Chapter 1

Review of literature
1.1. Applications of biosurfactants: Novel Antimicrobial & antiadhesive agents

1.1.1. Abstract:
Biosurfactants are a class of molecules produced by diverse microorganisms and are composed of hydrophilic part and lipophilic moieties. By virtue of their amphipathic nature, they are known to reduce surface and interfacial tension and exhibit excellent emulsification properties. Increasingly higher number of biosurfactants are reported to possess additional biological properties. Of major interest from the commercial point of view are the abilities of these biosurfactants to act as antimicrobial and antiadhesive agents. With the emergence of drug resistant bacteria and the concerns for the need of non-toxic biomedicines, biosurfactants offer a lucrative option. Different types of biosurfactants viz., lipopeptides, rhamnolipid and sophorolipids produced by innumerable bacteria have been reported to exhibit antimicrobial action against pathogenic bacteria, yeast and fungi. These can therefore be used in formulation of novel biomedicines. Additionally, biosurfactants have been reported to possess antiadhesive properties. They are known to retard biofilm formation on voice prosthesis, silicone rubber and innumerable other inanimate objects. Of major interest are antiadhesive lipopeptide and glycolipid biosurfactants produced by different bacteria like Lactobacillus, Bacillus, Streptococcus and Candida. These biosurfactants equipped with antimicrobial and antiadhesive capacity can be looked upon as possible alternative medicines and biofouling agents for the benefit of mankind. In the present review, we have attempted to compile and organize recent available information on diverse bacteria exhibiting antimicrobial and antiadhesive activity.

Keywords: Biosurfactants, antimicrobial, Surfactin, lipopeptide, glycolipid, antiadhesive, prosthesis, silicone rubber

1.1.2. Introduction:
Surfactants are amphipathic molecules, which reduce surface and interfacial tensions between liquids, solids and gases and confer excellent detergency, emulsifying, foaming and other versatile chemical properties. Biosurfactants are surface active molecules produced by various microorganisms and exhibit structural diversity, low toxicity and biodegradability (Xu et al, 2011;...
In addition to surface tension reduction, biosurfactants exhibit other activities of biological interest such as antimicrobial, antitumor, and antiviral activities (Rodrigues & Teixeira, 2010; Cameotra & Makkar, 2004; Lang & Wagner, 1993). Biosurfactants find wide range of applications in various areas including cosmetics, pharmaceutical formulations, agriculture, food industry, oil recovery and environment protection technology (Xu et al., 2011; Pacwa-Plociniczak et al., 2011; Nitschke et al., 2009; Kitamoto et al., 2002; Desai & Banat, 1997; Kosaric, 1992).

Increasingly large numbers of surface active agents are reported to possess antimicrobial and antitumor properties (Cameotra & Makkar, 2004). Few biosurfactants exhibit these activities against specifically Gram positive or Gram negative bacteria, others exhibit a broad range of antimicrobial action, while still others are antifungal. Some researchers also report production of biosurfactants with both antibacterial as well as antifungal potential. Thus these surface active agents may score as potential new drugs, replacing the antibiotics to which bacteria are rapidly acquiring resistance (Vatsa et al., 2010; Heerklotz & Seelig, 2001). Additionally with development of new species and resistant microbial forms like the Gram negative MRSA search for novel bioactive compounds is the need of the hour (Pardesi, 2009). Hence biosurfactants with their low toxicity and biocompatibility can be used as alternative biomedicines (Rodrigues & Teixeira, 2010; Gharaei-Fathabad, 2011). They can also be thus useful in formulation of antibacterial creams or lotions because of their inherent property to emulsify two immiscible liquids, thereby doing a dual function and reducing the production and formulation costs. Numerous studies on biological activities of biosurfactants have been initiated at the lab scale (Thanomsub et al., 2006). These studies can be scaled up to an industrial level for large scale synthesis of novel eco-friendly biomaterials. In this review, attempt has been made to organise information on applications of biosurfactants as novel natural antimicrobial and antitumor agents.

1.1.2.1. Antimicrobial activity of lipopeptide antibiotics:

Among the several categories of biosurfactants, lipopeptides are particularly interesting because of their high surface activities (Rodrigues et al., 2006a). A large number of cyclic lipopeptides, including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) are produced by diverse bacteria. These biosurfactants consist of a lipid moiety attached to a polypeptide chain (Muthusamy et al., 2008).

1.1.2.1.1. Surfactin:

Surfactin is a lipopeptide biosurfactant produced by cells of Bacillus subtilis ATCC 21332. It is the most powerful biosurfactant known for lowering the surface tension of water from 72 to 27 mN/m and is also a potent antibiotic inhibiting growth of large number of bacteria. It is composed of a seven amino-acid ring structure coupled to a fatty-acid chain via lactone linkage (Beven & Wroblewski, 1997; Nakano et al., 1988; Cooper et al., 1981, Arima et al., 1968).
Surfactin produced by \textit{B. subtilis} is effective against most multidrug resistant organisms like \textit{E. faecalis}, \textit{S. aureus}, \textit{P. aeruginosa} and \textit{E. coli} (Femandes et al, 2007). Recently, Bernue et al (2009) showed that a strain of \textit{B. pumilus} (SP21) isolated from a marine sediment sample collected in the Bahamas was able to produce five surfactin analogs each exhibiting antimicrobial activities. The surfactin analogues included glycocholic acid, amicoumacins A and B in addition to three new compounds named lipoamides A–C. The surfactin analogues showed good activity against \textit{P. vulgaris}, \textit{E. faecalis} and methicillin resistant \textit{S. areus}. Violeta et al (2011) isolated a \textit{Bacillus} strain B2 from oil spilled soil with the capacity of biosurfactant production. This biosurfactant surfactin exhibited strong antifungal potential against \textit{Alternaria tenuis}.

Surfactin is toxic to mycoplasmas which are known to contaminate mammalian cell lines. It is known to interact with cell membranes and change the cell permeability owing to its surfactant properties. Additionally low cytotoxicity of surfactin to mammalian cell is notable. Single treatment with surfactin showed improved proliferation rate and changes in morphology of contaminated cell lines. Treatment with surfactin is a fast and simple method of eradication of \textit{M. hyorhinis} contaminants from cell lines (Vollenbroich et al, 1997). Souto et al, 2004 have reported antifungal activity of surfactin and iturin like compounds from \textit{Bacillus amyloliquefaciens} BNM 122 against \textit{Fusarium, Rhizoctonia} and \textit{Sclerotinia} species. Bais et al (2004) using a sensitive plant infection model demonstrated that surfactin, a lipopeptide biosurfactant released by \textit{B. subtilis} strain 6051 was responsible for biocontrol against infection of \textit{Arabidopsis} roots by \textit{P. syringae}. The mutant \textit{B. subtilis} M1 strain incapable of synthesizing surfactin did not offer protection against infection. Their results indicate that upon root colonization, \textit{B. subtilis} 6051 forms a stable, extensive biofilm and secretes surfactin, which act in unison to protect plants against attack by pathogenic bacteria. Surfactin derived from Iranian native \textit{B. subtilis} isolates exhibited anti-phytoplasma towards \textit{Candidatus Phytoplasma aurantifolia}, the agent which is responsible for causing the lime Witches’ broom disease. The authors used an absolute quantitative real-time PCR system to monitor the phytoplasma population shifts in the lime phloem during 3 months following the injections with surfactin. Though lone application of surfactin was efficient in eradication of the pathogen, it was synergistically higher when co-injected with tetracycline (Askari et al, 2011). \textit{Bacillus mojavensis} an endophytic bacterium produced a cyclic lipopeptide surfactin which was mycotoxic to the fungus \textit{F. verticillioides}. Collisional ion dissociation (CID) analysis indicated that this surfactin was a cyclic heptapeptide linked to a β-hydroxy fatty acid. The peptide sequence consisted of two acidic amino acids and five hydrophobic amino acids with a sequence of Leu-Leu-Asp-Val-Leu-Leu-Glu (Snook et al, 2009).

Large number of other biosurfactants from the \textit{Bacillus} genera have been reported to be potent antimicrobial agents (Sen, 2010). Recently Pakpitchaoren et al, 2008, have reported a large number of bacteria of the genus \textit{Bacillus} to produce antibacterial and surface active compounds. Another biosurfactant from \textit{Bacillus coccus} NK1 also exhibited wide range of antimicrobial activity against...
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E. coli, E. fergussonii, Klebsiella varicolla, Bacillus subtilis, B. licheniformis, B. megaterium and Saccharomyces cerevisiae (Sriram et al, 2011). B. subtilis R14 strain has been investigated previously for agricultural application and has proven to be an effective biocontrol agent against important phytopathogens ( Monteiro et al, 2005).

Although surfactin exhibits strong antimicrobial potential with detergent action, the application of surfactin as a strong, biodegradable detergent for technical and household purposes can be envisaged but would require much cheaper production methods (Heerklotz & Seelig, 2001).

1.1.2.1.2. Other lipopeptide biosurfactants:

Lipopeptide biosurfactants have been reported from bacteria belonging to the genera other than Bacillus by different researchers. These biosurfactants are also effective antimicrobials and can be used to replace their synthetic counterparts. Gudina et al, 2010a have shown that crude biosurfactant form Lactobacillus paracasei demonstrated antimicrobial activity against E. coli, P. aeruginosa, S. aureus, S. epidermidis, S. agalactiae and S. pyogenes by the broth microdilution method. This biosurfactant was very potent and resulted in 83 to 100 percent reduction in growth of these microorganisms. The same group has reported antimicrobial and antiadhesive activity of another biosurfactant from a strain of Lactobacillus paracasei A20 (Gudina et al, 2010b). This biosurfactant at a very high concentration of 50 mg/ml inhibited almost completely growth of tested bacteria and fungi. It was effective against twelve different micro-organisms including pathogenic E. coli, S. aureus, S. epidermidis and S. agalactiae. It also was effective as an antifungal agent against Candida albicans and skin-associated pathogenic fungi T. mentagrophytes and T. rubrum.

Antimicrobial activity of a biosurfactants from marine strains are been studied by various researchers lately. Kiran et al (2010) studied a lipopeptide biosurfactant from a marine Brevibacterium aureum. This lipopeptide biosurfactant exhibited a broad range of antimicrobial activity against Gram positive and negative bacteria including E. coli, P. mirabilis, H. streptococcus, P. aeruginosa, S. epidermidis, E. faecalis, K. pneumoniae, M. luteus, S. aureus, Bacillus and against C. albicans. Candida was highly susceptible to the biosurfactant with an area of inhibition of around 600 mm², while M. luteus was most resistant with a zone of inhibition of just 100 mm². Biosurfactant produced by a marine B. circulans exhibited strong antibacterial activity against broad range of Gram positive and negative bacteria and multidrug resistant bacterial isolates. Antibacterial action was seen against M. flavus, B. pumilis, M. smegmatis, E. coli, S. aureus, P. vulgaris, C. freundii, K. aerogenes, B. bronchiseptica, E. cloacae, P. mirabilis, A. calcoaceticus and S. marcescens while antifungal activity was observed against C. albicans and A. niger. These properties largely depended on structure of the compound. On comparison of FTIR spectra with surfactin, the authors proved that the biosurfactant was a lipopeptide. The non haemolytic nature of B. circulans lipopeptide was most striking (Mukherjee et al 2009; Das et al, 2009; Das et al, 2008).

A lipopeptide biosurfactant produced by Bacillus natto TK-1 exhibited antibacterial activity against
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Rodrigues et al (2006c) reported that lipopeptide biosurfactant from Streptococcus thermophilus A exhibited antimicrobial activity against S. epidermidis, S. salivarius, S. aureus, R. dentocariosa, C. albicans and C. tropicalis at concentrations ranging from 2.5 to 40g/L. In their other study by Rodrigues et al (2004), the authors report antimicrobial activities of biosurfactant from Lactobacillus lactis. This biosurfactant inhibited the growth of different bacterial and fungal strains isolated from explanted voice prostheses viz., S. epidermidis, S. salivarius, S. aureus, R. dentocariosa, C. albicans and C. tropicalis. This attribute could be used for prolonging the life of voice prosthesis. Lichenysin A, a biosurfactant produced by B. licheniformis BASS50 exhibits antibiotic activity against A. calcoaceticus, A. eutrophus, B. subtilis, E. coli, Enterobacter sp. strain 306, P. fluorescens and P. proteofaciens (Yukimov et al, 1995).

Fluorescent Pseudomonas spp. isolated from soil produced cyclic lipopeptides which showed antagonistic activity against root pathogenic fungi, Rhizoctonia solani and Pythium ultimum. This biosurfactant could be used as specific biocontrol agent either as a single species or in synergism with other microbes to overcome the antagonistic effects of inoculum. The antimicrobial activity of purified CLPs seemed somewhat higher for at least three of the larger amphisin-like compounds (amphisin, lokisin, and tensin) than that for the smaller viscosinamide and viscosinamide-like compounds (Nielsen & Sorensen, 2003; Nielsen et al, 2002; Nielsen et al, 1998).

1.1.2.1.3. Glycolipids:

Glycolipids Biosurfactants are carbohydrates in combination with long-chain aliphatic acids or hydroxylaliphatic acids (Satpute et al, 2010). The linkage is by means of either ether or an ester group. Among the glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids (Muthusamy et al, 2008). Rhamnolipids are subclass of glycolipids which contain one or two rhamnose sugars linked to a lipid moiety (Salihu et al, 2009; Yin et al, 2009; Heyd et al, 2008) and are a source of novel antibacterials.

Rhodococcus erythropolis isolated from contaminated sites in Riyadh area, Saudi Arabia was found to produce glycolipid type of biosurfactant. This biosurfactant exhibited high inhibitory activity against E. coli, P. aeruginosa, A. niger and A. flavus. The microbes examined by scanning electron microscope (SEM), were totally deformed and exhibited severe destruction. Abnormal cell division was observed at high frequencies among cells that tried to divide in the presence of the R. erythropolis glycolipids. Many cells were enlarged, elongated, empty hosts, or fragmented, consistent with the extremely low viability. This study ascertained the value of use of these glycolipids for the development of new anti-microbial materials for medical applications as dental surgery equipments, pharmaceuticals and water purification plants, (Abdel-Megeed et al, 2011). In another study rhamnolipid produced by a Pseudomonas strain has been reported to be effective in controlling brown root rot disease caused by Phytophthora cryptogea in the hydroponic forcing
system of witloof chicory (Cichorium intybus var. foliosum). (De Jonghe et al, 2005). Rhamnolipid production by two strains of Pseudomonas B1 and B2 growing on molasses as substrate was studied. The antimicrobial activity of the cultures was noteworthy against B. subtilis and E. coli. Antimicrobial activity increased in a dose dependant fashion (Onbasli & Aslim, 2008).

L. lactis produces glycolipid type of biosurfactant which was purified in three fractions by Rodrigues and co-workers (2006d). All three fractions showed antimicrobial activity against three different pathogenic organisms namely C.tropicalis, S. aureus and C. albicans. Two fractions had very low activity. Abalos et al (2001) report antifungal activity of rhamnolipids from P. aeruginosa At10. The rhamnolipids excreted by this strain are a mixture of seven homologues and shows excellent growth inhibition of Gram positive and Gram negative bacteria, filamentous fungi and phytopathogenic fungi B. cinerea and Rhizosclonia solani at concentrations of 18 mg/L. RL-1 & 2 inhibited growth of B. subtilis at higher concentration of 10-35 mg/L. and also exhibited zoosporicidal activity against Pythium aphanidermatum, P. capsici and Plamopara lacticaeradicis (Lang & Wullbrandt, 1999).

1.1.2.1.4. Sophorolipids and other surface active agents from Candida sp. :

These glycolipids, which are produced mainly by yeasts consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxyl fatty acid by glycosidic linkage. Generally, sophorolipids occur as a mixture of macro lactones and free acid form. It has been shown that the lactone form of the sophorolipid is necessary, or at least preferable, for many applications (Mathusamy et al, 2008).

Sophorolipid biosurfactant produced by Candida bombicola ATCC 22214 exhibited broad spectrum antimicrobial activity against B. subtilis, S. xylosus, S. mutans and P. acne. The biosurfactant was not only antibacterial but also exhibited antifungal activity against the plant pathogenic fungus B. cinerea. They suggested that the antimicrobial activity was due to an alteration in membrane permeability. Treatment of B. subtilis with the sophorolipid increased release of the intracellular enzyme malate dehydrogenase confirming its target site as the cell membrane (Kim et al, 2002). Sudha et al (2010) reported production of a potent biosurfactant Candida tropicalis when grown in small scale bench top fermenter running on a fed-batch mode. This sophorolipid exhibited antimicrobial activity against E. coli and B. subtilis. Two sophorolipids viz., SL-1 & 2 inhibited growth of R. subtilis, S. epidermidis & S. faecium at concentrations of 6-29 mg/L. Additionally, SL-2 also inhibited germination of conidia of the fungal strain G. cingulata at a concentration of 50 mg/L. The lactone forms of sophorolipids produced by C. apicola IMET 43733 inhibited the growth of both Gram positive and Gram negative bacteria viz., Bacillus sp., E. coli and S. marcescens.

In a very recent study, Lunasan a biosurfactant derived from Candida sphaerica exhibited highest antimicrobial activity against Streptococcus oralis (68%), Candida albicans (57%), and Staphylococcus epidermidis(57.6%) at a biosurfactant concentration of 10mg/mL. It was also effective against different species of Lactobacillus and Pseudomonas (Luna et al, 2011). In yet another study conducted in 2011, Rufino et al have reported highest antimicrobial activity of Candida lipolytica 0988 biosurfactant against different species of Streptococcus viz. S. ugalactiae.
S. mutans, S. mutans NS and S. mutans HG, S. sanguis 12 and S. oralis 322 with a concentration of 12 mg/ml of the biosurfactant. The biosurfactant from Candida lipolytica 0988 was also effective against strains of the genera Pseudomonas, Lactobacillus, Staphylococcus and Candida.

The antimicrobial capabilities of biosurfactants are being explored largely by the scientific world. Also efforts for isolation of these compounds are evident from the recent research in this field. The problem of combating multidrug resistant species of pathogenic bacteria can be addressed with much better strategies using biosurfactants. Thus biosurfactants with antibacterial and antifungal potential can be looked upon as lucrative option for developing newer biomedicines.

1.1.2.2. Biosurfactants as antiadhesive agents:

Although microorganisms are often considered as simple creatures, they are capable of complex differentiation and behaviours (O'Toole et al, 2000). These complex structures are referred to as biofilms. Biofilms in simple terms are communities of microorganisms which offer them advantages over single cells in terms of offering protection against desiccation and drying, conferring resistance to antibiotics and hazardous materials and acting as a reservoir of nutrients and ions (Hughes et al, 1998; Allison, 1993).

Bacterial biofilms have a structurally complex and dynamic architecture and develop on many abiotic (plastic, glass, metal and minerals) and biotic (plants, animals and humans) surfaces (Thenmozhi et al, 2009; Hall-Stoodley et al., 2004; Stoodley et al. 2002). Biofilm-associated diseases caused by Gram-positive bacteria include caries, gingivitis, periodontitis, endocarditis and prostatitis (Hall-Stoodley et al, 2004). Development of surface-attached biofilm bacterial communities is considered an important source of nosocomial infections. Surface attachment is an initiating step in pathogenesis of most of the organisms. Microbial colonisation causes economic losses in clinical settings and medical implants, such as catheters, prosthetic heart valves and joint replacements (Pour et al, 2011; Falagas & Makris, 2009). Biofilm formation leads to blockage of prostheses, catheters and loss of function in implants. Such colonised implants must be immediately replaced. Thus biofilm formation reduces lifespan of such medical devices (Laccourreye et al, 1997; Van den Hoogen et al, 1996).

