniformis* BAS50 decreased the surface tension of water to 28 mN/m with a yield of 70-160 mg/L [26]. The maximum yield of surfactin was about 110 mg/L by the strain *B. subtilis* S 499 [21]. In a recent study the biosurfactant yields obtained from the recombinant strains BioS a, BioS b and BioS c were 2.13, 2.20 and 2.45 g/L, respectively, which was much higher than obtained from parent *Bacillus subtilis* SK320 i.e. 1.2 g/L. The total yield reported as g/L was calculated after purifying the biosurfactant using acetone and weighing the lyophilized powder (Figure 1). The recombinant strains not only showed enhanced biosurfactant production, but an increase in the esterase activity as well as surface tension values (Table 4) [3]. Similarly, *Bacillus clausii* 5B when grown on minimal medium containing 1% (w/v) glucose as carbon source gave the total yield of 2.11 g/L after 96 h [36], whereas *Kocuria turfanesis* BS-J and *Pseudomonas*

Low cost or waste raw material	Biosurfactant type	Producer microbial strain
Soybean oil refinery wastes	Rhamnolipids	*Pseudomonas aeruginosa* AT10
Curd whey and distillery wastes	Rhamnolipid	*Pseudomonas aeruginosa* BS2
Turkish corn oil	Sophorolipids	*Candida bombicola* ATCC 22214
Sunflower and soybean oil	Rhamnolipid	*Pseudomonas aeruginosa* DS10-129
Sunflower oil	Lipopeptide	*Serratia marcescens*
Soybean oil	Mannosyl erythritol lipid	*Candida* sp. SY16
Waste frying oils (sunflower and olive oil)	Rhamnolipid	*Pseudomonas aeruginosa* 47T2 NCIB 40044
Soybean soap stock waste	Rhamnolipid	*Pseudomonas aeruginosa* LBI
Sunflower oil soap stock waste	Rhamnolipid	*Pseudomonas aeruginosa* LBI
Oil refinery wastes	Glycolipids	*Candida ntarctica and/or Candida apicola*
Rapeseed oil	Rhamnolipids	*Pseudomonas* sp. DSM 2874
Babassu oil	Sophorolipids	*Candida lipolytica* IA 1055
Potato process effluents	Lipopeptide	*Bacillus subtilis*
Cassava flour wastewater	Lipopeptide	*Bacillus subtilis* ATCC 21332 and *Bacillus subtilis* LB5a
Olive oil	Lipopeptide	*Bacillus subtilis* SK320
Sludge Palm oil	Phospholipid	*Klebsiella pneumoniae* WMF02

Table 3: Use of low cost carbon sources for the production of biosurfactants by various microbial species.

Source	Biosurfactant activity (OD 550 nm)	Esterase activity (IU ml-1)	Growth (OD 600nm)	Biosurfactant recovery (g/L)	Surface tension (dynes/cm)
Bacillus subtilis SK320	0.859	4.382	0.514	1.2	40.1
BioS a	1.354	8.293	0.351	2.13	38.4
BioS b	1.259	8.521	0.296	2.20	35
BioS c	1.198	8.465	0.489	2.45	30.7
E. coli DH5α	0.157	0.101	0.132	0.054	11.2

† Results represented as mean of at least three replicate experiments (Sekhon et al, 2011)

Table 4: *Bacillus subtilis* SK320 and its recombinants showing enhanced activities after successful cloning of the biosurfactant genes.

aeruginosa BS-P when grown on combined distillery waste with other industrial wastes showed the biosurfactant production of 0.967 g/L and 1.976 g/L, respectively [37]. Research conducted on *Lactobacillus delbrueckii* cultured with peanut oil cake as the sole energy source gave the total biosurfactant yield of 5.35 mg/ml [38] whereas growth of *Corynebacterium kutscheri* [39] and *Pseudomonas aeruginosa* [40] on waste motor lubricant oil and peanut oil cake as carbon source showed the lipopeptide production of 6.4 mg/ml and 8.6 mg/ml, respectively.