Mechanical cleaning of the fouling can be very expensive and not always feasible. Hence, use of antimicrobial compounds like vancomycin against bacterial pathogens and fluconazole against fungal pathogens is in vogue. However, bacteria are increasingly acquiring resistance to antimicrobial agents (Pandess, 2009). Number of biosurfactants have been reported to reduce adhesion of pathogenic organisms and detrimental biofoulers to animate and inanimate surfaces. Prior coating of surfaces with biosurfactants have been shown to retard colonization and biofilm formation by large number of bacterial stains. Additionally, biosurfactants can also be used as flushing agents, by virtue of their ability to disrupt and eradicate preformed biofilms (Falagas & Makris, 2009). Rodrigues et al (2007) have elaborately described different strategies for the prevention of microbial biofilm formation especially on silicone rubber voice prostheses. Thus these
find applications as antiadhesive and antibiofouling agents. This can help in reducing economic and health losses due to colonization by bacteria. Biosurfactants thus find potential applications in medicine, pharmaceutical and healthcare industries, environment clean up and biofouling control (Gharafi-Fathabad, 2011; Cameotra & Makkar, 2004). The numerous advantages of biosurfactants, such as mild production conditions, lower toxicity, higher biodegradability and environmental compatibility make it a lucrative option for medical purposes (Rodrigues et al, 2006d; Cameotra & Makkar, 2004).

1.1.2.1. Antiadhesives for voice prosthesis:

Voice prostheses are a boon to patients who have undergone a laryngectomy due to a malignant laryngeal tumor and need to breathe through a tracheostomy, thus being useful in speech rehabilitation (Rodrigues et al, 2004). However, these prostheses have a tendency to become quickly colonized by microorganisms and are thus subjected to severe bacterial and fungal biofouling (Busscher et al, 1998; Neu et al, 1994, 1993 & 1992). This biofilm causes leakage of food and liquid, and may block the valves and increase resistance to airflow (Mahieu et al, 1986). This necessitates the need of replacement of these prosthesis every 3 to 4 months. This problem is overcome by using antibiotics. Bacteria are increasingly acquiring resistance to antimicrobial agents (Pardesi, 2009; Elving et al, 2000); hence search for novel biomedicines has become imperative. Biosurfactants exhibiting antiadhesive action can be looked upon as possible alternatives. Large number of reports appears on antiadhesive potential of biosurfactants from Lactobacillus strains.

Use of probiotic bacteria producing biosurfactants exhibiting antimicrobial and antiadhesive properties has also been explored as a method of choice for prolonging the lifetimes of the voice prosthesis by researchers and preventing nosocomial infections (Falagas & Makris, 2009). As early as 1998, Busscher et al. suggested that the deterioration of voice prostheses can be lessened by the daily intake of buttermilk through its inhibitory effects on biofilm formation. In a study by Rodrigues et al (2006b) it was found that conditioning of silicone rubber with rhamnolipids from P. aeruginosa DS10-129 resulted in reduction of S. salivarius and C. tropicalis adhesion rates by 66%. Minimal planktonic cell attachment and biofilm formation to the surface of voice prostheses was reported after conditioning. Rodrigues et al (2004) used a throat model to assess the efficiency of biosurfactants as probable antiadhesive agents. They cultured these bacteria on silicone rubber voice prostheses in presence of adsorbed biosurfactants from two probiotic bacteria viz., L. lactis 53 and S. thermophilus A. This lead to a substantial reduction in microbial numbers on prostheses and also induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation. Based on their results, the authors suggest biosurfactant application as an alternative strategy for prolonging the lifespan of the voice prostheses.

L. lactis and S. thermophilus produce biosurfactants which decrease the amount of bacteria in a multi-species biofilm on voice prosthesis (Rodrigues et al, 2004). The authors had postulated presence of O antigen (surface lipopeptide) as surface active agent. Surfactin can inhibit growth of
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preformed biofilm causing dispersal of colonies. It inhibits Salmonella enterica adhesion to PVC (polyvinyl chloride) sheets without inhibiting the growth. In another study, disruption of Bordetella bronchiseptica biofilms by rhamnolipids was demonstrated (Irie et al, 2005).

Busscher at al (1994) reported that thermophilic dairy streptococcal strains S. thermophilus produced surface active agents and might have great potential for fouling control in pasteurizers. In 1997, Busscher et al continued their studies and found that biosurfactant released by adhering strains of Streptococcus thermophilus B inhibited adhesion of Candida albicans and Candida tropicalis naturally colonizing voice prostheses. The biosurfactant was produced in response to the competition between bacteria and yeast for surface attachment and form a conditioning layer which interfered with yeast adhesion on the surface. The crude biosurfactants released were mixtures of various components, with a glycolipid-like component being the most surface active. The biosurfactant releases a powerful defense weapon to ward off post-adhesion competition of the bacterium with microorganisms such as yeasts. Pre-adsorption of biosurfactants to the silicone rubber prior to yeast adhesion was also as effective though only against C. albicans.

From all these studies, it can be said that biosurfactants represent biocompatible compounds that could be considered for developing future strategies for preventing microbial colonization of silicone rubber voice prostheses.

1.1.2.1.2. Antiadhesives from probiotic bacteria:

In more recent years, the use of probiotics per se and lactobacilli specifically has received great attention as an alternative, inexpensive, and natural remedy to restore and maintain health (Reid, 2001; Hamilton-Miller,1997; Shalev et al, 1996; McGroarty, 1993). Lactobacillus strains appear to be effective at colonizing and protecting the intestine (Biller et al, 1995; Saxelin et al, 1995; Isolauri et al, 1991; Gorbach et al, 1987) and urogenital tract (Marelli et al, 2004; Heinemann et al, 2000; Bruce & Reid, 1988; Hilton et al, 1995; Reid et al, 1994) against microbial infection.

Antiadhesive property has been attributed to the production of surface active agents by Lactic acid bacteria by various researchers. Velraeds et al have done tremendous studies on the antiadhesive potential of biosurfactants starting from 1996. Velraeds et al (1996) studied 15 isolates of Lactobacillus for their ability to produce biosurfactants. Further they showed that biosurfactants secreted by both L. acidophilus RC14 or L. fermentum B54 effectively inhibited the adhesion of uropathogenic Enterococcus faecalis to glass surfaces. The biosurfactant from L. fermentum B54 were richest in protein, while those from L. casei subsp. rhamnosus 36 and ATCC 7469 had relatively high polysaccharide and phosphate contents. Following which, in the same year they reported that stationary phase biosurfactants from L. casei subsp. rhamnosus 36 and ATCC 7469, L. fermentum B54, and L. acidophilus RC14 were able to inhibit the initial adhesion of uropathogenic E. faecalis 1131 to glass in a parallel-plate flow chamber. They found that the initial deposition rate of E. faecalis to glass with an adsorbed biosurfactant layer from L. acidophilus RC14 or L. fermentum B54 significantly decreased by approximately 70%, while the number of adhering
enterococci after 4 h of adhesion was reduced by an average of 77% (Velraeds et al, 1996). Heinemann et al (2000) in continuation with this work determined the nature and properties of this antiadhesive cell surface protein effective against Enterococcus faecalis 1131. They determined that this biosurfactant was a protein, with N-terminal sequence of the 29-kDa protein identical to that of a collagen-binding protein from Lactobacillus reuteri NCIB 11951, and exhibited close homology with a basic surface protein from L. fermentum BR11. This biosurfactant could be efficiently used in controlling biofilms of uropathogenic bacteria.

In the succeeding years, Velraeds et al (1998) studied the antiadhesive potential of a biosurfactant surlactin produced by Lactobacillus acidophilus RC14 in a parallel-plate flow chamber in filter-sterilised pooled human urine. Surlactin efficiently inhibited initial adhesion of various uropathogenic bacteria and two yeast strains to silicone rubber. Surlactin was highly potent antiadhesive and exhibited marked inhibition of the initial deposition rates and adhesion numbers for 11 of 15 strains tested. Inhibition was particularly effective against E. faecalis, E. coli and S. epidermidis. Thus, indicating its use in catheter and related explants.

Fracchia et al (2000) reported antiadhesive potential of biosurfactant derived from Lactobacillus sp. CV8LAC against two human pathogenic biofilm forming strains of Candida albicans (CA-2894 and DSMZ 11225) in pre-coating and co-incubation experiments. In pre-coating assays, biofilm formation of the strain CA-2894 was reduced by a whooping 82% at a concentration of 312.5 μg/ml and that of DSMZ 11225 by 81% at 625 μg/ml. In co-incubation assays, biofilm formation of CA-2894 and DSMZ 11225 was inhibited by 70% at 160.5 μg/well and by 81% at 19.95 μg/well concentration respectively. The authors suggest potential use of this biosurfactant as an antiadhesive product on medical devices like catheters, prosthesis and stents to prevent C. albicans infections.

Brzozowski et al (2011) studied in detail the structure and adhesive capability of biosurfactants produced by L. fennenti 126 and L. rhamnosus CCM 1825. They found both biosurfactants consisted of protein, polysaccharide and phosphate in varying concentrations. The authors found a positive effect of the impregnation of polystyrene surface with an aqueous solution of biosurfactants on the inhibition of adhesion of E. coli 22, K. pneumoniae 2 and P. aeruginosa W2 strains.

1.1.2.1.3. Antiadhesives for other biomedical applications:

Antiadhesive activity complemented with the antimicrobial activity exhibited by most of biosurfactants can be utilised to devise a very proficient infection preventive strategy. Bacteria residing in oral cavity are capable of releasing biosurfactants. Glycolipid type of biosurfactants from two streptococcus mitis strains (BA and BMS) found in oral cavity significantly reduced the glass surface adhesion of Streptococcus mutans NS with or without salivary coating. This suggests, that biosurfactants play a pivotal role in preventing oral biofilms (Van Hoogmoed, 2000).
Biosurfactants from the strains of the genera *Bacillus* have also been reported to have antiadhesive potential. A lipopeptide biosurfactant from *Bacillus natto* TK1 exhibited antiadhesive activity against *S. typhimurium*, *E. coli* and *S. aureus* (Cao et al, 2009). Another lipopeptide biosurfactant from *B. cereus* NK1 strongly inhibited biofilm formation by pathogenic microbes, *P. aeruginosa* and *S. epidermidis*. The highest biofilm inhibition was observed against *S. epidermidis* with inhibition percentage of upto 57% (Sriram et al, 2011). Biosurfactant from marine *B. circulans* exhibited antiadhesive activity against *E. coli, M. flavus, S. marcescens, S. typhimurium, P. vulgaris, C. freundii, A. faecalis* and *K. aerogenes*. The biosurfactant inhibited not only biofilm formation but also dislodged preformed biofilms of the test microorganisms (Das et al, 2009). Surfactin and rhamnolipids are also reported to inhibit adhesion of *Listeria monocytogenes* (de Araujo et al, 2011). The biosurfactant from two *Bacillus* spp. namely *B. subtilis* and *B. licheniformis* are very efficient in inhibiting the pathogenic biofilms especially of *E. coli* and *S. aureus*. It was observed that the surface adhesion is greatly reduced on precoating of biosurfactant to the surface as well as on addition to the inoculum. The lipopeptide biosurfactants consists of two fractions. The fraction belonging to fengycin-like family was mainly active as anti adhesive agent against pathogenic strains of *Escherichia coli* and *Staphylococcus aureus* (Rivardo et al, 2009).

Very recently in 2010, Zeraik & Nitschke studied the effect of surfactin and rhamnolipid biosurfactants on the attachment of *S. aureus* and *L. monocytogenes*. They preconditioned polystyrene surfaces with biosurfactants and then assessed their antiadhesive potential at different temperatures viz., 35, 25, and 4 °C. The results showed that surfactin was able to inhibit bacterial adhesion in all the conditions analyzed, giving a 63–66% adhesion reduction in the bacterial strains at 4 °C. Rhamnolipid promoted a slight decrease in the attachment of *S. aureus*. The anti-adhesive activity of surfactin increased with the decrease in temperature, showing that this is an important parameter to be considered in surface conditioning tests. Their studies indicated that Surfactin showed good potential as an anti-adhesive compound that can be explored to protect surfaces from microbial contamination. Nitschke et al (2008) in another study showed that preconditioning of stainless steel and polypropylene surfaces caused a reduction on the number of adhered cells of the food pathogens *Enterobacter sakazakii* and *Listeria monocytogenes*.

Rodrigues et al, 2006c reported antiadhesive property of biosurfactant from by *S. thermophilus* A. These authors reported a maximum adhesion inhibition of 40±4 and 7±4 by the crude biosurfactant and its purified fraction respectively against *S. epidermidis* GB 9/6 at 40 g/L of the biosurfactant. The authors have reported that the biosurfactant from *S. thermophilus* A exhibited antiadhesive activity against *S. salivarius, S. aureus, R. dentocariosa, C. albicans* and *C. tropicalis* in addition to *S. epidermidis* GB 9/6. The same group has also demonstrated antiadhesive activity against the same microorganisms by another surfactant derived from *L. lactis* 53 (Rodrigues et al, 2006d). In a more recent study, Gudnina et al (2010a & b) have reported a dose dependant antiadhesive activity of a biosurfactants from *Lactobacillus paracasei* and *L. paracasei* A20. Promising antiadhesive activity was observed against several pathogenic micro-organisms such as *S. aureus, S. epidermidis* and *S.
agalactiae, with less activity against E. coli, C. albicans and P. aeruginosa by biosurfactant from Lactobacillus paracasei A20.

Biosurfactant from Candida exhibits excellent antiadhesive activity against strains of the genera Lactobacillus and Streptococcus (Rufino et al, 2011). Recently Lunasan a biosurfactant from Candida sphaerica UCP 0995 at concentration of 10 mg/ml inhibited adhesion between 80 and 92% of P. aeruginosa, S. agalactiae and S. sanguis. Inhibition of adhesion with percentages near 100% occurred for higher concentrations of biosurfactant used (Luna et al, 2011).

Rhamnolipid preconditioned PTFE surface was able to reduce Listeria monocytogenes attachment at rates greater than 90%. These surface active molecules were derived from Pseudomonas fluorescens (Meylheuc et al, 2001). Further work demonstrated that the prior adsorption of P. fluorescens surfactant on stainless steel also favoured the bactericidal effect (Meylheuc et al, 2006). Studies on Pseudomonas fluorescens surfactant proved potential of biosurfactant as corrosion preventing agent for stainless steel (O’Toole & Yak, 2005).

These studies prove the huge potential of biosurfactants as anti adhesive agents. The various types of biosurfactant molecules produced by variety of micro organisms can be specifically used for eradication and control of detrimental biofilms. Due to increasing reports on understanding of formation and regulation of biofilms, the scope of application of biosurfactant as antiadhesive agents has broadened.

1.1.3. Conclusions:

Studies on biosurfactants have opened a new horizon for novel biocompatible antimicrobial and antiadhesive agents. In last decade most of the pathogenic bacterial and fungal microorganisms have developed resistance towards the modern synthetic antibiotics. This has urged the scientists to explore different natural roles of biosurfactants produced by several organisms. Their role as antimicrobials and antiadhesives makes them favourable candidates for application in drug development. The only hurdle in the application of biosurfactants on commercial scale is the cost effective production. Further studies on cheaper methods for biosurfactant production would overcome the hurdle of biosurfactant commercialisation. The non conventional applications are increasing exponentially and biosurfactants can be looked upon as novel biomaterials for health and well being. These robust bioactive biosurfactants can thus find applications in varied fields like cosmetics, medicine, pharmacy and environmental clean-up.

1.1.4. References:


Abstract

Biosurfactant (BS)/bioemulsifier (BE) produced by varied microorganisms exemplify immense structural/functional diversity and consequently signify the involvement of particular molecular machinery in their biosynthesis. The present chapter aims to compile information on molecular genetics of BS/BE production in microorganisms. Polymer synthesis in *Acinetobacter* species is controlled by an intricate operon system and its further excretion being controlled by enzymes. Quorum sensing system (QSS) plays a fundamental role in rhamnolipid and surfactin synthesis. Depending upon the cell density, signal molecules (autoinducers) of regulatory pathways accomplish the biosynthesis of BS. The regulation of serawettin production by *Serratia* is believed to be through non ribosomal peptide synthetases (NRPSs) and N-acylhomoserine lactones (AHLs) encoded by QSS located on mobile transposon. This regulation is under positive as well as negative control of QSS operon products. In case of yeast and fungi, glycolipid precursor production is catalyzed by genes that encode enzyme cytochrome P450 monooxygenase. BS/BE production is dictated by genes present on the chromosomes. This chapter also gives a glimpse of recent biotechnological developments which helped to realize molecular genetics of BS/BE production in microorganisms. Hyper-producing recombinants as well as mutant strains have been constructed successfully to improve the yield and quality of BS/BE. Thus promising biotechnological advances have expanded the applicability of BS/BE in therapeutics, cosmetics, agriculture, food, beverages and bioremediation etc. In brief, our knowledge on genetics of BS/BE production in prokaryotes is extensive as compared to yeast and fungi. Meticulous and concerted study will lead to an understanding of the molecular phenomena in unexplored microbes. In addition to this, recent promising advances will facilitate in broadening applications of BS/BE to diverse fields. Over the decades, valuable information on molecular genetics of BS/BE has been generated and this strong foundation would facilitate application oriented output of the surfactant industry and broaden its use in diverse fields. To accomplish our objectives, interaction among experts from diverse fields like microbiology, physiology, biochemistry, molecular biology and genetics is indispensable.

Introduction

Enormous structural and functional diversity are implicated in biosurfactant (BS)/bioemulsifiers (BE) produced by microorganisms. BS/BE possesses remarkable applications in diverse fields. With the need for green chemicals, their study is becoming imperative. Therefore, BS/BE
studies have been focused on by large number of researchers. However, commercial production of these compounds is quite expensive. Use of cheaper and renewable substrates is a necessity. However, a great deal of monetary input is required in purification processes. Thus, it represents two faces of a coin; so to overcome this dilemma and subsequently economize and commercialize BS production a better understanding at molecular level is mandatory.

Literature survey illustrates that detailed studies of BS/BE production have been carried out in *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Serratia*, *Candida* spp. BS producing microbes from different resources, viz., fresh water, soil, marine, oil wells and industrial effluents have been studied extensively. Among these natural resources, marine environment is attracting interest from many researchers due to its vastness and novelty with respect to products that can be obtained. However, this survey clearly illustrates that the maximum reports are focused on rhamnolipid and surfactin production from *Pseudomonas* and *Bacillus* spp. respectively. Few researchers have reviewed the enormous data generated on BS/BE production in microorganisms, briefing molecular biological aspects. However, it is important to note that, before and after Sullivan’s review on molecular genetics of BS not a single review is devoted exclusively to molecular biology of synthesis BS in microorganisms. A gap of ~10 years indicates that a compilation of molecular mechanisms involved in BS/BE production is essential. Enormous molecular and biotechnological developments have taken place in this decade and therefore, our understanding on the present topic has improved greatly. Therefore, present review is focused at compiling valuable developments in this area. To the best of our knowledge, this chapter would give comprehensive information on molecular genetics of BS/BE production in microorganisms.

Important Aspects Pertaining to Biosurfactant Production in Microorganisms

The mystery why microbes produce BS/BE is still unknown. Justifications include survival on various hydrophobic substrates and desorption from the hydrophobic substrates allowing direct contact with cell, thereby increasing the bioavailability of insoluble substrates. However, few microbes produce BS/BE on water soluble substrates. Different biosynthetic pathways and specific enzymes are involved. Synthesis takes place by de novo pathway and/or assembly from substrates. Based on the four assumptions proposed by Syldatk and Wagner, diagrammatic representations for biosurfactant synthesis in microorganisms is given in Figure 1. Induction/repression of BS/BE production are dependent on presence of carbon, nitrogen, phosphate, trace elements and multivalent cations. BS/BE production is controlled by environmental parameters. Literature survey suggests that complex pathways are involved in BS/BE production. BS/BE producing microbes may harbour plasmids. However, genes responsible for BS production are located on chromosomal DNA. Interacellular communication and production of enzymes, pigments and BS occurs by QSS which depends on the production of diffusible signal molecules termed autoinducers. The regulatory machinery is different for different BS/BE producers.

Molecular Genetics of Biosurfactant Production in Bacteria

**Acinetobacter Species**

*Acinetobacter* spp. are ubiquitous in nature, being isolated from various sources like soil, mud, marine water, fresh water, meat products etc. and reported for production of BE. *Acinetobacter* species are the most promising bacteria producing high molecular weight BS/BE. The first description of the best known marine BE, now exploited commercially as ‘Emulsan’ appeared in 1972. This emulsifier is produced by *A. calcoaceticus* RAG-1, isolated from the Mediterranean Sea. Emulsan produced by RAG-1 has a heteropolysaccharide backbone with a repeating trisaccharide of N-acetyl-D-galactosamine, N-acetylgalactosamine uronic acid and an unidentified N-acetyl amino sugar. Fatty acids (FA) are covalently linked to the polysaccharide through o-ester linkages. Different species of *Acinetobacter* are known to produce protein polysaccharide complexes. Proteoglycan type bioemulsifier is produced by *Acinetobacter junii*
Synthesis of both biosurfactant moieties dependent upon substrate

1. Both moieties independently synthesized by two different de novo pathways
2. Hydrophilic moiety
   Synthesis by De novo pathway & hydrophobic moiety
3. Hydrophobic moiety synthesis by De novo pathway and hydrophilic moiety is induced by substrate
4. Synthesis of hydrophobic moiety is induced by substrate
5. Synthesis of hydrophilic moiety is induced by substrate

Hydrophobic moiety
- Either a long-chain fatty acid, a hydroxy fatty acid, or α-alkyl β-hydroxy fatty acid
- Either a long-chain fatty acid, a hydroxy fatty acid, or α-alkyl β-hydroxy fatty acid

Hydrophilic moiety
- Carbohydrate, carboxylic acid, phosphate, amino acid, cyclic peptide, or alcohol

SC14. This bioemulsifier is made up of protein (50.5%), polysaccharide (43%) and lipid in a minor fraction (3.8%). 88.7% of the polysaccharide consisted of reducing sugars. About 16% of patents on BS have been reported from Acinetobacter spp. alone, which indicates the tremendous market potential of exopolysaccharide (EPS).

Emulsan
- It is a complex polysaccharide (9.9 × 10^7) produced by A. calcoaceticus RAG-1 and stabilizes oil-water emulsions efficiently. In spite of structural complexity, researchers have succeeded in identifying genes implicated in emulsan synthesis and emulsification phenomena. Polymer biosynthesis is accomplished by a single gene cluster of 27 kbp with 20 open reading frames (ORFs) called as wee regulon which contains weeA to weeK genes that accomplish polymer biosynthesis. Putative proteins encoded by the wee cluster have been tabulated by Nakar and Gutnick in detail.
These genes lead to the formation of polysaccharide containing amino sugars, with O-acyl- and N-acyl-bound side chain of FA. Further addition of intermediates takes place as follows: WeeA converts UDP-N-acetyl-D-glucosamine into UDP-N-acetylmannosamine. Consequently, WeeB oxidizes the UDP-N-acetylmannosamine into UDP-N-acetylmannosaminuronic acid. This regulon possess wea and wnb genes which are responsible for biosynthesis of emulsan. Gene products Wzc and Wzb were over expressed, purified and a bulk of polysaccharide was produced successfully. The WeeA or WeeB are possibly involved in formation of UDP-N-acetyl-L-galactosaminuronic acid. The gene WeeY further catalyses the formation of diamine 2,4-diamino-6-deoxy-D-glucosamine, a component of the repeating unit, from UDP-4-keto-6-deoxy-D-glucosamine. The sequence of WeeY is similar to dTDP-glucose 4,6-dehydratase and therefore could possibly be responsible for conversion of UDP-D-glucosamine into UDP-4-keto-6-deoxy-D-glucosamine. The overall process is summarized in detail by Nesper, et al. The monomers gather on a lipid carrier on the cytoplasmic face of the inner membrane. Subsequently, they are transferred by Wzx protein to the periplasmic face of the membrane. Wzy polymerase further catalyzes the polymerization process. Finally, lipid intermediates lead to the formation of a protein-polysaccharide complex which is transported across the periplasm to the outer membrane. This assembly gets accumulated on cell surface and is further excreted as polymer complex in the exterior.

Due to complex nature of exopolymers, genetic studies remained at a nascent level for a long period. However, with the advent of recent technologies and innovations, bioengineering of BE producing microorganisms has become possible. Complex polysaccharide backbone of emulsan was altered by modifying the culture conditions for *A. venetianus* RAG-1. The emulsan structure was modified by transposon mutagenesis of FA moiety. Analysis of various factors viz., yield, FA content, molecular weight and emulsification behavior demonstrated that parent strain yielded high emulsan as compared to mutant strain. The factors are dependent on the type of FA supplemented during the production process. However, cloning and sequencing of mutants with enhanced emulsifying activity indicated that they were involved in biosynthesis of emulsan. The presence and composition of long chain FAs on the polysaccharide backbone influenced emulsification behaviour. Such studies are highly significant and open newer avenues for applications of amphiphiles in diverse fields. Based on similar kind of studies, an interesting US patent (20040265340) on "Emulsan adjuvant immunization formulations" was filed by Kaplan, et al. The emulsan analog and mutants of *A. calcoaceticus* RAG-1 were produced in presence of different FA sources. Different molecular tools have been employed to modify and improve quality of emulsan produced by *Acinetobacter* spp. (Table 1).

**Apoemulsan**

It is an extracellular, polymeric lipoheteropolysaccharide produced by *A. venetianus* RAG-1. Purified deproteinized emulsan (apoemulsan, 103 kDa) consists of D-galactosamine, L-galactosamine uronic acid (pKa, 3.05) and a diamino, 2-desoxy N-acetylglucosamine. It retained emulsifying activity towards certain hydrocarbon substrates but was unable to emulsify relatively nonpolar, hydrophobic, aliphatic materials. It is now known that polymers are synthesized from Wzy pathway. However, there also appears a differing report which claims that the process is based on presence of polysaccharide-copolymerase (PCP). However, recently Dam-Kozlowska and Kaplan proved that synthesis of this polymer was dependant on Wzy pathway where, PCP protein controlled the length of the polymer. This was proved by inducing defined point mutations in the proline-glycine-rich region of apoemulsan PCP protein (Wzc). Five of the eight mutants produced higher weight BE than the wild type while four had modified biological properties. This study demonstrated the functional effect of Wzc modification on molecular weight of polymer and the genetic system controlling apoemulsan polymerization. It has been suggested that emulsifying activity and release of polymer is mediated via esterase gene est (34.5 kD). A study carried out by Leahy in 1993 proved that lipase is responsible for enhanced emulsification properties. Lipase negative mutants exhibited less emulsification activity. The gene est has been cloned and over expressed in *E. coli* BL21 (DE3) behind the phage T7 promoter with His tag system. Further Alon and Gutnick, also showed that est gene encodes protein that is located on the outer membrane.
Table 1. Employment of molecular tools for construction of recombinant/mutant strains of Acinetobacter and Pseudomonas spp.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mutation</th>
<th>Objective</th>
<th>Significant Feature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. calcoaceticus</td>
<td>CTAB (Cetyl trimethyl ammonium bromide)</td>
<td>Enhance the yield</td>
<td>High yield achieved</td>
<td>56, 57</td>
</tr>
<tr>
<td>RAG-1 (Emulsan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. venetianus</td>
<td>Recombinant point mutations in proline—glycine-rich region of the apoenzyme PCP protein (Wzc)</td>
<td>Alteration in Wzc gene</td>
<td>Molecular weight of polysaccharide was modified</td>
<td>58</td>
</tr>
<tr>
<td>RAG-1 (Emulsan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. calcoaceticus A2</td>
<td>N-methyl-N-nitrosoamine (NTG)</td>
<td>Alteration of wild type strain</td>
<td>Mutant produced equal/higher polysaccharide; rich protein also secreted along with it</td>
<td>59</td>
</tr>
<tr>
<td>(Biodispersan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A. calcoaceticus</td>
<td>Transposon</td>
<td>Disruption of genes involved in biosynthetic pathways of biotin, histidine, cysteine or purines</td>
<td>Modification in fatty acid (FA) metabolism influenced level and types of FA incorporated into emulsan</td>
<td>54</td>
</tr>
<tr>
<td>RAG-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A. venetianus</td>
<td>M13 and Primers for parent esterase gene</td>
<td>The pET system for over expression of esterase in E. coli BL21 carrying pESTAL-14b/pESTAL-11c and plasmid constructed by ligation of the Kpnl-Ndel-BamH1 fragment from parent est gene</td>
<td>Overproduction of HsO tagged recombinant esterase and affected protein confirmation that enhanced apoenzyme-mediated emulsifying activity</td>
<td></td>
</tr>
<tr>
<td>RAG-1 (Emulsan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A. lwoffii RAG-1</td>
<td>Mini Tn10Km</td>
<td>One region, transcribed in the upstream direction appears to encode for 3 complete ORFs, while the second region is transcribed in the opposite direction and encodes 17 complete ORFs</td>
<td>Identification of wee gene (27 kb) containing 20-ORFs for polysaccharide synthesis; Detect in emulsan production with specific activities of 5-14% of parental emulsifying activity</td>
<td>48</td>
</tr>
<tr>
<td>(Emulsan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>N-methyl-N-nitrosoamine (NTG)</td>
<td>Enhance yield of BS</td>
<td>Similar type of biosurfactant with 10 times more BS production</td>
<td>61</td>
</tr>
<tr>
<td>(Rhammolipid)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P. fluorescens</td>
<td>Tn5</td>
<td>Transposon integration into condensation domains of peptide synthetases</td>
<td>Rhammolipid reduced surface tension effectively</td>
<td>62</td>
</tr>
</tbody>
</table>
The same gene was sequenced and expressed in *E. coli*. High amount of esterase was found to be associated when cell was grown in presence of nitrogen. Variants resistant to cetyl trimethyl ammonium bromide (CTAB) showed enhanced emulsan production. Site directed mutagenesis revealed that esterase-defective mutants could not release emulsan. Mutant proteins defective were capable of enhancing apoenulans-mediated emulsifying activity. Bach, et al carried out studies on emulsan from *A. venetianus* RAG-1. It was seen that apoenulans and esterase are essential for the formation of stable oil-water emulsions.

**Alasan**
The polymer produced by *A. radioresistens* KA53 is designated as 'Alasan' and finds significant application in bioremediation. Alasan is an alanine containing complex heteropolysaccharide and protein polymer that stabilizes oil in water emulsions in n-alkanes with chain length 10 or higher and alkyl aromatics, liquid paraffin, soybean oil and crude oils. The proteins of alasan have been identified as AlnA, AlnB and AlnC. One of the alasan protein (AlnA) of 45 kDa exhibiting highest emulsification activity was purified and denoted high sequence homology to an OmpA-like protein from Acinetobacter spp. Four hydrophobic regions in AlnA forming specific structure on the surface of hydrocarbon are responsible for surface activity. The AlnB protein exhibited strong homology to peroxiredoxins (family of thiol—specific antioxidant enzymes). It was proposed that all three proteins may be released as a complex with AlnA entering the oil phase and AlnB forming a compact shell around the hydrocarbon, thereby forming stable emulsions. *A. calcoaceticus* RA57 grown on crude oil sludge possesses three plasmids, one of which pSR4, a 20 kb fragment was found to be essential for growth and emulsification of crude oil in liquid culture.

**Biodispersan**
It is an extracellular, anionic polysaccharide produced by *A. calcoaceticus* A2 which acts as a dispersing agent for water-insoluble solids. It is nondialyzable, with an average molecular weight of 54,400 and contains four reducing sugars, namely, glucosamine, 6-methylaminohexose, galactosamine uronic acid and an unidentified amino sugar. Rich protein was also secreted along with the extracellular polysaccharide. Protein defective mutants produced equal/enhanced biodispersion as compared to the parent strain.

**Exopolysaccharide (EPS)**
*A. calcoaceticus* BD4, BD413 produces EPS with rhamnose and glucose. EPS production is mediated by proteins like Ptk (protein tyrosine kinases) and was also found in *A. johnsonii*. These proteins encode for virulence factors and may serve as a target for the development of new antibiotics.

**Pseudomonas Species**
Glycolipid BS production was first discovered by Jarvis and Johnson in 1949. They reported production of an acidic, crystalline glycolipid L-rhamnose and L-β-hydroxydecanoic acid from *P. aeruginosa*. This compound was found to be quite similar to a compound of polymer and higher rhamnose-hydroxyacid ratio which was isolated previously by Bergstrom, et al. Later, Hauser and Kornovsky demonstrated the biosynthetic pathway for rhamnolipid production in *Pseudomonas* spp. Burger, et al and Lang and Wagner demonstrated that *P. aeruginosa* synthesizes mono as well as di-rhamnolipid. Similarly, *P. aeruginosa* synthesizes different rhamnolipid derivatives which include 3-(3-hydroxyvalerolactyl-3-hydroxydecanoic acid (HAA), mono-rhamnolipid (L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoic acid) and di-rhamnolipid (L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoic acid). Details of different intermediates have been accounted by Pamp and Tolker-Nielsen. However, studies on regulatory mechanisms came very late with the work of Ochse, et al and Latifi, et al who proposed the involvement of quorum sensing system (QSS) for rhamnolipid biosynthesis in *Pseudomonas* spp. Various components involved in rhamnolipid biosynthesis are
Biosurfactants

Q. excretion
rhamnolipid synthesis in Pseudomonas spp. by two quorum sensing systems. Pictorial representation of two quorum sensing systems (QSS) present at different regions of Pseudomonas spp. chromosome. Thick black bold arrows: Genes on chromosome of Pseudomonas; Black arrows: Protein synthesis from gene; Dotted oval indicates inactive regulatory protein; Continuous oval: Active complex of regulatory protein and autoinducer. Formation of mono and di-rhamnolipids is mediated through two different transferases viz., rhamnosyltransferase I and II. Rhamnolipid synthesis is coupled with nitrogen limitations to the cell. Phosphate limiting conditions are found to enhance biosynthesis. Detailed studies have been reported on rhamnosyltransferase I, which contains four genes viz., rhlA, rhlB, rhlR, rhlI. Plasmids encoding four genes are sufficient to produce rhamnolipid in heterologous hosts. Genes rhlA, rhlB are located upstream while rhlR, rhlI are located downstream of the structural genes. The rhlA and rhlB genes code for active rhamnosyltransferase I and are transcribed as a bicistronic RNA. Structural proteins are encoded by rhlB and present in the periplasm. Inner membrane proteins required for synthesis, transport or solubilization of rhamnosyltransferase are encoded on rhlA. In first QSS, genes rhlA, rhlB are positively regulated by rhlR. Transcriptional activator and autoinducer are encoded by rhlR and rhlI respectively. Two signal molecules viz., N-butanoyl-L-homoserine (PAI-2) and hexanoyl-L-homoserine lactone are produced by rhlA. Transcriptional activator produced by rhlR binds to autoinducer PAI-2 and this active complex causes transcriptional activation of rhlA and rhlB that encode

Figure 2. Rhamnolipid synthesis in Pseudomonas spp. by two quorum sensing systems: Pictorial representation of two quorum sensing systems (QSS) present at different regions of Pseudomonas spp. chromosome. Thick black bold arrows: Genes on chromosome of Pseudomonas; Black arrows: Protein synthesis from gene; Dotted oval indicates inactive regulatory protein; Continuous oval: Active complex of regulatory protein and autoinducer.
rhamnosyltransferase I. The second QSS contains two genes namely lasR and lasL. In this system autoinducer is encoded by lasI namely N-(3-oxododecanoyl)-l-homoserine-Lactone (PAI-1) RhlR regulatory protein requires autoinducers N-butyryl-HSL and N-(3-oxohexanoyl)-HSL autoinducer for its activity. Induction of second QSS occurs by cyclic AMP levels as indicated by the presence of lasR promoter region of both lux box and binding consensus sequence for cyclic AMP receptor protein. The transcription of rhlR system is positively regulated by las system. The rhl system is posttranslationally controlled by las system by hindrance of PAI-2 by PAI-1 from binding to RhlR. This situation is created till enough PAI-2 and/or PAI-1 are produced to create blockage effect. Figure 2 illustrates the regulation of rhamnolipid synthesis in *Pseudomonas* spp.

It is proved that rhlR expression is strongly influenced by environmental factors and is partially LasR-independet under certain culture conditions. Different regulatory proteins viz. Vir sigma factor O74 and RhlR itself regulates expression of rhlR.

The rhl negative mutant is unable to produce rhamnolipid on its own. However, addition of synthetic N-acylhomoserine lactone (signal molecule) initiates BS production by mutant. Holden, et al. carried out studies to find out whether the BS genes are expressed in unsaturated porous media contaminated with hexadecane and play role in biodegradation process. For this purpose, the gfp reporter gene was integrated with either the promoter region of *pra*, which encodes for the emulsifying PA protein and/or to the promoter of the transcriptional activator rhlR. It was found that GFP was produced in culture, which indicated that the rhlR and pra genes are both transcribed in unsaturated porous media. The expression was localized at the hexadecane-water interface. Other interesting studies carried out by Pamp and Tolker-Nielsen demonstrated the BS produced by *P. aeruginosa* has additional role in structural biofilm development. Genetic evidence showed that mutant deficient in *rhlA* lack the ability to synthesize BS and could not form microcolonies. This indicates significant role of *rhlA* in BS biosynthesis and biofilm development. The protein AlgR2 responsible for regulation of nucleoside diphosphate kinase also down regulates rhamnolipid production in *P. aeruginosa*. Lequette and Greenberg in 2005, worked on identifying the role of QSS responsible for rhamnolipid biosynthesis on biofilm architecture. They introduced a rhlA-gfp fusion into a neutral site in the *P. aeruginosa* genome and highlightened the activity of rhlAB promoter in rhamnolipid-producing biofilms. Campos-Garcia, et al. identified a new gene ghG which is a homologue of the fabG gene encoding NADPH-dependent β-ketoacyl acyl carrier protein (ACP) reductase. This is necessary for synthesis of FA. This gene rhlC is obligatory for synthesis of β-hydroxy acid moiety of rhamnolipids and partly contributes to production of poly-β-hydroxyalkanoate (PHA). This study proved that different pathways are involved in synthesis of FA moiety of rhamnolipids than those for general FA synthetic pathways.

Till the year 2001, it was obvious that, rhamnosyltransferase I (RhlAB) catalyses the synthesis of mono-rhamnolipid from dTDP-1-rhamnose and P-hydroxydecanoate, whereas di-rhamnolipid is produced from mono-rhamnolipid and dTDP-l-rhamnose. For the first time, Rahim, et al. in 2001, reported dependance of di-rhamnolipid synthesis on rhamnosyltransferase gene. Gene rhlC encode for rhamnosyltransferase which catalyses di-rhamnolipid (l-rhamnose-l-rhamnose-β-hydroxydecanoyl-β-hydroxydecanoate) production in *P. aeruginosa*. RhlC is a protein consisting of 325 amino acids (35.9 kDa). The rhlC gene is located in an operon with an upstream gene (PA1131) of unknown function. A σ^54-type promoter for the PA1131-rhlC operon was identified and a single transcriptional start site was mapped. Biological role of RhlC was confirmed by insertional mutagenesis studies and allelic replacement. Inhibition of QSS was demonstrated by work with mutants. Deletion mutants, complementation studies and northern blot analysis on *P. aeruginosa* strain PR1-E4; a lasR deletion mutant revealed that overproduction of the *P. aeruginosa* DksA homologue down regulated transcription of the autoinducer synthase gene rhl thereby inhibiting QSS.