In many other recent studies the total biosurfactant yield has been improved by more innovative methods. Biosurfactant production by *Bacillus subtilis* SPB1 was effectively improved by response surface methodology, giving the total concentration of 2.93 (±0.32) g/L when 15 g/L glucose, 6 g/L urea and 1 g/L K_2HPO_4 was added in the media [41]. In yet another interesting study conducted on *Bacilus subtilis* SPB1, it was reported that inoculum age and density can drastically influence the yield and total cost of the fermentation process. After optimizing the inocula conditions, the yield of the lipopeptide produced by *Bacilus subtilis* SPB1 was enhanced to 3.4 g/L [42]. *Pseudomonas aeruginosa*

RS29 produced a rhamnolipid after 48 h of incubation at 370C and pH 7-8 and the yield obtained after optimizing the various environmental factors was 0.80 g/L [43].

Since the arrival of biosurfactants, the cost of production and selection of the appropriate cheap material are the main criteria and concern for manufacturers. But with the recent advancements in this field and global awareness among consumers for the bio-based products, it appears inevitable that in the coming few years the high quality microbially produced biosurfactants will completely replace the chemical emulsifiers in many industrial applications.

Correlation Between Biosurfactant Production and Esterase Activity

Acknowledging the advantages and applications of biosurfactant-esterase complex, the correlation in their production during the growth of microbes seemed to have caught attention of many researchers [3,14,44-48]. Studies undertaken on biosurfactant-esterase complex are given in chronological order in table 5.

More than two decades ago the first report on bioemulsifier-esterase complex showing effective emulsification of hydrophobic substrates appeared. Since then many researchers have tried to exploit this unique correlation. An esterase activity was observed both in the cell-free growth medium and on the cell surface of petroleum-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) [48]. It was shown that the enzyme released from the cell surface was either emulsan free or associated with the bioemulsifier. Because of this reason the role for cell bound esterase in the release of emulsan (mol. wt. 106) from the surface of *A. calcoaceticus* was proposed and in turn suggested that the release process of emulsan involves the cleavage of the ester bond involved in the association of emulsan with the cell surface. The extracellular polyanionic, heteropolysaccharide bioemulsifier emulsan produced by oil-degrading microorganism *A. venetianus* RAG-1 formed and stabilized oil-water emulsions with a variety of hydrophobic substrates. Removal of the protein fraction yielded a product, apoemulsan, which exhibited much lower emulsifying activity on hydrophobic substrates such as n-hexadecane. Part of this activity could be recovered by the addition of larger amounts of the crude denatured protein [49]. Purified deproteinized emulsan (apoemulsan, 103 kDa) consisted of D-galactosamine, L-galactosamine uronic acid (pKa, 3.05), and a diamino, 2-deoxy n-acetylglucosamine [50]. The key protein associated with the emulsan complex was later found to be a cell surface esterase [14]. The amphipathic properties of this biopolymer were due in part to the presence of 15% fatty acids covalently bound to the polysaccharide backbone in both ester and amide linkages [51]. When recovered from the growth medium, crude emulsan contained a complex consisting of about 10-20% protein, which also contributed to its amphipathicity and to the hydrocarbon substrate specificity [48,52]. Emulsan initially accumulated on the cell surface of RAG-1 cell as a minicapsule [12,13,53] and subsequently gets released into the medium as an active emulsifier when the cells approach the stationary phase [12]. The release of the bioemulsifier depends on the presence of a suitable carbon and nitrogen source in the medium [11]. These requirements were presumable due to the *de novo* synthesis of amino sugar precursors and subsequent polymer synthesis which accompanies release of emulsan minicapsule. It was shown that some of the properties of an exocellular esterase activity from RAG-1 interact with emulsan and that emulsan was acting as a substrate for the release of the esterase enzyme from the cell surface [48].