*Pseudomonas* species are known to produce different types of BS viz., rhamnolipids, cyclic lipopeptides- putisolvins, lipopolysaccharide. Two types of cyclic lipopeptides (putisolvins I and II) are produced by *P. putida* PCL1445, which possess surfactant activity and also plays survival advantage in the presence of surfactant components in the environment. For the first time, Rahim, et al. in 2001, reported dependence of di-rhamnolipid synthesis on rhamnosyltransferase gene. Gene rhlC encode for rhamnosyltransferase which catalyses di-rhamnolipid (l-rhamnose-l-rhamnose-β-hydroxydecanoyl-β-hydroxydecanoate) production in *P. aeruginosa*. RhlC is a protein consisting of 325 amino acids (35.9 kDa). The rhlC gene is located in an operon with an upstream gene (PA1131) of unknown function. A σ^54-type promoter for the PA1131-rhlC operon was identified and a single transcriptional start site was mapped. Biological role of RhlC was confirmed by insertional mutagenesis studies and allelic replacement. Inhibition of QSS was demonstrated by work with mutants. Deletion mutants, complementation studies and northern blot analysis on *P. aeruginosa* strain PR1-E4; a lasR deletion mutant revealed that overproduction of the *P. aeruginosa* DksA homologue down regulated transcription of the autoinducer synthase gene rhl thereby inhibiting QSS.

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Biosurfactants

Wild type strain

Natural mutations:
- (N-methyl-N'-nitro-N-nitrosoguanidine)
- Radiation
- Resistance to ionic detergents (CTAB)

Transposon

Biosurfactant negative mutants

Chesed mutagens:
- Hydrophobic substrate

Figure 3. Effect of biosurfactant production on growth in presence of hydrophobic substrates.

significant role in biofilm formation and degradation. Mutants from Tn5luxAB library of strain PCL1627 defective in BS production contained transposon inserted in adaA homologue located downstream of gpeE and upstream of divA indicating positive regulation of these genes in BS synthesis. Two-component signaling system GacA/GacS was involved in BS synthesis. \(^{108}\) Studies on co-existence of *Burkholderia cepacia* and *P. aeruginosa* in lungs of cystic fibrosis (CF) patients as mixed biofilms correlated the formation of biofilms to cep-regulated BS production. \(^{110}\)

Generally hydrocarbon utilizing microbes produce BS. *P. aeruginosa* degrades hexadecane only if it can produce rhamnolipid. \(^{118,119}\) Mutated *Pseudomonas* spp. produce low rhamnolipid BS. \(^{116}\) \(^{116}\) Whereas, rhamnolipid defective mutants grow very poorly on hydrocarbons. \(^{117}\) Pictorial representation is given in Figure 3. Ability of hydrocarbon uptake can be improved by addition of BS in the growth medium. This concept was proved by various studies viz., Koch, et al. \(^{119}\) constructed a transposon TNS-3 GM induced mutant of *P. aeruginosa* PG201 which could not grow on minimal medium with hexadecane. It was found that the same culture grew well with rhamnolipid supplementation. Al-Tahhan, et al. \(^{118}\) showed that emulsifier makes the cell surface more hydrophobic through release of lipopolysaccharide (LPS). *P. aeruginosa* grew well on paraffin in presence of emulsifier in the production medium. All these observations clearly suggest role of BS/BE in survival of microbes on hydrophobic substrates. Natural or chemical mutations are employed to improve quality and yield of BS/BE from microorganisms. \(^{116}\) In the year 1995, Iqbal, et al. \(^{118}\) demonstrated hyper—production of BS, high biodegradation and emulsification of crude oil by an EBN-8 a gamma ray induced mutant of *P. aeruginosa*. The same mutant produced 4.1 and 6.3 of rhamnolipids (g/L) when grown on hexadecane and paraffin oil respectively. \(^{118}\) \(^{118}\) Another gamma ray induced *P. putida* 300-B mutant gave high yield of rhamnolipid (4.1 gL^{-1}) on soybean waste frying oil as carbon source and glucose as growth initiator over the wild type strain. \(^{118}\) A research team of Koch, et al. \(^{118}\) constructed a lactose utilizing strain of *P. aeruginosa* by insertion of *E. coli* lac Y genes. Two reporter systems, lacZY and luxAB, were incorporated into chromosome of *P. aeruginosa* UG2. This recombinant strain could utilize lactose and produced BS efficiently. Similar studies were also carried out by Flemming, et al. \(^{118}\) Their work proved to be efficient in sensitive detection and quantitative enumeration of *P. aeruginosa* UG2Lt (spontaneous rifampin-resistant derivative) using supportive data from antibiotic resistance, bioluminescence and PCR analyses. Oechsner, et al. \(^{118}\) constructed recombinant strains of
Molecular Genetics of Biosurfactant Synthesis in Microorganisms

P. putida and P. fluorescens by knocking down genes responsible for pathogenicity thereby produce harmless BS producing stains. This is the best example of application of molecular knowledge in producing biotechnologically improved stains.

Bacillus Species

Surfactin is a cyclic lipopeptide BS produced by Bacillus spp. The first report on surfactin production dates back almost to 4.5 decades. Arima, et al were the pioneer researchers who reported production of surfactin from Bacillus species. Surfactin the most effective BS reducing surface tension efficiently has low CMC (critical micelle concentration) value and finds potential applications in biotechnology and medicine. It is important to note that more than 70% of research on BS is accounted for Bacillus spp. alone. Surfactin production, structure, enzymes involved in biosynthesis, organization and genetics of production has been reviewed in great detail. Due to great potential of surfactin and its diverse applications, it became necessary to study the underlying genetic mechanisms. However, the advent of these studies was not until 1988. Kluge, et al laid the foundation for molecular studies by proposing a non ribosomal mechanism of surfactin synthetase. A brief summary of genetic machinery involved in surfactin synthesis is tabulated in Table 2.

Surfactin contains β-hydroxyl FA, usually β-hydroxytetradecanoic acid, synthesized by a 27 kb srfA operon. It is under regulation of QS. First QS involves nonribosomal peptide synthetases with four open reading frames (ORFs) in the srfA operon. Operon srfA catalyses three multi-functional enzymes for surfactin synthesis. These modular building blocks are called as surfactin synthetases encoded by srfA, srfB and srfC. The srfA locus plays a key role in surfactin production; Nakano and coworkers isolated srfA locus by cloning the DNA flanking srfA::Tn917 insertions followed by chromosome walking. This region was an operon (>25 kb) and the gene srfA codes for template enzymes while; another gene Sfp located downstream of the srfA operon encodes for 4'-phosphopantotheinyl transferase. This gene product modifies enzymes to their functional forms for their transcription. A third locus within srfA operon, the srfB gene is required for surfactin production. srfB is also necessary for expression of srfA-lacZ and is identical to an early competence gene comA. Surfactin production is under ComA (5r/5)-dependent regulation operating at the transcriptional level. srfA is positively regulated by product of 5r/5. Subsequently, SrfD stimulates the initiation process. However, release of surfactin is still unknown. There is an assumption that passive diffusion releases surfactin across the cytoplasm membrane. Once the cell density attains a maximum level, ComX get accumulated in the medium and interacts with membrane bound histidine kinase ComP and the response regulator ComA. Further, after phosphorylation, by ComP; ComA binds to promoter srfA and transcription begins. Competence stimulating factor (CSF), a signal peptide influences srfA expression. It is transported across the membrane and interacts with at least two different intracellular receptors depending upon its concentration. Mutation in ComA inhibits development of competence indicating that, comA gene is responsible for expression of srfA and other com genes. In addition to all these proteins, ComR and SinR also influence srfA expression. ComA is regulated positively as well as negatively by ComP under the control of the ComX pheromone. The authors also suggested that srfA expression requires SpoOK and another, as yet unidentified, extracellular factor under variable pH conditions. The gene spoOK codes for an oligopeptide permease that functions in cell-density-dependent control of sporulation and competence. Thus molecular machinery ensures appropriate surfactin synthesis.
Table 2. Genetic machinery involved in surfactin synthesis from Bacillus spp.

<table>
<thead>
<tr>
<th>Operon/Genes/Operator/Protein</th>
<th>Function</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Quorum sensing system I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>srfAA*</td>
<td>Amino acid activating domain for Glu, Leu, ß-Leu Expression of comS gene*</td>
<td>128,129</td>
</tr>
<tr>
<td>srfAC</td>
<td>Encodes a thioesterase of a Type I motif responsible for peptide termination</td>
<td>130</td>
</tr>
<tr>
<td>sfp</td>
<td>Surfactin production</td>
<td>131</td>
</tr>
<tr>
<td>Sfp'</td>
<td>Activation of surfactin synthetase by post translational modification</td>
<td>132,133</td>
</tr>
<tr>
<td><strong>Quorum sensing system II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ComQ</td>
<td>Modification of comX to form signal peptide ComX</td>
<td>133,134</td>
</tr>
<tr>
<td>ComP (Membrane bound protein)</td>
<td>Gets autophosphorylated upon stimulation and transfers its phosphate group to ComA</td>
<td>135</td>
</tr>
<tr>
<td>Phosphorylated ComA ComS</td>
<td>Binds comA-box and initiate transcription of surfactin peptide synthetase, srfAA-AD operon and comS Development of competence</td>
<td>136</td>
</tr>
<tr>
<td>ComX (Signal peptide)</td>
<td>Controls expression of srfA and interaction with • Membrane bound histidine kinase ComP • Response regulator ComA</td>
<td>137</td>
</tr>
<tr>
<td>SpoOK (Oligopeptide permease)</td>
<td>Transfer of Competence stimulating factor (CSF) through the cell membrane: Phosphotransferase activity</td>
<td>138</td>
</tr>
<tr>
<td>RapC</td>
<td>Enhances srfA expression posttranscriptionally</td>
<td>139</td>
</tr>
<tr>
<td>SmR (Transcriptional regulator)</td>
<td>Negatively controls srfA possibility by regulating comK</td>
<td>140</td>
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*: Multifunctional subunit of surfactin synthetase; †: Part of peptide synthetase; ‡: Embedded within but out of frame with srfB.

The sfp locus plays a significant regulatory role at the transcriptional level. The sfp locus from a producer strain *B. subtilis* ATCC 21332 was transferred to a standard *B. subtilis* 168 and further subjected to transposon mutagenesis. Studies suggested that, *B. subtilis* with a sfp genotype contains some genes required for surfactin synthesis; sfp locus responsible for surfactin production alters the transcriptional regulation of srf. A gfp gene with sequence homology to sfp gene from Gramicidin operon of *B. brevis* complemented in trans, a defect in the sfp gene and was able to initiate surfactin synthesis in a non producer strain *B. subtilis* JH642 with an sfp' phenotype. Additionally, Sfp gene is also responsible for hydrocarbon degradation. A sfp gene was successfully integrated in chromosome of *B. subtilis* to enhance bioavailability of hydrophobic liquids. Sequencing of sfp gene revealed 100% sequence homology to amino acid sequence reported earlier by Nakano, et al. A research team of Morikawa, et al worked on cloning and nucleotide sequencing of regulator gene in *B. pumilis*. Studies indicated that out of three large
ORFs (ORF1, 2, 3), ORF3 was essential for surfactin synthesis. Additionally, production of antimicrobial substances or other secondary metabolites is associated with resistance to the producing organism. Tsuge, et al. proposed function of gene yerP as a determinant of self resistance to surfactin in *B. subtilis* 168. YerP was homologous to the resistance, nodulation and cell division (RND) family of proteins, which confers resistance to wide range of noxious compounds to the secreting organism. Mutagenesis with mini-Tn10 transposon indicated that the transposon had inserted itself in the yerP gene in surfactin susceptible mutant. The molecular machinery for BS synthesis in *B. licheniformis* is similar to that in surfactin synthesis. A recombinant strain of *B. licheniformis* KGL11 was constructed by inserting the surfactin synthetase enzyme. This mutant produced 12 times the BS of parent strain. With better understanding of the molecular phenomena, many attempts were aimed to enhance BS/BE production. Mulligan, et al. were successful in obtaining a threefold higher BS production over wild type employing recombinant *B. subtilis* with modified peptide synthetase. A plasmid pC112 with lpa-4, a gene was used to construct a recombinant strain of *B. subtilis* MI113. High yield of surfactin was achieved by fermentation technology. Another recombinant strain of *Bacillus subtilis* MI113 (pC115), was constructed from *B. subtilis* RB14C. This recombinant strain had a gene responsible for surfactin, iturin production and produced new surfactin variants along with usual surfactin when cultured in solid-state fermentation employing soybean curd residue (okara) as substrate. Along with large number of research papers published, enormous patents on BS production appear to date. Carrera, et al. filed US. patents (5,264,363; 5,227,294) on *B. subtilis* ATCC 55033 mutant strain which produced 4-6 times better BS over wild type. Another US patent (7,011,969) on *B. subtilis* SD901 strain mutated with N-methyl-N’-nitro-N-nitrosoguanidine resulted in 4-25 times more surfactin production. Such studies are opening arrays for improved BS production technologies. Various mutant/recombinant strains of *Bacillus* spp. have been constructed for better quality and optimum quantity of surfactin production (Table 3).

**Serratia Species**

Followed by *Acinetobacter, Pseudomonas* and *Bacillus* strains, *Serratia* is one of the well-studied bacterium in terms of molecular genetic studies of BS production. *Serratia*, a Gram-negative organism is known to produce extracellular surface active and surface translocating agents. *S. marcescens* produces a cyclic lipopeptide BS ‘Serrawettin’ which contains 3-hydroxy-C10 FA side chain. BS production is correlated with populational surface migration. The mobility (swarming/sliding motility) and cell density of a population is monitored; depending on this information, regulatory systems control gene expression. This helps the microbial community in interacting with its surrounding. The SplR QSS is responsible for regulation of flagellum-independent population surface migration and synthesis of BS (prodigiosin) in *S. marcescens* SS1. Later on, Wei, et al. confirmed that *splR* quorum-sensing genes were located on a Tn3 family transposon, *Tn7*. They also proved that SplR negatively regulated transposition frequency of *Tn7*. This group for the first time reported direct evidence of involvement of a luxIR-type QSS in regulation of transposition frequency.

BS production is controlled by auto-induction system which subsequently helps in swarming of cells. *S. marcescens* ATCC 274 produces temperature dependant serrawettin W1[cyclo-(d-3-hydroxydecanoyl-L-seryl)]<sub>2</sub>. Presence of *swrW* gene encoding serrawettin W1 aminolipid synthetase was identified in *S. marcescens* 274 by transposon mutagenesis. The swrW had all four domains of nonribosomal peptide synthetase (NRP), responsible for condensation, adenylation, thiolation and thioesterification. The swrW NRP is unimodular and specifies only lysine. The authors also proposed a pathway for serrawettin synthesis based on their findings. Parallel production of serrawettin and pigment production in *S. marcescens* 274 is coded by an ORF namely *pswP*. Synthesis of serrawettin is believed to be through non ribosomal peptide synthetases (NRPSs) system which is a product of the *prod* gene. A single mutation in the gene is responsible for parallel disruption of both, pigment as well as BS production in *S. marcescens*. In another study, screening of serrawettin W1 overproducing mini *Tn5* insertion mutants
Table 3. Employment of molecular tools for construction of recombinant/mutant strains of Bacillus spp.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mutation</th>
<th>Objective</th>
<th>Significant Feature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus</td>
<td>Transforms</td>
<td>Integration of sip gene in chromosome</td>
<td>Improved availability of hydrocarbon.</td>
<td>156,157</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Recombinant</td>
<td>Modification of peptide synthetase</td>
<td>3 fold yield than the parent</td>
<td></td>
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<tr>
<td>ATCC21332</td>
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<tr>
<td>B. subtilis M113</td>
<td>Recombinant</td>
<td>Insertion of a plasmid pC112 with lpa-14</td>
<td>High yield</td>
<td>164</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Recombinant</td>
<td>Modification in surfactin synthetase</td>
<td>Surfactin without hemolytic activity of erythrocytes</td>
<td>169</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>Recombinant</td>
<td>Nucleotide sequence of regulator gene: 3 large ORFs; ORF3 pse-1 sequences</td>
<td>Surfactin synthesis Similar with operon of B. brevis</td>
<td>158</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>Recombinant</td>
<td>Enzyme complex with surfactin synthetase</td>
<td>Similar with that of surfactin synthetase (srA)</td>
<td>159,160</td>
</tr>
<tr>
<td>Lichenysinis</td>
<td>strain</td>
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</tr>
<tr>
<td>B. subtilis</td>
<td>M13</td>
<td>The mutated ParA fragments were inserted into pH-Klac for construction of plasmids. Promoter activity, pMMN102 was derived from ms333 and pMMN97-101 and -103 were constructed from ms29. pMMN104 with a 10-bp insertion between two ComA boxes. ParA of pMMN103 (5-bp insertion between the ComA boxes).</td>
<td>Found that transcription of srA operon is dependent on the transcriptional activator ComA.</td>
<td>170</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Recombinant</td>
<td>Gene responsible for biosurfactant synthesis cloned in B. subtilis RB14C</td>
<td>Production of new surfactin variants on soya-bean curd residue (okara)</td>
<td>165</td>
</tr>
<tr>
<td>(Surfactin and</td>
<td>M113[pC115]</td>
<td></td>
<td></td>
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<td>bufalin)</td>
<td></td>
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<tr>
<td>B. subtilis</td>
<td>Recombinant</td>
<td>Campbell-like recombination at pHI222 homology present in both Sphac2d12::fr97::pSK10A6 DNA and pMMN derivatives srB located between aroG, ala in genome.</td>
<td>Competence and surfactin production resorted by a single DNA fragment of 1.5 kbp namely srB gene is comA.</td>
<td>148</td>
</tr>
<tr>
<td>(Lipopeptide</td>
<td>Tr917</td>
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<tr>
<td>Antibiotic</td>
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<tr>
<td>surfactin)</td>
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<tr>
<td><em>B. subtilis</em> (lipopeptide surfactin)</td>
<td>In vivo and in vitro recombination</td>
<td>Peptide synthetase modified by eliminating large internal region of the enzyme with complete amino acid incorporating module. Permissive fusion sites identified for the engineering of peptide synthetase genes</td>
<td>Surfactin with altered antimicrobial activity. The selection of the recombination site is of crucial importance for a successful engineering.</td>
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<tr>
<td><em>B. subtilis</em></td>
<td>The srA operon of surfactin synthetase; competence regulatory protein ComS</td>
<td>Construction of <em>B. subtilis</em> strain expressing epitope-tagged srB. Plasmid pOH9 with carboxy-terminal end of srB fused to the DNA encoding the influenza virus hemagglutinin 1 (HA1) epitope was constructed.</td>
<td>Found that Sr expression alters with changes in culture pH. ComP acts both positively and negatively in the regulation of ComA and that both activities are controlled by the ComA pheromone.</td>
<td>153</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>Oligonucleotide directed mutagenesis</td>
<td>Ser-to-Ala substitutions made in the amino-acylation site of each domain</td>
<td>Part of the srA contains the region required for competence development and is composed of the first four amino acid-activating domains responsible for the incorporation of Glu, Leu, α-Leu and Val into the peptide moiety of the lipopeptide surfactin. Fourth, Val-activating domain is required for competence, suggesting that some activity, other than amino-acylation and perhaps unrelated to peptide synthesis, possessed by the fourth domain is involved in the role of srA in regulating competence development.</td>
<td>139</td>
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<tr>
<td><em>B. subtilis</em> 168 and 801</td>
<td>Transposon mini-Tn10</td>
<td>Deletion of internal region of the yep gene of <em>B. subtilis</em> strain 168 yielding a yep deficient strain 802</td>
<td>The gene yep determines surfactin self-resistance.</td>
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<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>Expression of sfp gene in <em>E. coli</em>, plasmid-amplified in <em>B. subtilis</em></td>
<td>No effect on surfactin production and repression of a lacZ transcriptional fusion of the srfA operon, which encodes enzymes that catalyze surfactin synthesis. The sfp represents an essential component of peptide synthesis and directly or indirectly affects regulation of surfactin biosynthesis.</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>Recombinant strain</td>
<td>To create sinR and degr mutant strains bearing multicopy comS, plasmid pMMN284 DNA in combination with DNA from strain LAB2274 (DsinR::Phleo) or LAB2275 (degr::lacZ::Ner0::Phleo) DNA To create a comS::9lacZ DsinR strain, DNA from LAB1874 (comS9::9lacZ Cmr) and LAB2274 (DsinR Phleo) was used to transform JH642 cells with selection for Cmr and screening for Phleo.</td>
<td>It was found that sinR is required for optimal comS expression but not transcription from the srf promoter and that SinR at high concentrations represses srf transcription initiation.</td>
<td>138</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>The peptidyl carrier protein (PCP) domains of surfactin synthetase by transferring the 4-phosphopantetheinyl moiety of coenzyme A (CoA) to a serine residue conserved in all PCPs.</td>
<td>Several residues of Sfp involved in CoA interactions are not conserved and CoA binding may vary between members of this super family.</td>
<td>171</td>
<td></td>
</tr>
</tbody>
</table>
suggested a down regulating mechanism for BS production. The transposon was inserted between the \textit{hexS} gene. \textit{hexS} is a suppressive gene controlling production, therefore insertion and deactivation resulted in enhanced production of exolipids. Thus, target specific repression of \textit{hexS} gene product in transcription is elucidated.^^ Such abortion of repression can be useful for large scale and economical production of surface active agents. Production of BS and thereby surface migration in \textit{S. marcescens} SS-1 is controlled by N-acylhomoserine lactones (AHLs) of QSS located on a mobile transposon.\textsuperscript{173,177} Production of BS is under negative control. \textit{S. marcescens} SS-1 produces four AHLs via \textit{spnI}. The production is regulated by \textit{SpnR} in \textit{spnI/ spnR} QSS. The \textit{SpnR} is a homologue of the transcriptional regulator LuxR.\textsuperscript{179} Furthermore, deletion of this \textit{spnR} gene to produce an isogenic mutant strain \textit{S. marcescens} SMAR was found to enhance BS activity.\textsuperscript{178} Upstream of \textit{spnI} is a gene \textit{spnT} encoding a 464 amino acid protein.\textsuperscript{179} The \textit{spnT} is cotranscribed with \textit{spnI} and also functions as a negative regulator of BS production and sliding motility. Thus mobility and horizontal transfer of these genes was proved by Wei, et al.\textsuperscript{179} Similar correlation of genes \textit{swrA/QS} and enzyme involvement in BS production and swarming motility exists in \textit{S. liquefaciens}.\textsuperscript{180,183} This interdependency is obligatory for \textit{S. liquefaciens} MG1 to develop swarming colony. The gene \textit{swrA} encodes a similar putative AHL synthase for synthesis of extracellular signal molecules N-butanoyl-L-homoserine lactone (BHL) and N-hexanoyl-L-homoserine lactone. Expression of \textit{swrA}, encoding serrawettin synthetase, is a homoserine lactone (HSL) and is dependent on QSS.\textsuperscript{179,183} The flagellar master operon (\textit{flhDC}) and AHL are involved in flagellar mobility and cell density regulation.