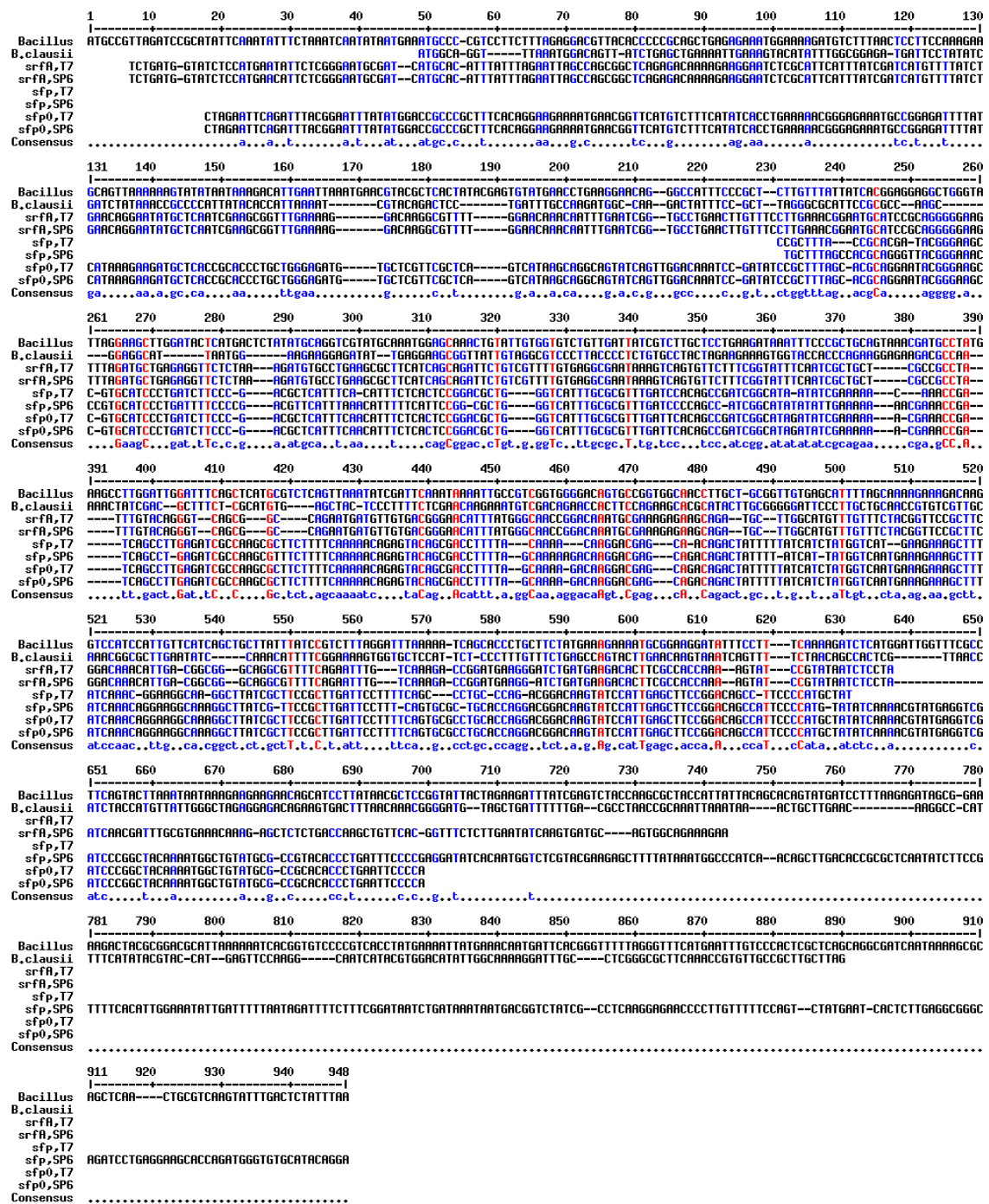It was repeatedly emphasized that the process of emulsan release

Figure 1: MultAlin (Multiple Sequence Alignment) of *sfp*, *sfp0* and *srfA* gene sequences with esterase gene sequences from NCBI database. The consensus sequences are shown in the last line of the alignment table. *Bacillus*=*Bacillus* sp. NK13 esterase gene (Liu et al. 2005, PubMed Accession number: DQ196347). *B. clausii*=*Bacillus clausii* KSM-K16 esterase gene (Hakamada et al. 1994, PubMed Accession number: AP006627). *sfp*, *sfp0* and *srfA* are gene sequences responsible for biosurfactant production in *Bacillus subtilis* SK320. (Sekhon et al, 2011)

requires the presence of an active esterase on the cell surface of *A. calcoaceticus* RAG-1 in presence of chloramphenicol [48]. In the absence of a carbon source, the esterase was released, whereas emulsan was retained on the cell surface. Once esterase was removed the emulsan was no longer released in the complete chloramphenicol system supplemented with a carbon source. Therefore, the release of esterase required the presence of nitrogen, but not necessarily a carbon source. Subsequently, the esterase enzyme was partially purified from the cell bound emulsan of *A. calcoaceticus* RAG-1. The partially purified enzyme catalyzed the hydrolysis of acetyl and other acyl groups from triglycerides and alkyl esters. Gel-filtration confirmed that the cell-free enzyme released from the cell surface was either emulsan free or associated with the bioemulsifier. The partially purified enzyme was found to interact specifically with the esterified fully active

Substrate/Carbon source used	Biosurfactant type	Producer strain	Finding	References
Absolute ethanol	Heteropolysaccharide bioemulsifier	*Acinetobacter calcoaceticus* RAG-1	Emulsan acts as substrate for esterase enzyme	[Shabtai and Gutnick, 1985]
Ethanol or Sodium acetate or Hexadecane or Crude oil	Heteropolysaccharide bioemulsifier	*Acinetobacter calcoaceticus* RAG-1	Emulsan producing RAG-1 capable of growing whereas emulsan deficient mutant failed to grow on crude oil	[Pines and Gutnick, 1986]
Glucose	Mannoprotein emulsifier	*Saccharomyces cerevisiae*	Mannoprotein acting as effective bioemulsifier	[Cameron et al, 1988]
Glucose or ethanol	Heteropolysaccharide bioemulsifier	*Acinetobacter calcoaceticus* RAG-1	Esterase coding gene from RAG-1 cloned-expressed in *E.coli. E.coli* able to grow on triglycerides such as TAC.	[Reddy et al, 1989]
Sodium acetate	Polysaccharide	*Acinetobacter radioresistens* KA53	Protein moiety essential for structure and emulsifying activity of bioemulsifier, alasan	[Navon-Venezia et al, 1995]
Absolute ethanol	Heteropolysaccharide bioemulsifier	*Acietobacter venetianus* RAG-1	Exocellular protein esterase enhances emulsifying activity of emulsan. Deproteinized product, apoemulsan, failed to form/stabilize oil-water emulsions.	[Bach et al, 2003]
Ethanol	Heteropolysaccharide bioemulsifier	*Acietobacter venetianus* RAG-1	*his*-tagged recombinant from RAG-1 formed hexadecane-in-water emulsions with 18 different polysaccharides	[Bach and Gutnick, 2006]
Olive oil	Lipopeptide	*Bacillus* sp. SK320	Esterase and biosurfactant activity exhibiting *Bacillus* sp. and its recombinant, capable of emulsifying olive oil completely	[Khanna et al, 2009]
Olive oil	Lipopeptide	*Bacillus* sp. SK320	Enhanced biosurfactant production through cloning of three genes and role of esterase in biosurfactant release	[Sekhon et al, 2011]

Table 5: Studies undertaken on biosurfactant-esterase complex.

emulsan, but not with the de-esterified polymer. A role for esterase in emulsan release from the cell surface was indicated when the enzyme was preferentially depleted from the cell surface under conditions in which emulsan was not released. Such cells lost the capacity to release the biopolymer.