Mutant strain of \textit{S. liquefaciens} was developed by transposon mutagenesis to construct a non-swarming mutant deficient in serrawettin W2 production. Sequence analysis indicated homology with gene \textit{swrA} that encodes a putative peptide synthetase. Expression of \textit{swrA} is controlled by QSS. Transposon mutagenesis involving the promoter less \textit{luxAB} reporter confirmed action of \textit{swrA} gene via QSS in production of the lipopeptide BS. The gene \textit{swrA} encodes a putative peptide synthetase.\textsuperscript{183} Microbes are able to change their cell surface hydrophobicity during different growth phases, morphogenesis and differentiation.\textsuperscript{184} Cell surface hydrophobicity is affected by cell bound and extracellular factors viz., serraphobin (capacity to bind with hexadecane) and serratamolide (act as wetting agent). Serratamolide negative mutants revealed that serratamolide increases cell surface hydrophobicity.\textsuperscript{185} Various BS producing, mutant/recombinant strains of \textit{Serratia} have been constructed employing molecular approaches (Table 4).

**Molecular Genetics of Glycolipid Synthesis in Fungi and Yeast**

**Candida**

Sophorolipids (SLs) are one of the most common glycolipids produced by \textit{Candida} species.\textsuperscript{191,196} SL is composed of sophorose disaccharide glycosidically linked to a hydroxy FA. Genes involved in biosynthesis of SLs were identified, characterized and cloned by several workers.\textsuperscript{191,192} Mono-oxygenase enzyme, cytochrome P450 dependant on NADPH (nicotinamide adenine dinucleotide phosphate) is essential for FA conversion. The \textit{CPR} (cytochrome P450 reductase) gene of \textit{Candida bombicola} was isolated using degenerate PCR and genomic walking. The \textit{CPR} gene is made up of 687 amino acids. Heterologous expression in \textit{Escherichia coli} proved functionality of the gene. The recombinant protein had NADPH-dependent cytochrome c reducing activity.\textsuperscript{193,194} The genes of cytochrome P450 are diverse among them and also within the genome of a single organism. The phenomenon responsible for induction and expression of these genes was unknown.\textsuperscript{194} Specific glycosyltransferase I leads to the coupling of glycosidic linkage of glucose and FA. Glycosyltransferase II carries out subsequent glycosidic coupling. Both glycosyltransferases have been partially purified.\textsuperscript{195,197} Like other microorganisms \textit{C. bombicola} produces glycolipid when grown on alkanes. Cytochrome P450 monoxygenase obtains reducing equivalents from NADPH cytochrome P450 reductase (CPR). The \textit{CPR} gene of \textit{C. bombicola} was isolated, sequenced and expressed in \textit{E. coli}. The recombinant protein shows NADPH-dependent ‘cytochrome c’ reducing activity.\textsuperscript{195,196}
Table 4. Employment of molecular tools for construction of recombinant/mutant strains of Serratia spp.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mutation</th>
<th>Objective</th>
<th>Significant Feature</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>S. marcescens</em> SS-1</td>
<td>SpnR-defective isogenic mutant, SMAR</td>
<td>Hemologous recombination</td>
<td>Isogenic spnR insertion deletion mutant of <em>Serratia marcescens</em> SS-1 where a 2 kb Sm-resistant DNA</td>
<td>174</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>Mini-Tn5</td>
<td>Purified protein encoded in his (6)-hexS bind to DNA fragments of the upstream region of pigA and swrW genes and not to that of the pswP gene.</td>
<td>Over production of exolipids; Plasmid carrying hexS yielded low prodigiosin and serrawettin W1 with reduced activity of exoenzymes (protease, chitinase and DNAse) except phospholipase C.</td>
<td>181</td>
</tr>
<tr>
<td><em>S. liquefaciens</em> MG1</td>
<td>Tn5</td>
<td>Transposon carrying a promoterless luxAB reporter the luxAB transposon most likely been integrated into a gene, designated swrA, is essential for surfactant production.</td>
<td>The gene swrA encodes a putative peptide synthetase. Expression of swrA is controlled by quorum sensing.</td>
<td>183</td>
</tr>
</tbody>
</table>

**Mycobacterium, Corynebacteria, Rhodococcus**

Trehalose lipid (TL) contain carbohydrates and long-chain aliphatic acids/hydroxy aliphatic acids and are most effective BS produced by *Mycobacteria, Corynebacteria* and *Rhodococcus* species. Finerty studied genes responsible for glycolipid biosynthesis in *Rhodococcus* sp. H13-A. A Genomic library was generated using *E. coli-Rhodococcus* shuttle vector pMVS301. Tn917 transpositional mutagenesis in *Rhodococcus*, was employed for isolation and analysis of sporulation and developmental genes in strains of *Bacillus*.

**Pseudozyma, Ustilago maydis**

Mannosylerythritol lipid (MEL) are produced by genus *Pseudozyma*. A yeast strain *P. antarctica* produces MEL. Genetic study was conducted on prospective genes involved in MEL production. Under nitrogen limitation, *Ustilago maydis*, a dinomorphic basidiomycete produces...
two different classes of glycolipids, ustilagic acids and ustilipids. Ustilagic acids contain celllobiose linked O-glycosidically to 15, 16 dihydroxyhexadecanoic acid, while ustilipids are derived from β-D-mannopyranosyl-D-erythritol and belong to the class of mannosylerythritol lipids. The first report of molecular characterisation of glycolipid production using mutants came very recently in 2005 by Hewald, et al. They identified two genes emtl and cypl responsible for production of extracellular glycolipids by the fungus. Gene cypl codes for cytochrome P450 monoxygenase and is involved in synthesing 15, 16 dihydroxyhexadecanoic acid. U. maydis Emtl codes for a protein which resembles eukaryotic prokaryotic glycosyltransferases and transfers GDP-mannose to form mannosyl-D-erythritol. DNA micro-array analysis revealed that emtl is part of a gene cluster which comprises five open reading frames. Three proteins namely Mac1, Mac2 and Mat1, contain short sequence motifs characteristic for acyl- and acetyltransferases. Mac1 and Mac2 are essential for MEL production and are involved in acylation of MEL. Enzyme Mat1 acts as an acetyl coenzyme which is dependent on acetyltransferase. Mat1 displays relaxed regioselectivity and is able to acylate MEL at both, the C-4 and C-6 hydroxyl groups. Fifth protein is an export protein of the major facilitator family. This is the first report on presence of a gene cluster for production of extracellular glycolipids in a fungus. With these studies, authors introduce the possibility of transfer of genes between species or recent progenitors, for secondary metabolite production in fungal species.

Exploitation of Biosurfactant Molecular Genetics in Biotechnological Applications

The inherent genetic machinery controls phenotypic expression for any particular organism. Understanding of this molecular machinery and its mechanism will play pivotal role in tailoring efficient microbes for potential, economic products. There has been an ever increasing progress in biotechnology in recent years, which has generated enormous opportunities. Initially biotechnological tools were aimed at hyperproducing mutant/recombinant strains. Mutant of P. aeruginosa PTCC 1637 produced 10 times BS to that of wild type. Those of B. subtilis M113 and B.licheniformis KGL1 enhanced production by 8 and 12 times respectively. Remarkably B. subtilis SD901 mutant produced 4-25 times higher yield. Recombinant and/or mutant strains provide huge impetus for further studies (Tables 1, 3 and 4). Biotechnological applications have been recently extended to initial screening methodology of BS producers. The best example is represented from the work by Hsieh, et al. The sfp locus was used for PCR based detection of BS producing B. amyloliquefaciens and B. circulans. Such methods would authenticate the conventional screening methods enlisted in the brief review of Bodour and Miller-Maier. On similar lines, P. rugulos a NBRC 10877 was identified as MEL producer on the basis of rDNA sequence. Direct search for genes involved (Fig. 4) would be faster and less laborious. Newer invention like those of Whiteley, et al could be used to identify modulators and genes of QSs signals in bacteria. Novel indicator strains and vectors have been engineered. Techniques like electroporation are useful in transformation studies and have been used successfully in Pseudomonas. The cationic liposome bearing MEL (produced by C. antarctica) has been demonstrated to increase dramatically gene transfection efficiency into mammalian cells. Similar studies have been reported by Inoh, et al in 2004. Thus, molecular tools would help to regulate and modify biosynthetic pathways to improve BS production technologies. Such significant findings can be used to upgrade lab scale studies towards field application. Advent of techniques in identification, isolation and manipulation of structural genes involved in BS biosynthesis has made it easier to improve existing BS production technologies. The first genetically engineered bioluminescent strain P. fluorescens HK44, with a plasmid containing pUTK21 (naphthalene catabolism), transposon and introduced lux gene fused within a promoter for naphthalene catabolic genes was released for bioremediation process. The strain HK44 was capable of generating bioluminescence in response to soil hydrocarbon bioavailability. Authors suggested that lux-based bioreporter microorganisms can prove a practical alternative in determination of biodegradation in situ, with the process being well-monitored and controlled.
Biosurfactants

To be identified as a probable BS producer (possessing gene encoding BS production (Pseudomonas spp. : rhlA gene or Bacillus spp. srfgene))

Expression of rhlA gene in suitable host

Cloning in E. coli

Vector carrying biosurfactant gene

Obtain single PCR product of the expected size and ligate into vector

Single product of PCR reaction

Subject to PCR amplification using primers designed to identify and amplify specific BS producing (rhlA/ sfp ) gene

Genomic DNA isolation

rhlA

PCR

rhlA

PCR primer

PCR primer

• Sequence the PCR product
• Run a BLAST search
• Match sequence similarity of PCR product particular base pairs gene with the reported sequence gene characterized by other investigators
• Possibility of BS production

Figure 4. Molecular approach for screening of biosurfactant producers.

It is possible to use naturally occurring molecular tools for investigation purpose. Three cryptic plasmids from both A. calcoaceticus BD413, BD4 were isolated, characterized, sequenced and used in the construction of E. coli shuttle plasmids. Studies were done to clone and express the alcohol dehydrogenase regulon from A. lwoffii RAG-1. Gene expression and transformation in emulsan production and cell surface esterase activity in A. lwoffii RAG-1 were also analyzed. The gene (alnA) was cloned, sequenced and over expressed in E. coli. The recombinant emulsifier protein (AlnA) exhibited 70% emulsifying activity as compared to that of native protein and 2.4 times more than that of the alasan complex. Thus, for the first time Toren, et al. in the year 2002, successfully produced a recombinant surface-active
protein using a defined gene. The existing molecular knowledge has opened gateways in drug discovery and manipulations. Protein products from microbes can be used for formulation of newer antibiotics and/or life saving drugs. Dams-Kozlowska and Kaplan, introduced a promising and new approach for bioengineering emulsan analogs which has novel application in the field of medicine as biological adjuvants for vaccine and drug delivery. Research team of Symmank, et al introduced genetically tailored peptide synthetase, which produced surfactin with reduced haemolytic activity. Rhamnolipid was synthesized in a heterologous host of P. putida by cloning rhlAB with rhlRI from the pathogenic producer strain P. aeruginosa. These discoveries are highly commendable and certainly provide promising approach towards conversion of pathogenic to avirulent strains. It appears that, although there is no dearth to the data accumulated which is constantly building up; its actual filed implementation is in a stage of infancy. Thus, maximum exploitation of molecular mechanisms will not only add to our existing understanding of BS production; but will also help bridge the gap between research and actual application.

**Conclusion**

Irrespective of structural complexity, molecular mechanisms involved in polymer synthesis have been revealed. Among the low molecular BS, the genetic mechanisms in *Pseudomonas* and *Bacillus* have been clearly elucidated. The BS production in both microbes is under the influence of QSS. Different genes are involved and interplay of these genes ensures efficient BS synthesis. Mere choosing of substrates, optimization of physicochemical parameters are not enough. Understanding the genetic mechanisms will help in accelerating research towards achieving economical production. Continued research is adding to the ever expanding knowledge of this field and will certainly prove to be a boon for surfactant industry. Although the utility of genetically modified organisms seems to be farfetched due to environmental constraints; Nevertheless, an understanding of the genetic mechanisms and molecular biology of production of biosurfactants will help us in better understanding of the production phenomena. This will form the basis for further manipulation of conditions resulting in optimal and faster production of these surface active agents. More concerted efforts are needed for an optimal exploitation of generated information. A strong foundation of molecular mechanisms will help in an application oriented outlook at the surfactant industry.

**Future Prospects**

Over the decades, valuable information on molecular genetics of BS/BE has been generated and this strong foundation would facilitate application oriented output of the surfactant industry. Promising biotechnological advances have expanded the applicability of BS in therapeutics, cosmetics, agriculture, food, beverages and bioremediation. Interaction among experts from diverse fields like microbiology, physiology, biochemistry, molecular biology and genetics is necessary. With the knowledge at hand, BS with desired qualities can be produced. Mutants and recombinants can be generated to achieve desired yield and properties of BS. Potent but harmless strains can be constructed by employing biotechnological advances. However, meticulous and concerted efforts in unfolding the molecular phenomena of BS production in yeast and fungi are essential. PCR based detection methods can be used to authenticate newer BS producers obtained by conventional screening methodologies. Additionally, switch on/off regulatory mechanisms if involved in BS production need to be discovered and investigated.

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References


Biosurfactants

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1.3.1. Abstract

*Acinetobacter* spp. are the predominant group of Gram-negative bacteria in the marine environment. Oceans provide an exotic but potentially fertile medium for this aerobic, non-motile, oxidase negative, coccobacillary *Acinetobacter* spp. It produces large number of novel and commercially important biological products. Due to its distinctive properties, marine *Acinetobacter* find large number of environmental and biotechnological applications. The present review deals with occurrence and different physiological and biochemical properties of marine *Acinetobacter* species.

*Acinetobacter* spp. have been isolated from seawater, sediments, estuaries as well as hypersaline areas. It has been reported as a free-living bacterium in marine water and sediment. It is also found attached to different substrates such as sea grass, algae or other material. It contributes to the normal flora of marine macro organisms like fishes, crabs, prawns etc. Different marine organisms like Indian mackerel, oil sardine, croaker and pink peach have *Acinetobacter* as an important normal gut flora. Different enzymes such as DNase, gelatinase and protease are produced by marine *Acinetobacter* sp. *Acinetobacter* species are one of the leading spoilage causing organisms of processed and unprocessed seafoods. Some of the interesting properties such as resistance to antibiotics and heavy metals like mercury, lead and arsenate and resistance to radiations are also elucidated. Marine *Acinetobacter* utilize and grow on a wide range of hydrocarbons and are useful for bioremediation purposes. One of the biotechnologically important activities of marine *Acinetobacter* is in the production of biological surface-active components or bioemulsifiers.

The growing interest in this novel system is adequately demonstrated by novel metabolites recently reported from it and number of research laboratories working in this field globally. For a total chemical potential of marine *Acinetobacter* to be effectively assessed, a continued research is needed. Further investment in this area will enhance the utility of this important organism of this potential environment. Thus study of marine *Acinetobacter* is not only of academic interest, but also of great practical importance commercially, environmentally, ecologically and from health point of view.

Keywords: *Acinetobacter*, marine, hydrocarbons, biodegradation, seafoods, enzymes, halophilic.
1.3.2. Introduction:

The oceans cover more than 71% of the earth’s surface and taking this into account, by volume represent greater than 90% of the biosphere. The marine environment is a rich and untapped resource of novel natural products (Satpute et al, 2010, Shete, 2003). While a total appreciation of the true potential of the biological resources in the ocean are yet to be completed, the sampling till date throws up extremely important potentials (Fenical, 1993). The magnitude of Indian ocean with its distribution of over 7,40,00,000 Sq. Km, an average depth of 4000 metres and an 8000 kilometer perimeter with India’s coastline, underlines the absolute treasure trove that lies at our doorstep. These Oceans provide an exotic and potentially fertile medium for biotechnology. The microflora of our seas offers a great potential to develop highly flexible bioremediation methodologies which can be used to the deteriorating environment. Today, marine bacteria are being recognized as an important resource of microbial products (Kokare et al, 2007, 2004a & b). There exists a rich biodiversity with respect to flora and fauna in the marine environment. Amongst different communities present in the marine environment, species of Acinetobacter, a Gram negative, catalase positive, oxidase negative, nonmotile coccobacilli (Bouvet & Grimont, 1987 &1986) exist as predominant organisms. They also appear as a predominant organism of the microflora of sea foods (Choi, 1995; Chen-Hsing-Chen, 1995). Acinetobacter spp. are known to have a reservoir of a large number of plasmids, which allow genetic manipulation (Pardesi, 2009). All three methods of gene transfer are also very well known in Acinetobacter, which make it a novel system for genetic studies. In addition, they are resistant to large number antibiotics and metals (Deshpande & Chopade, 1994; Dhakephalkar & Chopade, 1994). Due to all these properties they have been attracting great deal of attention in environmental and biotechnological applications (Abdel-El-Haleem, 2003)

1.3.2.1. Occurrence of Acinetobacter in the marine environment:

Large number of researchers have reported presence of different species of Acinetobacter from the marine environment. Acinetobacter sp. have been isolated from seawater, sediment, estuaries as well as hypersaline areas. It has been reported as a free-living bacterium in marine water (Satpute, 2008, Shete, 2003; Baxter & Sieburth, 1984). There appear a large number of reports of Acinetobacter being isolated from seawater, sediment, estuaries as well as hypersaline areas (Kang et al, 1997; Lee & Lee, 1990; Park et al. 1991; Lee & Taga, 1985; Simidu, et al, 1982; Duong-van-Qua et al, 1981; Austin et al, 1979) during different periods of the year.