The cell surface esterase (molecular mass 34.5 kDa) associated with the emulsan was later characterized, cloned and over-expressed in *Escherichia coli* BL21 (DE3) behind the phage T7 promoter with the His-tag system and after over-expression, recovery of about 80 to 90% of the protein in the inclusion bodies was reported [14]. The over-expressed esterase was recovered from the inclusion bodies by solubilization with deoxycholate and, after slow dialysis, was purified by metal chelation affinity chromatography. Mixtures containing apoemulsan and the recombinant esterase isolated from cell extracts formed stable oil-water emulsions with very hydrophobic substrates such as hexadecane under conditions in which emulsan itself was ineffective. Similarly, a series of esterase-defective mutants were generated by *site-directed* mutagenesis, cloned, and over-expressed in *E. coli*. Mutant proteins defective in catalytic activity as well as others apparently affected in protein conformation were also active in enhancing the apoemulsan-mediated emulsifying activity. Other proteins, including a His-tagged over-expressed esterase from the related organism *A. calcoaceticus* BD4, showed no enhancement. Various other microbial proteins have been reported to play a constructive role in emulsification. The *his*-tagged recombinant esterase from *Acenitobacter venetianus* RAG-1 has been shown to confer emulsifying activity on 18 different polysaccharides from microbial, plant, insect and synthetic sources and formed stable hexadecane-in-water emulsions. Emulsions in presence of 7 polysaccharides exhibited 80% stability [54].

In another study conducted in 1986 [55] researchers have shown that emulsan-deficient mutants of *A. calcoaceticus* RAG-1 grew very poorly on crude oil when compared with the parent strain, regardless of whether emulsan was supplemented in the medium or not. Results emphasized that the capability of producing emulsan facilitated the cells to grow on crude oil. Furthermore, a putative esterase gene (est) from

A. calcoaceticus RAG-1 was cloned into *Escherichia coli* [47]. Esterase-positive clones exhibited high levels of esterase activity even in intact cells. In addition, expression of the *est* gene conferred on *E.coli* the ability to grow on simple triglycerides such as triacetin (TAC), utilizing it as a source of carbon and energy. Mutants of RAG-1 defective in esterase were found to be defective in emulsan production and release. These two studies were in accordance with the formation of emulsion in the presence of olive oil by the recombinant cells of *Bacillus subtilis* SK320 emphasizing that the cloning of the biosurfactant gene conferred on the *E.coli* cells the ability to utilize olive oil as a sole carbon source [3,44,45]. The genes *sfp* (1210 bp), *sfp0* (642 bp) and *srfA* (707 bp) (DDBJ/EMBL/GeneBank accession numbers: EU822921, EU822922, EU822923) of the recombinants strains BioS a, BioS b and BioS c were cloned and used to infer functional and evolutionary relationship between sequences in the database. The amino acid sequences from *sfp, sfp0* and *srfA* biosurfactant genes were aligned with two of the esterase gene sequences: *Bacillus* sp. NK13 esterase gene, complete cds [56] (PubMed Accession number: DQ196347) and *Bacillus clausii* KSM-K16 DNA, complete genome, esterase gene [57] (PubMed Accession number: AP006627). The results revealed similarity as well as conserved family characteristics between biosurfactant genes and esterase genes taken from the database for multiple alignments, concluding that the biosurfactant production genes had some role to play for the release of esterase protein in the culture medium (Figure 1) [3].

The role of a protein (or an enzyme) in the emulsifying activity of biosurfactants is difficult to rule out. This was recognized when the mannoprotein of *Saccharomyces cerevisia* (a major component of the cell wall and an effective bioemulsifier) after treatment with a proteolytic enzyme pronase lost its ability to stabilize emulsions [58], just as treatment with proteinases or phenol, the protein component of alasan, a bioemulsan, isolated from *Acinetobacter radioresistens* KA53 is left with no emulsifying activity [59,60]. Alasan activity was shown to be sensitive to pronase and 95% emulsifying activity was lost when protein component (apoalasan) was removed with hot phenol treatment. In case of *A. calcoaceticus* BD4, both fractions of the protein-

Hydrophobic substrate	Average emulsifying activity (SD)	
	Emulsan (U/mg)	Apoemulsan+esterase (U/mg of apoemulsan/ mg of esterase)
Anthracene	0	970 (170)
Crude oil	1,100 (140)	4,900 (800)
Dicyclohexane	310 (50)	2,800 (500)
Diesel oil	730 (130)	4,200 (700)
Eicosane	0	1,800 (260)
Fluoranthene	0	590 (150)
Heptadecane	120 (25)	2,400 (380)
Immersion oil	75 (15)	780 (100)
2-Methylnapthalene	0	2,000 (400)
Mineral oil	450 (80)	3,000 (400)
Octadecane	0	2,200 (320)
Petroleum refinery sludge	580 (95)	1,200 (220)
Pyrene	0	420 (75)
Soya oil	0	1,300 (190)
Squalene	0	600 (150)
n-Tetracosane	0	510 (75)