The most widely studied strain of Acinetobacter especially for its industrial importance by virtue of producing a potent bioemulsifier, Acinetobacter strain RAG-1 (5ATCC 31012) was isolated from seawater near a beach (Tel Baruch, Israel) after enrichment of mineral medium with sterilized crude paraffin oil (Rosenberg et al, 1979a & b; Reisfeld et al, 1972). This strain has been most often referred to as Acinetobacter lividans (eg. Alon & Gutnick, 1993) or Acinetobacter calcoaceticus (Sar & Rosenberg, 1983; Leahy et al, 1993). Di Cello et al (1997) isolated Acinetobacter venetianus as a part of a bacterial oil-degrading consortium from the Venice lagoon.
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(Ch. 1) This isolate was found to grow with fuel oil, n-alkanes and their oxidation products as sole carbon sources thus useful for pollution clean-up. This strain was interestingly found to harbour plasmids (Vaneechoutte et al, 2009, Di Cello et al, 1997). Acinetobacter have been found to the predominant bacteria in the Suyeong Bay, Korea (Kang et al, 1997). In a study conducted during the cruise on board R. V. Skypjack, to record the aerobic heterotrophic bacteria found in the Arabian Sea, Acinetobacter was one of the predominant microorganisms. It was found that gram-negative population dominated the flora. These heterotrophic bacteria play a very crucial role in the decomposition of particulate matter and regeneration of nutrients. (Alavandi, 1990). Simida et al (1982) have reported that Acinetobacter were the most dominant species encountered during red tides in the Bengal Bay and the South China Sea; while Lee et al (1986) have reported that Acinetobacter were amongst the predominant microbial species from the Masan Bay located in southern part of Korea. Both Acinetobacter species were responsible for decomposition of organic matter and recycling of nutrients.

Acinetobacter species are not only found to be free living organisms but also are associated with different animate and inanimate substances. Marine sandy beaches can be regarded as buffer zones between the sea and the land. They represent dynamic environments shaped by the action of the wind, sand, and water and are of recreational value (Węclawski et al. 2000; Koop et al, 1982). Bacteria play a key role in the functioning of sandy beach ecosystems, since they carry out processes of decomposition and transformation of organic matter (Ołaciucznik-Ceyman & Jankowska 1998; Mudryk 1997). Large number of researchers have reported presence of Acinetobacter associated with marine sediment, oil coated sand, mud and gravel particles (Al-Awadhi et al, 2002a & b; Radwan & Al-Hasan, 2001; Rontani et al, 1999; Venkateshwaran et al, 1991, Lee & Lee 1990). Jan Mudryk & Podgorska (2005) reported isolation of A. calcoaceticus strains from marine sandy beaches in the southern Baltic sea while Shete (2003) have reported them from sediments from coastal marine areas from the west coast of India.

Large number of microbes exist as surface attached communities surrounded by matrix materials called biofilms. Biofilms can be formed by a single bacterial strain. However, most natural biofilms are actually formed by multiple bacterial species. Microorganisms compete, communicate, and protect each other in multispecies biofilms (Yang et al, 2011). Biofilms are found in natural and industrial aquatic environments, tissues, and medical biomaterials and devices (Gaddy & Actis, 2009; Costerton et al, 1994). Acinetobacter species are known for their ability to form biofilms on urinary catheters and in inanimate objects on hospital settings (Pour et al, 2011). Lobova et al in 2004 isolated different species of halotolerant Acinetobacter from a brackish Lake Shira in Khakasia, Russia. In 2005, another group of scientists, Mogilnay et al (2005) reported that these halotolerant species of marine Acinetobacter existed singly but interestingly also had the ability to form structured communities or biofilms under stressful conditions.
The ability to form biofilm was triggered by conditions of increased salinity presence of high levels of copper ions. Under these conditions, halotolerant *Acinetobacter* formed multispecies biofilms with other bacteria, this coexistence under stressful conditions was beneficial to both the groups of bacteria. Erable et al. (2010) reported that *A. johnsonnii* formed dense biofilms on stainless steel electrodes dipped in sea water and resulted in current density of 3% when used individually. When present as a part of the marine microbial biofilm synergetic effects occurred giving higher current densities. The authors proposed that these polymicrobial biofilm containing *A. johnsonnii* could be used in formulation of biocathode catalysts.

Members of the genus *Acinetobacter* have also been reported to be found attached to different substrates such as sea grass, algae or other material. Kang, 1982 reported presence of *Acinetobacter* species on the seaweeds of *Undaria pinnatifida*. The number of infecting *Acinetobacter* were higher in diseased fronds than those in the healthy ones, indicating a probable pathogenesis role of these bacteria in the plant community as well. In another study in Aqaba Jordan, Wahbeh & Mahasneh (1984) found that *Acinetobacter* species were associated with Seagrass *H. ovalis*. *Acinetobacter* sp. have been found to be associated with the seaweed, *Undaria pinnatifida*. Kim & Lee (1993) studied the relationship between marine bacteria and phytoplankton in Suyeong Bay in May when the phytoplankton bloom occurred and found *A. calcoaceticiis* as one of the dominant species of bacteria during the phytoplankton bloom.

Macro-organisms are continually bathed in an aqueous suspension of microbes. Therefore their external surfaces have frequent contact with microorganisms. It is therefore obvious that these microorganisms become intimately associated with the external surface(s) of the plants or animals. Here, the potential exists for the microorganisms to colonise the surface and become a part of the hypothetical microflora. This microflora could inhibit or retard the colonization by other microorganisms (Austin, 1998). *Acinetobacter* occurs as a predominant organism of the microflora of fresh seafood including shellfish and finfish (Chen-Hsing-Chen, 1995) and mackerels (Barile et al., 1985). *Acinetobacter* species also have been found to be a part of the normal flora of the marine horse mackerels *Trachurus japonicus*, marine fishes and prawns (Bari, 2002; Surendran et al, 1983; Okuzumi et al, 1980). While several other investigations have shown that *Acinetobacter* is a normal flora member of gut of marine organisms like Indian mackerel, oil sardine, croaker, pink perch and sea urchins (Shetty et al, 1992; Surendran & Gopakumar, 1981; Nickelson et al, 1980; Unkles, 1977). According to yet another study, *Acinetobacter* also contributes to the normal flora of skin, gills and intestines of oil sardines *S. longiceps* (Surendran & Gopakumar, 1983).

Much emphasis has been placed on microbiology of the gastrointestinal and digestive tracts tract of marine fishes (Zhou et al, 1997). Numerical taxonomy of bacteria isolated from the hepatopancreas of giant tiger prawn *Penaeus monodon* revealed *Acinetobacter* to be one of the predominant organisms of the microflora (Chang et al, 1996). *Acinetobacter* strains were isolated from foregut, hindgut, coelomic liquid and body surface of *Stichopus japonicus*. (Sun & Chen,
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1989) *Acinetobacter* is also regarded as autochthonous in the intestines of *Oncorhynchus* sp. of salmonids (Ringo et al., 1995). Coz-rakovak et al. (2002) conducted a survey in the northern Adriatic Sea over a period of one year that included comparative parasitological, bacteriological, virological, histological and biochemical studies of the cultured and wild sea bass (*Dicentrarchus labrax* L). The authors isolated members of the genus *Acinetobacter* from reared fish.

In a study carried out by Nickelson et al. (1980) on fresh and frozen fish flesh prepared from sheepshead, black drum, croaker, sand trout and mullet, *Acinetobacter* was found to be the most prevalent microbial type in the fish before, during and after processing into the minced fish flesh. *Acinetobacter* sp. have also been found to be adhering to the processing equipments during production and cleaning and disinfection of fish processing plants. *Acinetobacter* spp. were predominant among the process microflora obtained in processing plants during production and after cleaning and disinfection of cold-smoked salmons, semi-preserved herrings and caviars in different processing units. The salmon used was farm (ocean)-raised from Norway and the Faeroe Islands. Herring caught in the North or Atlantic Sea near Norway and caviars were from the roe of lumpfish, caught from, Canada, Iceland or Greenland (Bagge Ravn et al., 2003).

1.3.2.2. Properties exhibited by marine *Acinetobacter*:

1.3.2.2.1. Production of Enzymes:

The commercial value of enzymes has increased substantially, with uses including the confectionery, detergent and other industries. The search for novel metabolites is a lucrative business for which the marine environment should not be overlooked. Already it has been established that some marine bacteria produce copious amounts of important enzymes (Satpute, 2008; Austin et al., 1988). Marine *Acinetobacter* has been reported to produce a variety of enzymes. Protease production has been reported from *Acinetobacter* species isolated from diverse habitats viz., intestinal microflora of sardines. (Chang et al., 1984; Van Qua et al., 1981). *Acinetobacter* from shrimp *Mepanaropis harbatus* produced copious amounts of DNAse and gelatinase. (Wu & Chen, 1980) Different species of *Acinetobacter* isolated from habitats of two saltmarsh burrowing detritivore thalassiniid prawns exhibited alginase, gelatinase and lipase activity (Harris et al., 1991). Gelatinase, lipase and protease producing *Acinetobacter* strains have been also isolated from marine shrimps caught off the west coast of India by Bari (2002). While Shete (2003) reported production of an array of enzymes including lipase, gelatinase, phosphatase and L-asparaginase from marine *Acinetobacter* strains isolated from water and sediment samples from the west coast of India.

1.3.2.2.2. Spoilage:

India is the largest exporter of marine prawns and *Acinetobacter* is one of the leading spoilage causing organisms of processed and unprocessed prawns (Surendran & Gopakumar, 2003).
It has been reported to cause spoilage of EDTA treated and untreated prawns, sardines and mackerel and other bacteria (Thampuran & Iyer, 1990; Surendran & Gopakumar, 1982). Recently marine Acinetobacter species isolated from marine shrimps caught off the west coast of India had been identified to be potent producers of enzymes namely gelatinase, lipase and protease at low temperatures of 4°C indicating their role in spoilage of frozen shrimps (Bari, 2002). Acinetobacter has also been harvested from spoiled Penaeus indicus (Chandrasekharan et al, 1987). In a study to determine the effect of storage at 5°C in the bacterial flora and the physical properties of Malaysian fishballs Yu & Lee concluded that there was no change in the texture at 3 days of storage but bacterial spoilage rendered the fishballs unacceptable by fourth day. Acinetobacter was found to be one of the spoilage causing organisms (Yu & Lee, 1995). The changes in quality of bacterial flora in M. barbatus during fresh, storage at 3°C or -28°C were studied (Wu & Chen, 1980). Acinetobacter was one of the predominant organisms and was suspected to be the putrefactive bacteria in the shrimp texture because of their potential to produce the enzymes DNAse and gelatinase.

In a study conducted to determine succession of bacterial genera during iced storage of three species of tropical prawns Penaeus indicus, Metapenaeus dobsoni and M. affinis. Surendran et al (1985) found that the native flora was dominated by Pseudomonas, Acinetobacter, Moraxella and Arthrobacter. A definite succession of bacterial genera during iced storage was observed in these prawns. As days of ice storage increased, the proportion of Acinetobacter and Moraxella increased considerably and constituted 70-78% of the flora at time of spoilage. Members of the genera Acinetobacter were also involved in the spoilage of horse mackerels, Trachurus japonicus, during storage by partial freezing. After 12 days of storage, count of the normal flora decreased and those of Moraxella, Acinetobacter increased tremendously (Okuzumi et al, 1980).

Chandrasekharan et al (1985) devised a fish flesh agar medium for detection of spoilage bacteria. The spoilage characteristics of bacterial strains were studied by growing them at 28 ± 2°C in agar and broth media prepared with sterile fish and prawn flesh homogenates. Acinetobacter exhibited faster growth in flesh media than in the usual artificial media. Acinetobacter has also been reported to cause saddle-shaped skin lesions in both wild and captive Bermudan reef silverside fishes along with other microorganisms (Rand & Wiles, 1988).

1.3.2.3. Tolerance to Heavy Metals :

The universal concern caused by the diffusion of toxic metals into our living environment by industrial emissions and leaching from hazardous wastes has motivated survey of microorganisms potentially useful for biological monitoring (Dhakephalkar & Chopade, 1994). Heavy metal contamination in marine environment is also a cause of concern. Marine heavy metal pollution increases due to anthropogenic activities. These cause accumulation of heavy metals in aquatic ecosystems and alter the macro and microbiological communities (Ivorra et al, 1999). Additionally, Heavy metals found among hydrocarbons and increase the difficulty of
biodegradation. Consequently several isolated microorganisms from sites contaminated by hydrocarbons show low sensitivity to heavy metals existing at these sites, such as chromium, mercury and cadmium. (Fleck et al, 2000) Multiple metal resistance has been observed in Acinetobacter spp. by Dhakephalkar & Chopade (1994). The toxic action of heavy metals includes inducing oxidative stress and interfering with protein folding. Bacteria therefore develop variety of resistance mechanisms to counteract heavy metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Nies, 2003; Harrison et al, 2004). Shete, 2003 reported that A. haemolyticus and A. jiunii strains isolated from water and sediment samples from the west coast of India were resistant to an alarming number of metal salts. All strains were resistant to salts of lead, cobalt, antimony, mercury, sodium arsenic, tellurium, nickel, strontium, copper, nickel, gold, platinum, stannous and palladium. Highest resistance was seen against salts of lead, arsenic, strontium, nickel and stannous.

Selenite and arsenic resistant marine Acinetobacter species have been isolated from marine shrimps by Bari (2002). Mercury resistant marine Acinetobacter isolated from the Chesapeake Bay were found to volatilize mercury. Interestingly, these strains also harbored plasmids (Olson et al, 1979a & b). In yet another study Babich & Stotzky (1979) found that marine Acinetobacter were much resistant to cationic mercuric compounds. In a study conducted by Nieto et al in 1989 to evaluate the tolerance patterns of moderately halophilic eubacteria. Tested marine Acinetobacter strains showed tolerance to lead, mercury, silver and zinc. Since Acinetobacter species commonly inhabit the marine environment, they have become resistant to most metal contaminants of this environment and could be used as bioreporters to assess the level of contamination of marine water bodies.

1.3.2.2.4. Radiation Resistance:

Radiation resistance is an interesting property which has not been studied in detail and needs further investigation since this property of the marine strains could be of great importance.

The radiation resistance of 504 strains of Moraxella-Acinetohacter isolated from marine fish was studied by Munzer in 1977. The D0 values of 97 % of the strains was 70 Krad. For 13 strains irradiated in the log phase the D0 values ranged from 95-190 Krad. When the strains were irradiated at 80 °C the D0 values ranged fro 220 to 290 Krad. Bari (2002) studied the effect of gamma radiations on Acinetobacter sp. isolated from marine shrimps as a means to test its efficiency as a preservation technique. It was observed that the D0 (0.54 KGy) were highest for A. baumannii WS17. The D0 values of other Acinetobacter species viz. A. junii, A. johnsonii and A. haemolyticus also isolated from marine shrimps was much lower (around 0.12 KGy). Bari also suggested that an irradiation dose of 6 KGy was sufficient to eliminate natural contamination of marine shrimps by Acinetobacter species. These studies could prove potentially useful in defining new strategies for marine food preservation.
1.3.2.2.5. Biosurfactants:

There is global concern about the release of hydrocarbons to the environment, either from industrial activity or from accidental oil spills. Many of those hydrocarbons present hydrophobic structures, being insoluble in water and are toxic handicapping their biodegradation and causing serious environmental impacts. One of the remediation alternatives is the addition of chemical surfactants (dispersants) that emulsify the hydrocarbons, allowing microbial action on these compounds (Viramontes-Ramos et al., 2010). However, many synthetic surfactants have toxic effects and usually they are not biodegradable contradictory to all current policies of environmental preservation techniques. An alternative technology is the use of biosurfactants produced by microorganisms, since these compounds are biodegradable and much less toxic. (Cameotra & Makkar, 2004; Kosaric, 2001; Fleck et al., 2000). Growth of microorganisms on water-insoluble carbon sources is often accompanied by the emulsification of the water-insoluble substrates in the culture medium by virtue of production of bioemulsifiers (Patil & Chopade, 2001). These are attracting considerable attraction in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries (Ayanwuj et al., 2011; Gharaei-Fathabad, 2011; Mulligan, 2005 & Desai & Banat, 1997). The use of this remediation technique involves the direct use of the biosurfactant as well as the microbial producers of biosurfactants, which would produce this compound directly in situ (Banat, 1995; Harvey et al., 1990).

The terms biosurfactants and bioemulsifiers though slightly different are often used synonymously. Biosurfactant(s) spontaneous release and function are often related to hydrocarbon uptake; therefore, they are predominantly synthesized by hydrocarbon degrading microorganisms. (Banat, 1995). Numerous studies have been done to determine novel and efficient hydrocarbon utilizing, degrading and biosurfactant or bioemulsifier producing microorganisms (Muthusamy et al., 2008; Kitamoto et al., 2002).

The biosurfactants produced by bacteria in marine ecosystems are also involved in the degradation of hydrocarbons. (Coelho et al., 2003). The first description of the best known marine biosurfactant, now exploited commercially as Emulsan, appeared in 1972 (Yakimov et al., 1998, Austin et al., 1988). This emulsifier is produced by Acinetobacter calcoaetici RAG-1, isolated from the Mediterranean Sea. This extracellular emulsifying factor emulsified light petroleum oil, diesel oil and a variety of crude oils. It also emulsified efficiently all binary mixtures containing an aliphatic and aromatic hydrocarbon. It also emulsified kerosene and gasoline in presence of additional aromatic compounds such as 2-methylphenalene or hexadecane. Great deal of studies have been carried out concerning isolation and emulsifying properties, chemical and physical properties, specificity of hydrocarbon substrate enhancement of Emulsan release and other properties of this isolate (Rosenberg et al., 1979a & b). Emulsan produced by Acinetobacter calcoaeticius RAG-1 is a high molecular weight bioemulsifier containing a heteropolysaccharide backbone containing a repeating trisaccharide of N-acetyl-D-galactosamine. N-
acetylgalactosamine uronic acid, and an unidentified N-acetyl amino sugar. Fatty acids are covalently linked to the polysaccharide through o-ester linkages. It finds potential applications in cleaning of oil-contaminated vessels, oil spills and microbial enhanced oil recovery (MEOR). This bioemulsifier is patented by Gutnick and is presently marketed by Petrofirm Inc. (Desai & Patel, 1994). After this initial discovery, innumerable *Acinetobacter* species have been reported for production of bioemulsifiers or biosurfactants. Table 1 gives a comprehensive summary of surface active agents produced by members of the genus *Acinetobacter*.

### 1.3.2.6. Miscellaneous Properties:

In addition to above properties marine *Acinetobacter* spp. also shows several other interesting properties such as nitrate reduction, sulphur oxidation, antibiotic production and resistance to antimicrobial agents.

Sulfur-oxidizing *Acinetobacter* were isolated from samples of vent water, invertebrates, and chimney rocks collected at two deep-sea hydrothermal vents (2000 m) in back-arc basins from the southwestern Pacific, the North Fiji Basin and the Lau Basin. They oxidized thiosulfate either to sulfate or to polythionate (Durand et al, 1994). It has also been reported as a nitrate reducer. In a study done on sediments from Kingoodie Bay and from North Sea off the Tay Estuary, *Acinetobacter* species were able to reduced nitrate. Continuous culture enrichments of sediments showed that in media containing no added NaCl, *Acinetobacter* predominated anaerobically in the presence of nitrate and acetate (Dunn et al, 1980). In addition *Acinetobacter* isolated from intestines of the trumpet shell *Charonia sauliae* produced tetrodotoxin and or related substances (Narita et al, 1989). *A. Johnsonii* and *A. lwofi* were detected in trout intestinal contents and frozen shrimps and were found to be resistant to antimicrobial agents (Guardabassi et al, 1999).