[Gutnick et al, 2003]

Table 6: Enhancement of apoemulsan activity on different hydrophobic substrates by recombinant esterase.

polysaccharide complex are absolutely required for emulsification [61], while in *Acinetobacter radioresistens* KA53 the protein itself is the active emulsifying agent [62]. Boiling or incubating the lipopeptides purified from *Bacillus subtilis* SK320 and its recombinants BioS a, BioS b and BioS c with proteinase K led to a decrease in emulsifying activity of the lipopeptide biosurfactant [3]. Further it was shown that the protein in emulsan is not an active bioemulsifier by itself but rather enhances the activity of the polysaccharide component, apoemulsan. The portion of the esterase protein that is responsible for emulsification enhancement still needs to be identified [14] and possibly a challenging field of research.

The genes responsible for the biosynthesis and control of emulsan heteropolysaccharide emulsan were also targeted [63]. The genes were cloned, sequenced and shown to exist within a single 27 kbp gene cluster termed as *wee* regulon in the *A. venetianus*, but little is known about the protein components of the emulsan complex. Emulsan released from bacterial cell surface is apparently mediated by one of the key proteins i.e by the action of cell surface esterase which is encoded by the est gene (909 bp). This *est* gene has been cloned, sequenced and over-expressed in *E. coli* [64]. Mutants deleted for several of these genes were defective in emulsification, indicating that the polysaccharide is absolutely essential for extracellular emulsifying activity. In support of this finding the apoemulsan recombinant-esterase mixture was investigated for emulsification of a wide range of pure and crude oil products from a variety of sources and the activities were compared with that of fully proteinated emulsan (Table 6) [14], and results revealed that the esterase-apoemulsan complex was actually more effective in emulsifying a variety of hydrophobic substrates that are normally not emulsified by crude emulsan itself [14].

There is increasing interest for isolating new enzymes and new enzyme producing strains for their use in industrial conversions [65]. Enzymes like lipases, esterases, cellulases, xylanases and pectinases play a significant role in many industrial operations. Bacteria produce and secrete esterases which typically show the highest activity towards the soluble state of its substrate [66]. According to recent findings, esterases play a role in degradation of natural materials and industrial pollutants like cereal wastes, plastics and some other toxic chemicals

[7]. Some esterases may be involved in plant pathogenicity, carbon source provision and biocide detoxification [67], others catalyze inter-esterification, aminolysis, and peracid formation [68]. Not only this; lipases and esterases have also been used successfully in organic synthesis of optically pure substances, perfumes and antioxidants [7,69-76].

The studies till date on the biosurfactant-esterase complex focused on the emulsifying capability of the complex which has been utilized for bioremediation purpose due to its extraordinary surface-active property. Future research must be focused on better understanding of the interaction between the biosurfactant-esterase complex and the hydrocarbon, and the degradation pathway of the hydrocarbon.

Future Prospects and Concluding Remarks

Due to their environmentally friendly nature and increased awareness among the masses for natural products, biosurfactants have carved a niche for themselves in the market today. Manufacturers are staking money on biosurfactants because of their promising properties. Using mutants or super active microbial strains with high yielding capacities and cheap renewable substrates as raw material the production of biosurfactants has been ameliorated at industrial level. Current market trends show that the demand of biosurfactants such as rhamnolipids, glycolipids, lipopeptides, phospholipids and sophorolipids is going to increase manifolds because of their utility in detergent, paint, cosmetics, textile, agriculture, food and pharmaceutical industries. Biosurfactants are put to maximum usage for their environmental applications such as bioremediation and dispersion of oil spills. The addition of microbial surfactants enhances the solubility and removal of some major soil contaminants. Biosurfactant (bioemulsifier) production with esterase activity in the culture media shows two-fold increase in the emulsification of hydrophobic substrates ascertaining that the biosurfactant-esterase complex could also be instrumental in the remediation of polluted sites.

Acknowledgements

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