To provide essential information of the role of marine bacteria on the dinoflagellate blooms, distribution of marine bacterial flora and dinoflagellate species was investigated in Chinhae Bay *Acinetobacter* strains were the most dominant in bacterial species found in these seawater samples (Lee et al., 1990). Salt tolerance studies on bacteria isolated from tropical marine fish and prawn and sediment samples revealed that *Acinetobacter* spp. were moderately halophilic (Surendran et al, 1983; Van Qua et al, 1981) Moderately halophilic *Acinetobacter* were found to precipitate calcium carbonate to calcite and aragonite in a study by Ferrer at al in 1988. Halophilic and halotolerant marine *Acinetobacter* species have also been reported from marine coastal areas (Satpute, 2008; Shete, 2003; Moral et al, 1988).
Table 1.3.1: Biosurfactants / bioemulsifiers produced by different strains of *Acinetobacter*:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Emulsifier</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. calcoaceticus</em> RAG-1</td>
<td>Emulsan</td>
<td>Heteropoly saccharide backbone with a repeating trisaccharide of N-acetyl-D-</td>
<td>Zackerberg et al, 1979 Rosenberg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>galactosamine, N-acetyl-D-galactosamine uronic acid, and an unidentified N-acetyl amino</td>
<td>et al, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sugar. Fatty acids are covalently linked to the polysaccharide through o-ester</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>linkages. Emulsifies light petroleum oil, diesel oil, variety of crude oils</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>and all binary mixtures containing an aliphatic and aromatic hydrocarbon.</td>
<td></td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> BD 413</td>
<td>Emulsan</td>
<td>Polysaccharide-protein complex; Stabilizes oil in water emulsions</td>
<td>Kaplan &amp; Rosenberg, 1982</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> BD4</td>
<td>Emulsan</td>
<td>Polysaccharide-protein complex; Stabilizes oil in water emulsions</td>
<td>Kaplan et al, 1987</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> A2</td>
<td>Biodispersan</td>
<td>Anionic polysaccharide surfactant; Effectively disperses limestone and titanium</td>
<td>Rosenberg et al, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dioxide, disperses calcium carbonate in water.</td>
<td></td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> sp. 2CA2</td>
<td></td>
<td>Whole cells act as emulsifiers, as well as extracellular lipopeptide emulsi</td>
<td>Neufeld &amp; Zajic, 1984</td>
</tr>
<tr>
<td><em>A. radioresistans</em> KA53</td>
<td>Alasan</td>
<td>Alanine containing heteropoly saccharide and protein complex; Stabilizes oil</td>
<td>Navon-Venezia et al, 1995; Barkay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in water emulsions in n alkanes with chain length &gt;10 or higher, alky ara</td>
<td>et al, 1999</td>
</tr>
<tr>
<td><em>Ac. calcoaceticus</em> MM5</td>
<td></td>
<td>Polysaccharide protein; Emulsifies heating oils</td>
<td>Marin et al, 1996</td>
</tr>
<tr>
<td><em>Acinetobacter</em> ADH-1</td>
<td></td>
<td>Grows with crude oil as the sole carbon source degrades n-dodecane. Decrease</td>
<td>Mac Cormack &amp; Fraile, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in the surface tension values observed in the culture broth from 68 dynes/cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>to 31 dynes/cm</td>
<td></td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> CECT 441</td>
<td></td>
<td>Lipoprotein type of biosurfactant produced by on olive oil and sunflower oil</td>
<td>Heba et al, 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with a reduction of surface tension to 42.5 and 38 mN/m respectively</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>almond oil, castor oil, olive oil and palm oil</td>
<td></td>
</tr>
<tr>
<td><em>A. johnsonii</em> JS5</td>
<td></td>
<td>Protein polysaccharide type of bioemulsifier, emulsified large number of</td>
<td>Bari 2002</td>
</tr>
<tr>
<td><em>A. junii</em> WS1?2</td>
<td></td>
<td>hydrocarbons including those with carbon length varying from C10-C18, toluene</td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em> WSI7</td>
<td></td>
<td>kerosene, benzene,</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Emulsifier</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------</td>
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<td>-----------------------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td><em>A. johnsonii</em> WS26</td>
<td>-</td>
<td>aniline and petrol, with highest emulsification activity with toluene.</td>
<td></td>
</tr>
<tr>
<td><em>A. baumannii</em> JS10</td>
<td>-</td>
<td>Polysaccharide-protein-lipid complex. Emulsified hydrocarbons carbon length varying from C10-C18, toluene kerosene, benzene, aniline and petrol and different mixture of pentadecane, hexadecane, toluene and octadecane. Also produces bioemulsifier when grown using molasses and whey as cheaper substrate, also containing Polysaccharide-protein-lipid; reduced the surface tension of Distilled water to 66.15 dynes/cm</td>
<td>Bari, 2002</td>
</tr>
<tr>
<td><em>A. baemolyticus</em> MW1*</td>
<td></td>
<td>Protein polysaccharide type of bioemulsifiers, emulsified hydrocarbons with chain length varying from C10-C18, toluene kerosene, benzene, aniline and petrol, with highest emulsification activity with toluene.</td>
<td>Shete, 2003</td>
</tr>
<tr>
<td><em>A. baemolyticus</em> MW11</td>
<td></td>
<td></td>
<td>Shete, 2003</td>
</tr>
<tr>
<td><em>A. junii</em> GW2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. junii</em> GW9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. baemolyticus</em> GSS</td>
<td></td>
<td>Produced protein polysaccharide type of bioemulsifier with molasses and whey as substrate. Bioemulsifier purified from whey reduced the surface tension of water to 58 dynes/cm and that of artificial sea water to 63 dynes/cm.</td>
<td>Shete, 2003</td>
</tr>
<tr>
<td><em>Actinobacter</em> sp. DS5-74</td>
<td>-</td>
<td>Produced 1.9 ± 0.2 g/l of biosurfactant in 96 h with a reduction in surface tension to 33.7± 0.9 mN/m. Emulsified xylene, benzene, n-hexane, Bombay High crude oil, kerosene, gasoline, diesel fuel and olive oil.</td>
<td>Rahman et al, 2003</td>
</tr>
<tr>
<td><em>A. eutrofexicus</em> subsp. anitratus SM1*</td>
<td></td>
<td>Crude bioemulsifier recovered from the culture supernatant by ethanol precipitation, yield of 2.94 g/l and critical emulsifier concentration of 0.04 g/ml. Emulsified n-hexadecane at a broad pH range (6-12), temperatures (30-121 °C) and in presence of NaCl up to 12% (w/v).</td>
<td>Phetrong et al, 2008</td>
</tr>
<tr>
<td><em>Actinobacter</em> sp*</td>
<td></td>
<td>Large number of marine <em>Actinobacter</em> sp. screened for their emulsification potential by different screening methods</td>
<td>Satpute et al, 2008</td>
</tr>
<tr>
<td><em>Actinobacter</em> sp. R4M4</td>
<td></td>
<td>Production of biosurfactant (surface tension value of 58.3 ± 7.6 ) and bioemulsifier (Emulsification index of 86.1 ± 2.2)</td>
<td>Viramontes-Ramos et al, 2010</td>
</tr>
</tbody>
</table>

* indicates marine origin; ~ no name allocated.
Acinetobacter sp. strain PHY9, isolated from hydrocarbon-polluted marine coastal sediments and foams collected from different sites in Golf of Fos (Mediterranean Sea, France) have been known to produce isoprenoid wax esters during the aerobic degradation of 6,10,14-trimethylpentadecan-2-one and phytol (Rontani, 1999). This study is very important since, bacterial production of isoprenoid wax esters has not been reported. In bacteria of the genus Acinetobacter, wax esters are generally considered to be energy storage components (Alvarez et al., 1997). The authors found that cells of the isolate Acinetobacter sp. 211 were able to synthesize and accumulate lipid inclusions during growth on acetate, ethanol, olive oil, hexadecanol and heptadecane. The composition of the lipid inclusions depended on the compounds provided as carbon source. Wax esters and acylglycerols occurred mainly during the cultivation on olive oil, and wax esters and free alcohols occurred during cultivation on hexadecanol. Palmitic acid was the main fatty acid in the lipids when the cells were cultivated on acetate or ethanol. The fatty acids present in the accumulated lipids consisted predominantly of saturated and unsaturated straight-chain fatty acids.

1.3.2.3. Acinetobacter: A potential organism for Bioremediating the marine environment:

The marine environments are burdened with a large number of pollutants from various industries. Petroleum hydrocarbons are introduced onto earth’s surface by a number of routes including: natural seeps, offshore oil production, transportation accidents, coastal refineries, industrial wastes and urban runoff (Viramontes-Ramos et al., 2010; Jagtap et al., 2009; Rahman et al., 2003; Perry, 1977). These pollutants may be removed from the environment in a variety of physical, chemical and biological ways. Bacterial attack can be an important process in some environments and a large number of strains have been investigated to assess their capabilities to degrade particular compounds. Today, bacterial bioremediation techniques offer new possibilities to accelerate the pollutant degradation, either by adding the appropriate nutrients that are required by the naturally occurring microorganisms or, when the bacterial populations are not adequate, by seeding pure or mixed cultures of bacteria having the metabolic pathways that permit a faster metabolism of the contaminant (Viramontes-Ramos et al., 2010; Jagtap et al., 2009; Head et al., 2006; Christofi & Ishina, 2002; Mac Cormack & Fraile, 1997).

Among microbial communities involved in different ecosystems such as soil, freshwater, wastewaters and solid wastes, several strains belonging to the genus Acinetobacter have been attracting growing interest from medical, environmental and a biotechnological point of view. Bacteria of this genus are known to be involved in biodegradation, leaching and removal of several organic and inorganic man-made hazardous wastes. It is also well known that some of Acinetobacter strains produce important bioproducts (Abdel-El-Haleem, 2003).

The large-scale production and transportation of different hydrocarbons has resulted in dispersion of oil in the marine environment through various activities. A diversified group of petroleum products are amenable to microbial degradation (Edlund & Jansson, 2006; Bordenave et al., 2004).
The activities of microorganisms involving various complex biodegradation pathways produces a series of primary metabolites, which act as synergistic intermediates that accelerate the process of biodegradation (Coelho et al, 2003). A large number of bacteria including *Acinetobacter* spp. have been known to metabolise and degrade hydrocarbons (Chikere et al, 2009; Bordenave et al, 2004). Psychrotrophic *Acinetobacter ADH-1* isolated from soil samples taken near the shoreline in Jubany Station (King George Island, South Shetland Islands) was able to grow with hydrocarbons as sole carbon and energy source and degrade them. This could be potentially useful to design bioremediation processes in temperate and cold climate areas (Mac Cormack & Fraile, 1997). Similar studies from marine environs could open up newer bioremediation technologies for the deteriorating environment.

Numerous *Acinetobacter* species are represented at cold, petroleum-contaminated sites and can survive solely on hydrocarbon compounds (Rosenberg et al, 1992). *Acinetobacter venetianus* isolated from the Venice lagoon was found to grow with n-alkanes their oxidation products as sole carbon sources. *A. venetianus* VE-C3 also isolated from the Venice Lagoon efficiently used diesel fuel containing n-alkanes or pure n-hexadecane as the sole carbon and energy source and degraded them (Baldi et al, 1999). *Acinetobacter* spp. isolated from the Arabian Gulf water at Kuwait and from surface water and sediment from north eastern Japanese coastal waters have been found to be efficient crude oil degraders (Al-Gounaim & Diab, 1998; Venkateshwaran et al, 1991). Studies on generic composition and degradation activity of hydrocarbon-degrading bacteria isolated from the seawater of the western North Pacific Ocean, eastern Indian Ocean and South China Sea revealed that *Acinetobacter* was one of the predominant organism capable of degrading hexadecane. (Higashihara & Sato, 1985) *A. calcoaceticiis* was able to utilize octane as the sole source of organic carbon. It was able to utilize and grow even at high-octane concentrations (Zawdzki et al., 1983).

Selective degradation of biphenyl and methylbiphenyls in crude oil by two strains of marine bacteria was studied by Fedorak et al in 1983. They showed that *Acinetobacter* spp. isolated from marine sediment and water could use crude oil as the sole carbon source. It selectively degraded biphenyl, 3-methylbiphenyl, 4-methylbiphenyl and was also able to grow on benzoic acid, 3 and 4-methylbenzoic acids, indicating that they could further metabolize the aromatic acid intermediates. In another study conducted to determine biodegradation of petroleum hydrocarbons under tropical estuarine conditions in the Lagos lagoon, *Acinetobacter* species were found to be one of the prominent hydrocarbon degraders. It was found to grow extensively on long chain alkanes such as n-dodecane, n-tetradecane, n-hexadecane and their corresponding alcohol and fatty acid derivatives. These studies were carried out at laboratory as well as field level and indicate that the hydrocarbon degraders conferred an inherent capability of self-purification to the lagoon in spite of frequent pollution incidents (Armand & Igiri, 1990). *A. venetianus* strain T-4 is known to degrade alkane and was found to be the dominant organism in the initial stages around 6 days in co-culture studies to determine the predominance of *Alcanivorax* in crude oil containing sea water with
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Biodegradation of Bombay high crude oil by *Acinetobacter* sp. A3 was affected by the carbon : nitrogen ratio. A C: N ratio (40:1) supported maximum degradation by the end of 120 h the fastest degradation in 24 h was obtained with C:N ratio (80:1). Unlike nitrogen sources, phosphorus sources supported degradation to varying levels and C : P ratio (200:1) was found to be optimum for biodegradation of crude oil by *Acinetobacter* sp. A3. Additionally crude oil degradation was also influenced by supplementation of nitrogen and phosphorous (Hanson et al, 1997 & 1996). All these studies indicate the potential of *Acinetobacter* as a potent candidate for biodegradation and thus in restoration and maintenance of environmental balance.

Radwan et al, 2001 reported *Acinetobacter* as a member of hydrocarbon degrading microbial consortia in the intertidal zone of the Arabian Gulf coast. This consortium was found to be immobilized in biofilms on gravel particles. They contributed to the self-cleaning of the coasts and, in addition, could potentially be used for cleaning oily industrial waste before its disposal. The most dominant hydrocarbon-degrading bacterium in this consortium was *A. calcoaceticus*. Further this group also described a method for artificially establishing microbial biofilms containing a consortium of hydrocarbon degrading *Acinetobacter calcoaceticus* and nocardioforms, in addition to cyanobacteria picoplankton and diatoms on gravel particles and glass plates. Thus *Acinetobacter* as a part of consortia could find potential application in preparing trickling filters and in bioreactors for attenuating hydrocarbons in oily liquid wastes prior to their disposal in the open environment. In a study along the coasts of Kuwait, Radwan S. S. et al (1999) found that littoral materials collected from the intertidal zone along the coast of Kuwait city were associated with much higher numbers of oil-utilizing microorganisms. *A. calcoaceticus* was the predominant indigenous oil-utilizing bacterium in this environment. Also coast-immobilized strains had higher hydrocarbon degradation potential than planktonic strains. It was also concluded that marine coasts have a much higher potential for oil biodegradation than the water body. In yet another study by Radwan et al. (2002) in the Arabian Gulf employing a solid mineral medium with crude oil as a sole source of carbon and energy, 10 different macroalgae were found associated with large numbers of oil-utilizing bacteria. *Acinetobacter* along with the nocardioforms was found to be predominantly associated with the microalgae. Further shake flask studies using seawater and individual hydrocarbons resulted in attenuation of the hydrocarbons. It was also found that relatively negligible numbers of bacteria were released into the seawater compared with the numbers immobilized on the macroalgal surfaces. These natural biological consortia therefore represent valuable tools that could be of high potential for phytoremediation of oily seawater.

In a study to determine whether the bioremediation potential of coastal materials for oil-polluted sea water depended on the numbers of hydrocarbon-utilizing bacteria they naturally harbor, (Radwan et al, 2002), found out that, Inshore water of the Arabian Gulf contained only a few hydrocarbon-utilizing bacteria. However, coastal sand, cyanobacterial mats and epilithic biomass were much richer in these bacteria, than in the water body. Predominant bacterium in all samples
was *Acinetobacter calcoaceticus*. Bioremediation effects depended on external addition of KNO₃. This information could be further useful in constructing technologies for oily seawater bioremediation employing *Acinetobacter* species as a potential bioremediation organism. Similar observations were made by Razak et al, 1999 in for *Acinetobacter* sp. E11, isolated from Port Dickson Beach, Malaysia. This isolate was able to utilize crude oil as the sole carbon and energy source. Nitrogen and phosphorous supplements were essential for growth and the isolate exhibited strong substrate specificity.

Equipped with a crude oil and hydrocarbon biodegradation potential and other exciting properties, the marine *Acinetobacter* can serve to bioremediate the fast deteriorating marine ecosystem, thereby leading to clean pollution free blue seas.

1.3.3. Conclusions and Future prospects:

The growing interest in the marine resource is adequately demonstrated by the novel metabolites recently reported from marine bacteria and the number of research laboratories working in this field throughout the world. There is a tremendous potential and scope for search of novel *Acinetobacter* strains possessing physiologically and biotechnologically important properties.

For a total chemical potential of the marine *Acinetobacter* to be effectively assessed, continued research is needed. It appears that, from a natural product perspective, marine *Acinetobacter* species are scarcely explored resource. Further investment at both academic and industrial perspective, will enhance the utility of this resource. With a clear understanding of the species of marine *Acinetobacter* inhabiting the marine expanse and their potential at hand, further venturing into their industrial applicability is possible. Even though there are certain ongoing programs on several aspects in this area, some important aspects still warrant further input and investigation.

1.3.4. References:


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1.4. Biology of the genus *Brevibacterium*

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1.4.1. Abstract:

The genus *Brevibacterium* comprises of Gram positive, non-sporing non-motile rods, belonging to the phylum *Actinobacteria*, with a G+C content in their DNA, ranging from 51 to 70%. Their cell-wall peptidoglycan contains mesodiaminopimelic acid (DAP) as the diamino acid. Absence of arabinose in the cell wall, absence of mycolic acids and large amounts of dehydrogenated menaquinone is another characteristic of this genera. *Brevibacterium* spp. exhibit a marked rod-coccus cycle during growth on complex media. The *Brevibacterium* genus is a heterogeneous genus comprising of large number of species. Production of enzymes, amino acids and surface active agents are a prominent characteristic of members of this genus. Recently members of this genera have been elucidated in nanoparticle synthesis. *Brevibacterium* species have been attracting attention by scientists worldwide due to their applications in cheese making industry and by virtue of production of industrially important products. This review describes the general taxonomy, occurrence, characters and potential industrial applications of members of the *Brevibacterium* genus.

**Keywords**: *Brevibacterium*, occurrence, taxonomy, glutamic acid, lysine, cheese, biosurfactant, nanoparticles

1.4.2. Introduction:

*Brevibacterium* belongs to the phylum *Actinobacteria* within the domain bacteria. In terms of number and variety of identified species, the phylum *Actinobacteria* represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria (Stackebrandt et al, 1997), including 5 subclasses and 14 suborders (Stackebrandt, 2000). This phylum comprises of Gram-positive bacteria with a high G+C content in their DNA, ranging from 51 to 70 % (Ventura et al, 2007).
Brevibacterium spp. exhibit a marked rod-coccus cycle during growth on complex media. During the exponential phase the cells are morphologically rod shaped and become coccoid as they enter the stationary phase. Both rod and coccoid forms are Gram-positive, but some strains and older colonies may tend to be Gram negative. These are non endospore forming, non-motile and have an optimum growth temperature of 20 to 30 or 37 °C. These are obligate aerobes and show slight or no acid production from glucose, exhibit extracellular protease production and are catalase positive. Their cell-wall peptidoglycan contains mesodiaminopimelic acid (DAP) as the diamino acid. Absence of arabinose in the cell wall, absence of mycolic acids and large amounts of dehydrogenated menaquinone is another characteristic of this genus (Jones & Keddie, 1986).

This bacterium has a long history of classification and reclassification and its members exhibit interesting properties like production of enzymes, amino acids and other agents of industrial importance. In this review we briefly summarize on each of these properties exhibited by different Brevibacterium species.

1.4.2.1. Taxonomy and Classification:

The genus Brevibacterium comprises of large number of species thus making it a heterogeneous genus. The genus was first proposed by Breed in 1953 with B. linens as the type species. (Onraedt et al, 2005; Breed, 1953). Later three more species were included in it comprising of B. epidermis, B. casei and B. iodinum (Collins et al, 1983; Collens et al, 1980). Bacterial strains previously classified to belong to the Brevibacterium genera have been classified and reclassified with the advent of newer molecular techniques for bacterial systematics. Liebl et al (1991) have reclassified Brevibacterium divaricatum DSM 20297T, “Brevibacterium avum” DSM 20411, “Brevibacterium lactofermentum” DSM 20412 and DSM 1412 to Corynebacterium glutamicum on the basis of rRNA gene restriction patterns. While Stackebrandt et al (1999) have reclassified Brevibacterium incertum (Breed 1953) as Desernzia incerta gen. nov., comb. A new species, B. lutescens was described by Wauters in 2003 which was later corrected to B. luteolum (Euzéby, 2004). Three more species namely Brevibacterium halotolerans, Brevibacterium frigoritolerans and Brevibacterium stationis, have not been subjected to genetic studies and their taxonomic positions therefore remain uncertain (Jones & Keddie, 1986).

1.4.2. Occurrence:

*Brevibacterium* belongs to the phylum *Actinobacteria*, members of which are widely distributed in both terrestrial, aquatic and marine ecosystems. They are especially found in soil, where they play a crucial role in the recycling of refractory biomaterials by decomposition and human formation, and are also found inhabiting activated sludge. (Juang & Chiou (2007); Lee, 2006; Stach & Bull, 2005; Goodfellow & Williams, 1983). Number of *Brevibacterium* species have been associated with dairy products like milk and cheese. These include *B. linens, B. casei* and *B. iodinum* (Mounier et al, 2007; Kircher et al, 1999; Salva et al, 1999; O'halloran et al, 1998; Gripon, 1987). Members of *Brevibacterium* genus have also been found in soil or saline soils and colonizing roots of plants (Tang et al, 2008; Faisal & Hasnain, 2006).

Strikingly, members of this genera have also been isolated from marine environments including marine water, sediments, mud and islands (Tong-Wei et al, 2010; Lee, 2006; Johnson et al, 1968). Bhadra et al (2008) reported isolation of *Brevibacterium oceani* from the deep sea sediments of the Chagos trench in the Indian Ocean. While Caton et al (2004), reported halotolerant species of *Brevibacterium* from the great salt plains of Oklahoma. *Brevibacterium* species have also been isolated from inanimate sources like damaged mural paintings. The three strains now identified as *B. picturae* were obtained from three European sites and were recovered from a sample of a paint layer, showing black discoloration, located in the chancel vault of the Saint-Catherine chapel of Castle Herberstein in Austria (Heyrman et al, 2004). Pepe et al, recently in 2011 have isolated *Brevibacterium* species by culture independent approach from an archaeological excavation site from a deteriorated wall painting of Herculaneum in Italy.

*Brevibacterium* species have also been isolated from human and environmental samples (Wauters et al, 2004, 2003 & 2001a & b). Wauters et al (2003) reported four novel species of *Brevibacterium* which they named *B. lutescens* sp. nov from peritoneal fluid, infected ear discharge, peritoneal dialysate fluid and the fourth one an environmental isolate. *Brevibacteria* are opportunistic pathogens, and several species have been isolated from clinical samples (Funke et al, 1997). Several studies have shown that *Brevibacterium* species have been isolated from human sources like ears, peritoneal fluids, skin, secretions and from patients undergoing dialysis. These species include *Brevibacterium casei, B. epidermidis, B. otitidis, B. mcbreinleri*, and *B. paucivorans* (Wauters et al, 2004, 2001a & b, 2000; Brazolla et al, 2000; Pascual et al, 1996; Funke & Carlotti, 1994; Gruner et al, 1994; Gruner et al, 1993; Collins et al, 1983). *Brevibacterium* species namely *B. celere* has been isolated from degraded thallus of a brown alga *Fucus evanescens* (Ivanova et al, 2004).
1.4.2.3. Industrial importance of the genus Brevibacterium:

_Brevibacterium_ species are known to produce different enzymes, amino acids, surface active agents and nanoparticles thus indicating a great commercial value. In the succeeding discussion we report different properties of the members of the genus _Brevibacterium_ that make it a lucrative option for applications as an industrial agent.

1.4.2.3.1. Production of amino acids:

Species of _Brevibacterium_ are known to be industrially important by virtue of their ability to produce important amino acids like L-lysine and glutamic acid. (Mosavi-Nasab et al, 2010; Roeva & Pencheva, 2005; Ruklisha et al, 2004; Das et al 1995; Nampoothiri & Pandey, 1995; Aida et al, 1986). L-amino acids find diverse commercial applications as food additives, feed supplements, infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agrochemicals (Nampoothiri and Pandey, 1999). Nampoothiri & Pandey (1999) have exploited strain of the genus _Brevibacterium_ for production of L-glutamic acid using a cheap and locally available starchy substrate, cassava (_Manihot esculenta_ Crantz). They have standardized a process for its fermentation and recovery of lysate by ion-exchange resin column. Choi et al (2004) reported that glutamic acid production by _Brevibacterium_ sp. TC452 was temperature dependant, an increase in the temperature from 30 to 38 °C in biotin limitation increased not only the rate of glutamic acid biosynthesis but also its specific production rate. Ali et al (2009) recently reported _Brevibacterium flavum_ for production of lysine. The authors were successful in producing a lysine concentration of 57.2 mg/100 mL with 2 % corn steep liquor by a _B. flavum_ auxotroph generated by mutation employing iso-propyl methane. Iskandar et al (2004) have also reported that mutant strains yield higher L-Lysine than the wild type strain. The authors have used UV irradiation for improvement of a _Brevibacterium_ sp. ATCC 21866 to yield higher L-lysine. The irradiation created a mutant increasing the L-lysine yield from 5.75 g/L to 11.29 g/L at the end of the 4 day fermentation process.

Shiratsuchi et al (1995) have reported fermentative and simultaneous production of L-lysine and L-glutamic acid by _B. lactofermentum_. Different enzymes and amino-acids have been produced by different bacteria by conventional methods or by a factorial approach (Jagtap et al, 2010). However, modeling in biology is a comparatively new approach for biosynthesis of metabolites. Roeva & Pencheva (2005) devised a generalized net model for fermentation process for manufacture of L-lysine by fed-batch cultivation from _B. flavum_ 22LD. In order to render the specific peculiarities of the fermentation processes, as well as to avoid the complexity of mathematical description with systems of differential equations, the elaboration of some new methods and approaches for their modeling and control are predetermined. The authors have used an application of generalized nets as a novel and alternative approach for modeling of the fermentation process.
1.4.2.3.2. Production of enzymes:

Enzymes are attracting attention worldwide due to wide range of industrial applications including organic synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. These are gradually replacing the use of harsh chemicals in various industrial processes (Mukhopadhyay et al., 2008). Proteases are one of the most important groups of industrial enzymes accounting for nearly 60% of the total enzyme sale (Abusham et al., 2009; Thumar & Singh, 2007; Rao et al., 1998). Rattray & Fox (1998) have reviewed the enzymes produced by *Brevibacterium* in great detail. Members of the genera *Brevibacterium* are known for production of extracellular enzymes. *B. linens* is one of the species which is known to produce copious amounts of enzyme while growth on surfaces of different cheese varieties. These extracellular enzymes secreted by *B. linens* include proteinase (Rattray et al., 1996 & 1995; Hayashi et al., 1990; Juhasz & Ska’rka, 1990) and aminopeptidase (Hayashi & Law, 1989; Foissy, 1978). The release of these extracellular enzymes results in the production of low molecular mass compounds which are capable of diffusing towards the interior of the cheese and are required for the development of the characteristic qualities of these cheeses (Rattray et al., 1996). Clancy & O’Sullivan (1993) partially purified and characterized proteinase from *B. linens* isolated from the surface of ripened cheese. This proteinase was thermostable and had a molecular weight of 18.5 kDa and was stable over a pH range of 2 to 12. Salva et al. (1999) reported production of the enzyme cholesterol oxidase from a *Brevibacterium* strain isolated from buffalo milk.

1.4.2.3.3. Cheese flavoring industry:

Large number of the *Brevibacterium* species have been associated with dairy products like milk and cheese (Salva et al., 1999; O’Halloran et al., 1998). Rattray & Fox (1999) have detailed comprehensively all aspects of enzymology and biochemical properties of *B. linens* relevant to cheese ripening. The value of any product for human consumption depends on its aesthetic value, its taste and the aroma. An important industry in western countries is the cheese making industry. *Brevibacteria* are industrially important microorganisms used in manufacture of Limburger, Roquefort, Stilton and other Trappist-type cheeses (Sharpe et al., 1977; Ades & Cone, 1969). High proteolytic, varied lipolytic activity, aminopeptidase activity, and diverse biochemistry of *Brevibacterium* especially *B. linens* extend their use as a novel flavor adjunct in the manufacture of low-fat Cheddar cheese (Weimer et al., 1997). *B. linens* predominant the surface flora, contributes to the final surface flavour, colour and aroma of different varieties of cheese not only due to its strong proteolytic activity but also due to the production of methanethiol (Ummadi & Weimer, 2001). Mounier et al. (2007) reported that *B. aurantiacum* found on the surface of cheese was responsible for its surface ripening. These species are able to grow in high salt concentration of 15%, a property useful in formulation of some special varieties of cheese.
1.4.2.3.4. Flavor defects in cheese:

Attempts to develop a desirable reduced fat Cheddar cheese are impeded by a propensity for flavor defects such as meaty brothy, putrid, fecal, and unclean off flavors in these products. Studies suggest aromatic amino acid catabolism of starter, adjunct, and nonstarter lactic acid bacteria significantly impact development of off flavor (Dunn & Lindsay, 1985). Ummadi & Weimer (2001) studied the catabolism of tryptophan by a strain of *B. linens* to study whether the catabolism products of the tryptophan catabolism by *B. linens* generated off flavours; based on their results, they reported that the catabolic enzymes of *B. linens* are not likely to be involved in the formation of compounds associated with off flavors in cheddar cheese.

1.4.2.3.5. Production of bacteriocins/ bioactivity:

Red smear cheeses are economically important in Austria, Germany, France, Scandinavia, and Switzerland. Surveys on the occurrence of *Listeria* spp. in dairy products have revealed that red smear cheeses are more frequently contaminated with *Listeria* than are any other soft cheeses (Eppert et al, 1997). It is, therefore, important to develop defined ripening starters which inhibit *Listeria* efficiently. *B. linens* M18, isolated from red smear cheese produces a bacteriocin named Linocin M18 in the stationary phase which inhibits growth of *Listeria* spp., several coryneform and other Gram-positive bacteria. Linocin M18 is heat labile and stable between pH 3 and 12. It was purified by ultrafiltration, ultracentrifugation, and gel filtration chromatography. Linocin M18 consisted of a single protein subunit with a molecular mass of 31 kDa and an isoelectric point of 4.5. N-terminal sequence analysis yielded Met-Asn-Asn-Leu-Tyr-Arg-Glu-Leu-Ala-Pro-Ile-Pro-Gly-Pro-Ala-Ala-Glu-Ile (Valdes-Stauber & Scherer, 1994). Antilisterial activities have also been reported from *B. linens* by Gori et al in 2010. Eikmanns et al in 1997 reported bacteriocin production from *B. linens* growing on the surface of cheese. This bacteriocin was in part responsible for reduction in growth of *Listeria* spp. growing in situ on soft cheese and could thus be useful in improving the quality of cheese. As early as 1997, Valdes-Stauber & Scherer also reported Linocin M18 production by *B. linens* with Linocin being composed of a single protein subunit with a molecular mass of 28.5 kDa. Beattie & Torrey (1986) reported antifungal activity of *B. linens* against *P. expansum* NRRL 877 by virtue of methanethiol produced by the bacterium.

*Brevibacterium* species are also known to be a part of marine biofilm as per a study conducted from Hong Kong waters. These strains were found to induce larval settlement in the marine polychaete *Hydroides elegans* (Lau Stanley et al, 2002).

1.4.2.3.6. Presence of plasmids and antibiotic resistance:

There are several reports on presence of plasmids in the genus *Brevibacterium*. These plasmids may be useful in gene transfer studies, few of them are cryptic while others may code for antimicrobial resistance. Dib et al (2009) isolated *Brevibacterium* sp. Ap13 from flamingo’s feces in Laguna Aparejos, a high-altitude lake located at approximately 4,200 m in the northwest of
Argentina. This strain was unusually resistant to multiple antibiotics which include colistin, ampicillin, cefazidime, ceftazidime, trimethoprim/sulfamethoxazole, and cefepime. Further when Dib et al (2010) studied this bacterium they detected two plasmids approximately 87 and 436 kb, designated pAP13 and pAP13c, respectively in Brevibacterium sp. Ap13. pAP13c was a circular megaplasmid, while interestingly, pAP13 was a linear megaplasmid, the first of its kind to be detected. Circular plasmids have been detected in different strains of Brevibacterium. Antibiotic resistance has also been reported in B. casei by Funke et al (1996). They showed that large number of B. casei strains were highly resistant to amoxicillin-clavulanic acid, cefuroxime, cephalothin, imipenem, oxacillin, ampicillin and ceftriaxone. Opportunistic species of the Corynebacterium and related coryneforms especially Brevibacterium isolated from different clinical materials were found to be highly resistant to vancomycin, teicoplanin and imipenem antibiotics and hence could be cause of infections (Chudnicka & Kozoli-Montewka, 2003). Babay & Kambal (2004) isolated Brevibacterium sp. from blood cultures of patients at King Khalid University Hospital (KKUH), Riyadh, Kingdom of Saudi Arabia. They found that Brevibacterium sp. exhibited high resistance to antibiotics especially erythromycin, clindamycin and ciprofloxacin. While, Troxler et al (2001) also report that Brevibacterium casei are alarmingly resistant to large group of antibiotics viz, tetracyclines, aminoglycosides, beta-lactam agents (subgroup: penicillins, cephalosporins, carbapenems, and monobactams), macrolides, lincosamides, streptogramins and antifolates.


Moore et al in 2003 showed that B. linens harbored four novel plasmids, they were also the first to determine the complete nucleotide sequence of a native plasmid of B. linens. They showed that plasmid pLIM is a cryptic plasmid of 7610 bp length, and belongs to a subfamily of theta replicating ColE2-related plasmids. Their investigation suggested that replication in pLIM requires two replicases, a primase (RepA) and a DNA binding protein (RepB), encoded by a single operon repAB and that the origin of replication is located upstream of repAB transcription.

All these studies on plasmids indicate the potential of the species of the Brevibacterium genera for use in construction of vectors and studying detailed mechanisms of gene transfer. These plasmid could also explain probable reasons for their economic importance due to their ability to synthesize essential amino acids, flavouring agents, surfactants, nanoparticles and antibiotic resistance.
1.4.2.3.7. Hydrocarbon and metal metabolism:

Members of the genus *Brevibacterium* have been elucidated in hydrocarbon metabolism. Pirmik et al. (1974) reported metabolism of branched- and straight-chain alkanes by *B. erythrogenes*. This strain metabolized n- alkanes like n-pentadecane and branched alkanes like pristane (2,6,10,14-tetramethylpentadecane) and 2-methylundecane. Lim & Halos (1995) reported excellent bioremediation potential of species of *Brevibacterium*. These species efficiently degraded motor oil and diesel oil. *B. linens* a garden soil isolate has been recently reported to degrade paranitrophenol (Ningthoujam, 2005).

*Brevibacterium* species may play important role in metal uptake and thus bioremediation. Abou-Shanab et al. (2008) examined effect of bacterial isolates for their ability to increase availability of water soluble Cu, Cr, Pb and Zn in soils and for their effect on metal uptake by *Zea mays* and *Sorghum bicolor*. They found highest concentrations of Pb (0.2 g/kg), Zn (4 g/kg) and Cu (2 g/kg) were accumulated in shoots of *Z. mays* grown on Cu-rich soil inoculated with *B. halotolerans*. Thus indicating that *Brevibacterium* species play an important role in increasing metal availability in soil, thereby enhancing Cr, Pb, Zn and Cu accumulation by plants. Additionally, *Brevibacterium* species have been recently reported for production of growth hormone Indole-3-acetic acid. These bacteria thus play important role in plant growth promotion and could be used for enhanced growth of plants (Nimnoi & Pongsilp, 2009; Faisal & Hasnain, 2006).

1.4.2.3.8. Production of Surface active agents and nanoparticles:

The surfactant industry is an important industry and studies on biosurfactants are fast gaining grounds due to their environment compatibility, low toxicity and ability to be synthesized from cheaper and renewable resources (Muthusamy et al, 2008; Maneerat, 2005). Members of the genus *Brevibacterium* have been recently elucidated for the production of biosurfactants. Kiran et al. (2010a) have reported production of a lipopeptide biosurfactant from a marine strain of *Brevibacterium aureum* MSA13 isolated from a marine sponge *Dendrilla nigra*. Interestingly this biosurfactant was produced using industrial and agro-industrial solid waste residues as substrates in solid state culture (SSC). This biosurfactant could find potential aplication in microbial enhanced oil recovery processes in the marine environments. This was a novel biosurfactant with a hydrophobic moiety of octadecanoic acid methyl ester and a peptide part predicted as a short sequence of four amino acids including pro-leu-gly-gly. Kiran et al. (2010c) also report production of another glycolipid biosurfactant from marine *B. casei*. Interestingly this biosurfactant was also antiadhesive in nature. Samadi et al. (2007) report production of glycolipid biosurfactant by a soil isolate *Brevibacterium* sp. strain S-34. The biosurfactant had a yield of 2.4 g/L and reduced surface tension from 69 mN/m to 30 mN/m.

The recent use of *Brevibacterium* species for synthesis of nanoparticles is lucrative and offers benefits of ecofriendliness and amenability for large-scale production. These studies have been initiated lately in 2010. Silver nanoparticles synthesis has been reported by Kiran et al (2010b)
from B. casei isolated from a marine sponge. They have used the glycolipid biosurfactant produced by this strain for biosynthesis of nano-scale silver particles in reverse micelles; these particles exhibited uniformity in size and were stable for 2 months. In another study, Kalishwaralala et al (2010) have demonstrated an unprecedented green process for the production of spherical-shaped gold and silver nanoparticles synthesized and stabilized using B. casei. They suggest possible presence of protein molecules as reducing and capping agent thereby increasing the stability of the synthesized silver and gold nanoparticles. These synthesized nanoparticles exhibited stable anti-coagulant effects and thus could be used in medicine. Interestingly, Pandian et al (2009) described direct use of dairy waste and sea water as potential sources for the production of polyhydroxybutyrate (PHB). They used this PHB to synthesize nanoparticles using solvent displacement technique.

All these studies indicate tremendous potential of Brevibacterium for synthesis of nanoparticles of different metals, having different shapes, sizes and tunable properties. This can be exploited further for biosynthesis of nanoparticles with applications in diverse fields.

1.4.3. Conclusions and Future prospects:

Members of the genera Brevibacterium have been isolated from several different niches and find wide applications in biosynthesis of compounds of high economic importance like enzymes, amino acids, plant growth promoting substances, surface active agents and nanoparticles. They harbor plasmids and show resistance to large number of antibiotics and are implicated in hydrocarbon and metal remediation. Though currently the use of this organism is restricted to only few industries like cheese making, due to its wide array of properties it could be applicable in innumerable fields. Research on this bacterium has gained importance in the recent years and with continued studies, this bacterium could find its way into the commercial market easily.

1.4.4. References:


