CHAPTER II

LITERATURE REVIEW

2.1 Microbial surfactant (Biosurfactant)

The term surfactant circumscribed diverse variation of compounds with surface active properties existing either in synthetic or biological state. These compounds have the ability to reduce surface and interfacial tension at the interfaces between solids, liquids and gases and hence, allowing them to disperse freely as emulsions (Mulligan, 2009). Being amphiphilic in nature, surfactant contains both hydrophilic and hydrophobic domains and thus capable to exist preferentially at the interfaces between two phases of different degrees of polarity and hydrogen bonding (Wang and Mulligan, 2004).

The effectiveness of a surfactant is determined by its ability to lower the surface tension. The term surface tension is a measure of the surface free energy per unit areas required to bring a molecule from the bulk phase to the surface (Vedaraman and Venkatesh, 2011). By lowering the surface tension at interfaces or surfaces, reduction of repulsive forces between dissimilar phases allows the two phases to mix and interact more easily (Seydlova and Svobodova, 2008)

Surfactant encompasses a paramount class of industrial chemicals highly used in almost every sector of current industry. Indeed, total surfactant output is utilized almost in household as laundry detergents while some amount is destined for industrial use. Most of these surfactants are petroleum based and are chemically synthesized at the present which however, often have detrimental ecological effects (Isa et al., 2007). So, the purpose of the surfactant itself is one of the most important factors that should be considered prior application. Other factors that should be considered including cost, public and regulatory perception, biodegradability and degradation products, toxicity to humans, animals and plants and ability to recycle (Mulligan et al., 2001). Thus, this leads the trend towards using and searching for environmental friendly biodegradable surfactants of natural origin.

Surfactant compounds which are produced by microorganisms and demonstrate particularly prominent surface activity and emulsifying activity are classified as biosurfactant (Ahimou et al., 2000; Cao et al., 2009). These microbial compounds constitute a diverse group of surface-active molecules and usually produced by hydrocarbon-utilizing microorganisms in various substrates such as sugars, oils, alkanes and wastes (Finnerty, 1994). The hydrophilic part of a biosurfactant usually consists of amino acids, anions or cations, or polysaccharides while its hydrophobic part consists of saturated or unsaturated long chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids (Seghal Kiran et al., 2010). Various types of biosurfactant including their respective microorganism producer are represented in Table 1.

Table 1: List of biosurfactant and their respective microbial origins

Type of biosurfactant	Organism	Reference
Lipopeptide (surfactin, iturin and fengycin)	*Bacillus subtilis* sp., *Pseudomonas libenensis*, *Rhodococcus* sp., *Azotobacter chroococum*, *Pseudomonas fluorescens*.	(Seydlova and Svobodova, 2008; Youssef et al., 2004; Mulligan, 2005)
Glycolipids (rhamnolipids, sophorolipids and trehalolipids)	*Rhodoccus erythropolis*, *Psedomonas aeruginosa*, *Psedozyma hubeiensis*, *Micrococcus luteus*,	(Kitamoto et al., 2002; Bogaert et al., 2007; Sullivan, 1998)
Phospholipids	*Thiobacillus thioxidans*	(Knickerbocker et al., 2000)
Fatty acids or neutral lipids	*Clavibacter michiganensis* subs. *insidious*	(Banat, 1994)
Polymeric biosurfactant (Emulsan and Alasan)	*A. calcoaceticus*, *A. radioresistens*	(Choi et al., 1996; Barkay et al., 1999)
Cellobiose lipids	*Ustilago maydis*	(Teichmann et al., 2007)
Serrawetin	*Serratia marcescens*	(Lai et al., 2009)

Recently, high attention has been focused towards biosurfactant since this surface-active compound has clear advantages compared with its synthetic counterpart including low toxicity, high biodegradability, low irritancy and compatibility with human skins (Najafi et al., 2010). Thus, biosurfactant could be a propitious alternative to the widely used chemically synthesized surfactants since it is consumable for the food, pharmaceuticals, and cosmetics industries (Haniyavarn et al., 2003).

Due to the amphiphilic characteristics of biosurfactants, they have been employed in industry as de-emulsifiers, penetrants, adhesives as well as flocculating, wetting and foaming agent. Diverse application of biosurfactants in industry is due to their abilities including low surface tensions, high solubility, detergency powder, wetting ability and foaming capacity (Mulligan, 2005). Most intendment on microbial surfactant applications has been directed toward their use in environmental purposes owing to their diversity, environmentally friendly nature and propriety for large scale production (Rodriguez et al., 2006).

2.2 *Bacillus sp.* as biosurfactant producer

The genus *Bacillus* can be considered as one of the prominent microbial used by the fermentation industry. Among them, well-known examples are *B.subtilis, B. amyloliquefacies, B. licheniformis, B. alkalophilus, Blentus and B. thuringiensis*. Some of this microbial species is capable to synthesize substantial amount of protein such as amylases and proteases (Sietske and Diderichsen, 1991).

Bacillus subtilis is a Gram-positive, spore forming bacterium. Generally, this type of bacteria resided in soil and on plant material and grows aerobically at moderate temperatures and pH (Vater et al., 2002). *B. subtilis* is considered as an important of most environments as well as other *Bacillus* species. Since *B. subtilis* exhibits no pathogenic potential to humans, it is regarded as an opportunistic microorganism and indeed, applicable for various purposes (Sietske and Diderichsen, 1991).

In conjunction with any other bacilli, *B. subtilis* also produce a broad spectrum of bioactive peptides with excellent surface active and antimicrobial properties. Among the reported class of such compounds are lipopeptides, comprising of surfactin, fengycin and iturin. The chemical and physical diversity of lipopeptide biosurfactants produced by *B. subtilis* earns them ideal candidates for various applications (Joshi et al., 2008).

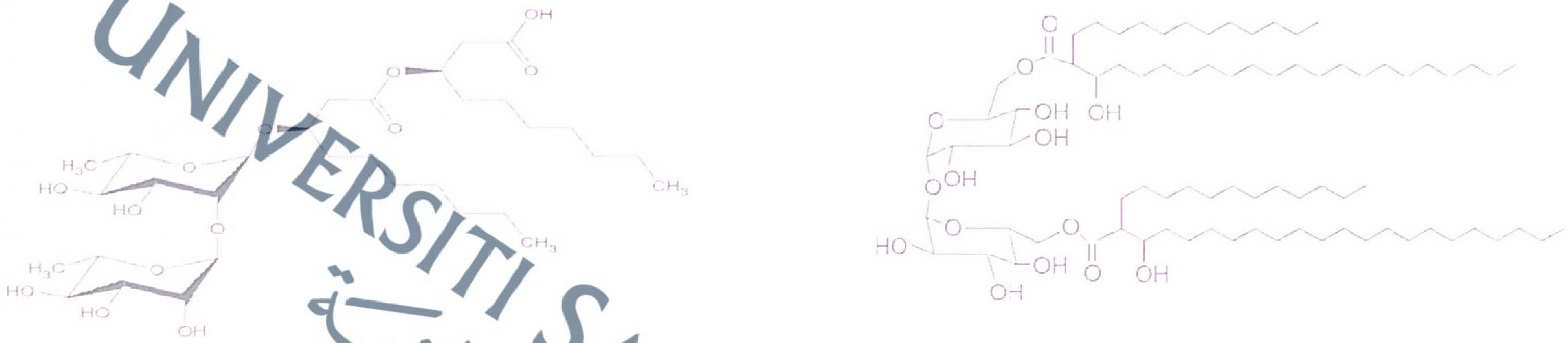
2.3 Classification of biosurfactant

Biosurfactants are yielded by various types of microorganisms mainly by bacteria, fungi and yeasts (Yeh et al., 2006). Those microorganisms are able to produce surface-active compounds with broad ranges of chemical structures as shown in Figures 1. A different type and structure of biosurfactants is plausible if the hydrocarbon chain which is the hydrophobic part of biosurfactant comprises of diverse chain length, branching or total number of saturated and unsaturated fatty acids (Sivapathasekaran et al., 2009). For hydrophilic part of biosurfactant, however, can be a carbohydrate, cyclic peptide, phosphate, amino acid, carboxylic acid or alcohol (Mulligan, 2009). Accordingly, biosurfactant can be categorized into four types which are glycolipids, phospholipids, polymeric biosurfactants and lipopeptides.

2.3.1 Glycolipids

Glycolipids are known as the most typical class of biosurfactants. These compounds are sugar-containing lipids in which a carbohydrate moiety is linked to a fatty acid moiety (Faria et al., 2014). The most effective glycolipids from the point of view of surface-active properties are the trehalose lipids produced by the *Mycobacterium* and related species, the rhamnolipids produced by *Pseudomonas* species and the sophorolipids obtained from the yeast (Rodrigues at al., 2006).

Figures 1: Representative structures of biosurfactants (Soberon-Chavez and Maier, 2001)



Rhamnolipid

Trehalose lipid

Saphorolipid

Lipopeptide

Rhamnolipids are the most comprehensively studied glycolipid biosurfactants since they exhibit relatively high surface activities and are presented in high yields merely after brief incubation periods (Kumar et al., 2012). The physicochemical property that caused rhamnolipids to receive extensive interest is because of their excellent surface activity. Hence, they show high potential in industrial and biotechnological applications such as synthesizing and stabilization of nanoparticles, preparation of microemulsion, as an antiagglomeration agent and as dispersing agent (Henkel et al., 2012).

Trehalolipids and sophorolipids are likewise similar in structure when compared to rhamnolipids. The structures of trehalolipids are quite diverse especially in hydrophobic moiety and can be varied from short simple to long complex fatty acids. They can be characterized by disaccharide trehalose linked at C_6 to two β-hydroxy-α-branched fatty acids (Desai and Banat, 1997). Moreover, they have been isolated from several strains of *Arthrobacter*, *Mycobacterium*, *Rhodococcus*, *Brevibacterium* and *Corynebacterium*. From the point of practical use as biosurfactant, the trehalose lipids from *Rhodococcus* and the genera are of high potential in application (Cameotra and Makkar, 2004).

On the other hand, sophorolipids structure consists of a disaccharide sophorose linked to a long chain hydroxylated fatty acid. Sophorose is a diglucose with an unusual β-1,2 bond and may contain acetyl groups (in the case of sophorolipids) (Mulligan et al., 2001). The sophorolipids obtained from the *C. bombicola* are in fact a mixture of related molecules with distinction in the fatty acid portion (chain length, saturation and position of hydroxylation) and the lactonization and acetylation pattern (Ron and Rosenberg, 2001).

2.3.2 Phospholipids and polymeric biosurfactants

Phospholipids are probably best known as a main component of all cell membranes. Basically, most phospholipids comprise of several fatty acids linked to a cationic phosphate group while their proportion depends on the production by various microorganisms. Phospholipids that contain specific fatty acids are usually desirable for some practical applications (Schiller et al, 2004; Seydlova et al., 2012).

On the other hand, polymeric biosurfactant consists of high molecular weight biopolymers which mainly a polysaccharide backbone linked covalently to fatty acid side chains. Liposan and emulsan are usually the most extensively studied polymeric biosurfactants (Satpute et al., 2010). This type of biosurfactant can emulsify hydrocarbon efficiently, even a very low concentration as well as a powerful emulsion stabilizer that having several applications in diverse fields.

2.3.3 Lipopeptides

Lipopeptides biosurfactant are an interesting class of metabolites that have a wide variety of applications in industrial and pharmaceutical sectors which are formed by surfactin, iturin and fengycin families (Athukorala et al, 2009). All these substances existed as families of closely related complexes and isoforms which differ in length and branching of the fatty acid side chains as well as in the amino acid substitutions in the peptide rings. *B. subtilis* strains have been proclaimed as the major source and producer of lipopeptide biosurfactant. Different strains of *Bacillus* sp can generate miscellaneous lipopeptide family and a single strain can also produce lipopeptides with different peptides cores. The diversity of lipopeptide is due to the combinatorial and precursor-directed biosynthesis (Liu et al., 2009; Zhao et al., 2012).

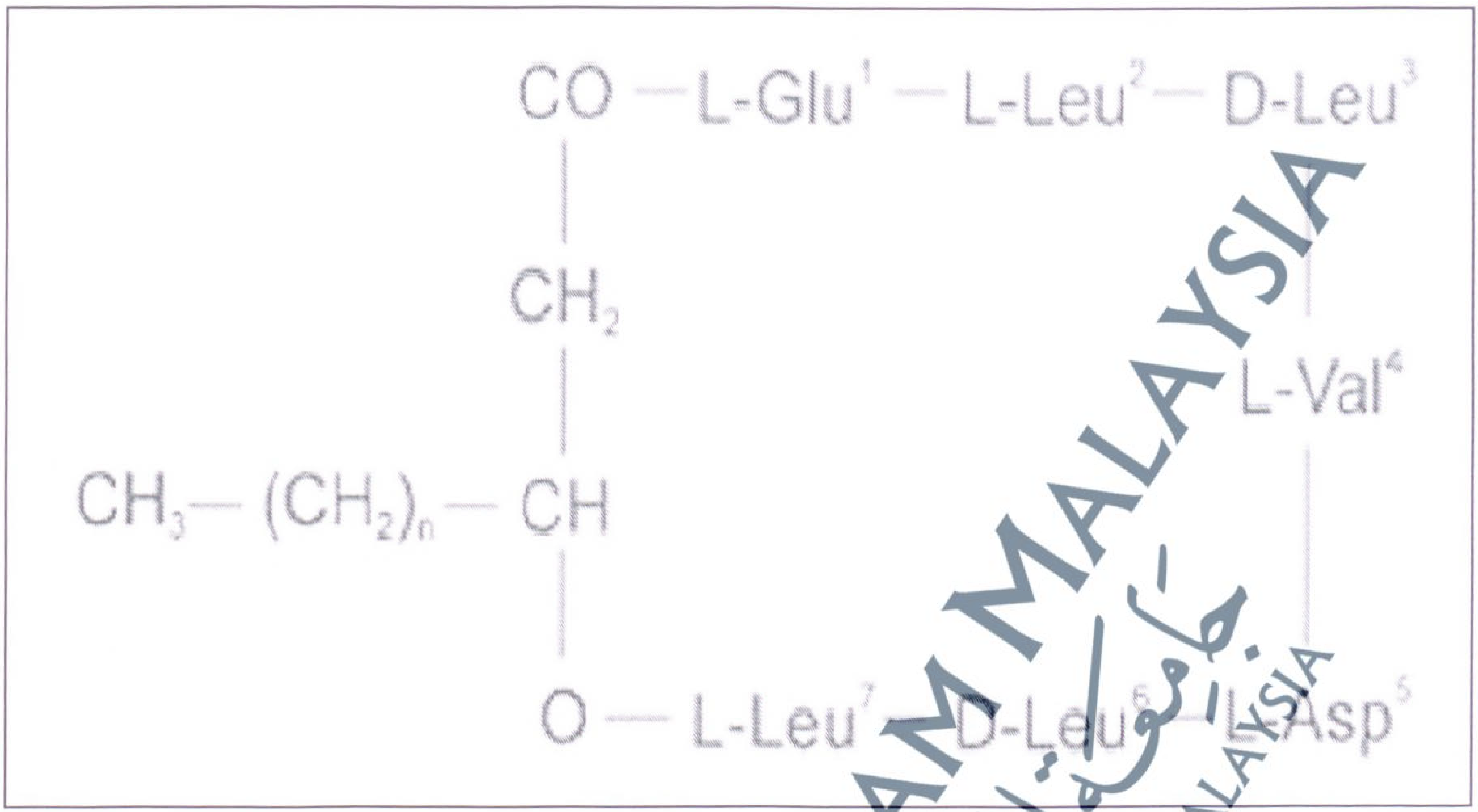
The surfactin and iturin compounds usually are cyclic lipoheptapeptides which include a β-hydroxy fatty acid and β-amino fatty acid respectively as their lipophilic constituent. However, fengycin is a lipodecapeptide with a β-hydroxy fatty acid in its side chain. Another difference between these types of biosurfactant situated at length of the fatty acid chains that can vary from C_{13} to C_{16} for surfactin, from C_{14} to C_{17} for iturin and C_{14} to C_{18} for fengycin (Vater et al., 2002).

2.4 Surfactin

Surfactin is a cyclic lipopeptide biosurfactant which is generally synthesized by several strains of the genus *Bacillus* in certain types of culture media (Ongena et al., 2007). This microbial surfactant has large variety of isoforms which differ by variation of the length and branching of their fatty acid component as well as by amino acid replacement in their peptide ring (Zhao et al., 2012; Liu et al., 2015).

Surfactin constitutes of seven amino acids bonded to the hydroxyl groups of carbon acid. The compound is structured and characterized by a heptapeptidic moiety ($_{L}$-aspartic acid, $_{L}$-leucine, $_{L}$-glutamic acid, $_{L}$-leucine, $_{L}$-valine and two $_{D}$-leucines) linked to a beta hydroxyl-fatty acid of the chain lengths 12 to 16 carbon atoms bring about its cyclic structure (Heerklotz & Seelig, 2001; Behary et al., 2011). Figure 2 shows the primary structure of surfactin that displaying the position of amino acids residues which is located at positions 2, 3, 4, 6 and 7, while the glutamyl and aspartyl residues at position 1 and 5, respectively introduce two negative charges to the molecule (Joshi et al., 2008). In solution, surfactin is illustrated exhibits in a "horse saddle" conformation that is a remark for its large spectrum of biological activity, making it very appealing for both industrial application and academic studies. Surfactin biosynthesis is catalyzed non-ribosomally by the action of a large multienzyme complex consisting of four modular building blocks, called the surfactin synthetase (Peyfoux, 1999).

Figure 2: Primary structure of surfactin (Seydlova and Svobodova, 2008)



Surfactin reduces the surface tension of water from 72 to 27 mN/m at a concentration as low as 10 μM, which is far below the critical micelle concentration in water of 23 mg/L and about two orders of magnitude smaller than those of most other detergents (Mulligan et al., 2001; Chen et al., 2007). Due to this attribute, surfactin displays antifungal and antibacterial properties and able to interact with artificial and biomembrane systems, for example bacterial protoplasts and enveloped viruses (Rodrigues et al., 2006).

2.5 Properties of biosurfactant

Several prominent physico-chemical properties have been proposed for biosurfactant due to its amphiphilic structure. Among them are pH stability, thermal stability, solubility and surface activity which are very attractive properties for industrial use. Furthermore, in contrast to routine synthetic surfactants, biosurfactant usually possesses short alkyl chains hence enhancing its aqueous solubility (Gong et al., 2009). Biosurfactant also enables to interact with the membrane hence disturbing its integrity thus explain their wide range of for biological properties such as antibacterial, antiviral, antifungal and anti-tumor (Cameotra and Makkar, 2004).

2.5.1 Surface tension

Biosurfactant tends to accumulate at the interface between two different phases because of its structure (Rodrigues et al., 2006). Therefore, at an interface of air-water, the hydrophilic part will be immersed within the water phase while the hydrophobic part approaching the air. Aggregation of biosurfactant at interfaces of surfaces results in the reduction of repulsive forces between opposite phases and grants the two phases to mix and interact at ease. Consequently, alignment of biosurfactant molecules at the interface between two different phases reduces surface and interfacial tensions.

The efficiency of surfactant surface tension activity is expressed by addition of surfactant monomers into the solution hence decrease its surface or interfacial tension until the surfactant concentration attain to the state called as critical micelle concentration (CMC) (Seydlova and Svobodova, 2008). Above the CMC, no further reduction in surface or interfacial tension is observed. However, surfactant monomers instigate to involuntary associate into structured aggregates such as micelles, vesicles and lamellae at the CMC. These aggregates establish as a repercussion of numerous weak chemical interactions between the polar head groups and the non-polar tail groups (Arutchelvi et al., 2014). CMC value can be varied for any surfactant depending on the surfactant structure, pH, ionic strength and temperature of the solution, (Wei et al., 2003).

2.5.2 Emulsification activity

Another physicochemical characteristic that describe a surfactant are its potential to apparent water solubility of hydrophobic compounds by forming emulsions (Slivinski et al., 2012). Emulsions are formed when one liquid phase (water) is dispersed as microscopic droplets within another (hydrocarbon). Addition of biosurfactant have lowering the surface tension of water solution and interfacial tension between water and hydrocarbon mixtures and thus forming stable emulsion of water-hydrocarbon solution (Saikia et al., 2011). For that reason, low molecular weight biosurfactants are more preferred by industries since they have been proved to form stable emulsions (Nitschke and Pastore, 2006).

The roles of biosurfactant as emulsifiers are well reported. For instance, in food industries, biosurfactants are employed as emulsifiers in the processing of raw materials. The rheological characteristics of flour or the emulsification of partially broken fat tissue can be influenced by applying biosurfactant in meat products and bakery (Wan Nawawi et al., 2010). For biomedical purposes, biosurfactants act as emulsifying agent for drug transport to the infection site (Makkar and Cameotra, 2002).

2.5.3 Foaming activity

Foaming is another plausible property that is capable by biosurfactant and is of prominent interest in the development of cosmetics, detergents and pharmaceutical applications (Chen et al., 2008). Basically, the latency of an amphiphile potential at the gas-liquid interface lowers the surface tension which is the driving force for the reduction of surface area and increases the stability of the foam. In many cases, stable foams with small bubble size are usually demanded by various industries (Razafindralambo et al., 1996).

2.5.4 Antimicrobial and antiviral properties

The antimicrobial and antiviral activity of several biosurfactants has been documented in various literatures for several different purposes. Biosurfactant manifests outstanding antimicrobial and antiviral action due to the membrane interactions of its amphiphilic properties which responsible for a variety of pharmacological activities of biosurfactant (Leclère, 2006; Seydlova and Svobodova, 2008).

The antimicrobial and antiviral activities potential of biosurfactant depends on the mechanism by which it inhibits or kills a target organism including on the cell structure of the target organism (Das et al., 2008). It was suggested that the efficacy of the biosurfactant with respect to both activities, is dependent on the chain length of the lipid as well as the charge of the hydrophilic head group. The membrane active surfactant induces permeability changes and at elevated concentrations leads finally to the disintegration of the mycoplasma membrane system by a detergency effect (Vollenbroich et al., 1997).

The biosurfactants that have been most intensively studied regarding its antimicrobial activities are rhamnolipids and surfactin produced by *P. aeruginosa* and *B. subtilis* respectively. Both of the biosurfactant showed excellent antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi (Vollenbroich et al., 1997;

Kracht et al., 1999). The target organisms in Table 2 reported to be susceptible to biosurfactant produced by *B. subtilis*. Among the bacteria *P. fluorescns, B. licheniformis, Rhodococcus globurulus and Staphylococcus aureus* develop a particularly resistant strain (methicilin-resistant *Staphylococcus aureus* or MRSA) that is known to cause postsurgical infections (Cushnie, 2005).

Concurrently, antiviral activity of biosurfactant against various types of viruses has been reported by a few literatures. A study by Vollenbroich et al. (1997) showed positive effect of surfactin and succinol-trehalose lipid against several viruses including Semliki forest virus, herpes simplex virus (HSV), suid herpes virus, vesicular stomatits virus, simian immunodeficiency virus, feline calicivirus and murine encephalomyocarditis virus. The report suggested that the antiviral action of surfactin commences due to a physicochemical interaction of the surfactant and the outer part of the virus lipid membrane (Vollenbroich et al., 1997).

Due to the antimicrobial and antiviral activities of various biosurfactants against many common pathogens, it was possible for them to be applied as antibiotic against known pathogenic microorganisms and viruses. For example, development and production of daptomycin biosurfactant (Cubicin®) by *Streptomyces roseosporus* in commercial scale which is an antimicrobial lipopeptide was approved for the treatment of complicated skin and skin-structure infections by the FDA (Seydlova and Svobodova, 2008).

Table 2: Organisms susceptible to antimicrobial action of biosurfactants

Type of bacteria	Microorganisms	References
Gram-negative bacteria	*Acenitobacter calcoaceticus*	(Yakimov et al., 1995; Fernandes et al., 2007; Bechard and Eastwell, 1998)
	Alcaligenes eutrophus	
	Pseudomonas Fluorescens	
	Psedommonas aeruginosa	
	Escheria coli	
	Serovar typhimurium	
	Pseudomonas corrugata	
Gram-positive bacteria	*Bacillus cereus*	(Yakimov et al., 1995; Fernandes et al., 2007)
	Bacillus licheniformis	
	Bacillus subtilis	
	Rhodococcus globerulus	
	Staphylococcus faecalis	
Fungi	*Chrysosporium indicum*	(Ongena et al., 2004; Joshi et al., 2008; Sabate et al., 2009)
	Alternaria burnsii	
	Fusarium oxysporum	
	Fusarium udum	
	Trichoderma herzanium	
	Rhizotonia bataticola	
	Botrytis cinerea	

2.6 Application of biosurfactant

Since surfactants should be both effective and environmentally appropriate, it is common then to turn to the microbial world to meet the demand from various industries. Due to their exceptional chemical characteristics, biosurfactants have been acknowledged for the commercial use in bioremediation; as antibiotics in the medical industry; for enhanced oil recovery in the petrochemicals industry; in the minerals processing industry and also in the food industry (Makkar et al., 2011). The uses of biosurfactant in these industries show significant advantages over chemically synthesized surfactants.

2.6.1 Biomedical and pharmaceutical applications

There has been growing interest in the effect of biosurfactants on human and animal cells. Some biosurfactants are applicable to be used as a substitute to synthetic medicine and antimicrobial agents. Since numerous biosurfactants have been documented to display antimicrobial activity against many common numerous pathogens, antimicrobial biosurfactants can possibly be applied as antibiotics against known pathogenic microorganisms (Rodrigues et al., 2006).

Biosurfactant have been actualized to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection site. This action causes biofilm forming bacteria unable to adhere to solid surface or to infection sites in the presence of biosurfactant. For other purpose, running the surfactin solution to the pre-coating vinyl urethral catheters resulted in a decrease in the amount of biofilm formed by *Salmonella typhimarium, Salmonella enteric, E. coli* and *Proteus mirabilis* (Seydlova and Svobodova, 2008).

Biosurfactant was also reported to have an antitumor activity against Ehrlich's carcinoma cells and an antifungal activity for various pharmacological applications. They are including inhibit fibrin clot formation as well as development of membrane ion channels. Furthermore, surfactin and surfactin analogues have also been reported as antiviral agents due to physicochemical interactions between the membrane-active surfactant and the virus lipid membrane (Banat et al., 2000).

2.6.2 Agricultural applications

Recent concerns regarding pesticide pollution have motivated global efforts to discover for any new alternative biological control technologies. There were report that *Pseudomonas aeruginosa* has been used to control fungal infections of cucumbers and peppers caused by *Phytium aphanidermatum* and *Phytophora capsici.* The finding concluded that this is due to the effect of biosurfactant in the nutrient solution which is later known as rhamnolipid (Banat et al., 2000). Biosurfactant can also be utilized in the biological control of post harvest diseases and insects. It has been reported that the supernatant of a *B. subtilis* culture inhibited the growth of *Aspergillus flavus, A.niger, Penicillium oxalicum* and *Botryodiploidia theobomae*. These organisms are all major spoilage organisms of intermediate moisture foods (Joshi et al., 2008).

Application of surfactants is possible as well for hydrophilization of heavy soils as a function to attain good moisture and also to deliver equal distribution of fertilizers and pesticides in the soil. For other purposes, biosurfactants also have been used in formulating poorly soluble organophosphorus pesticides in order to regulate a stable emulsion in the existence of the pesticide fenthion (Banat et al., 2000). On the other hand, a biosurfactant synthesized by *P. aeruginosa* has been proclaimed to solubilize toxic organic chemicals and amplify the solubility and recovery of hexachlorobiphenyl from oil slurries (Mulligan et al., 2005).

2.6.3 Bioremediation applications

Bioremediation is a procedure by which contaminants or pollutants are removed from certain environmentally sensitive field by enhancing the biodegradation of such contaminants. Generally, this process focuses at supplying cost effective operation and contaminant-specific treatments to lower the concentration of individual or mixed environmental contaminants. The capability of a biosurfactant to enhance biodegradation of slightly soluble organic compounds depends on the extent to which it increases the bioavailability of the compound (Ron and Rosenberg, 2002).

For instance, many cases of oil spill accidents from the production of petroleum culminated in critical pollution of oceans and shoreline environments. Beside from accidental spills, intentional discharges of oil have also resulted in considerable amount of contamination. Such events have increased attempts to develop various chemical, procedures and techniques for confronting oil pollution (Ron and Rosenberg, 2002). There are many existing oil decontamination processes yet mostly are expensive, have limited use and only partially effective (Das and Mukherjee, 2008). Hence, bioremediation has been introduced to be an alternative to existing techniques. The ability of biosurfactants to emulsify hydrocarbon-water mixtures manages to enhance the degradation of hydrocarbons in the environment. Moreover, biosurfactants also offer distinct advantages over synthetically derived compounds such as low toxicity, biodegradability and high specify. Exxon Valdez spill in the Prince William Sound in 1989 was an example where biosurfactant was used successfully for bioremediation (Mulligan, 2005).

2.7 Process conditions

Biosurfactant production by various types of microorganism has been investigated mainly from the perspective of theirs biotechnological potential. These microorganisms are not just widely distributed but they also produce different type of biosurfactants. Specifically, each of the microorganisms has exclusive preferred environmental conditions which are necessary for achieving optimal growth and product synthesis (Trejo-Tapia et al., 2003). Accordingly, this section of literature will focus on the process conditions required for growth and enhanced surfactin production by *B. subtilis* bacteria. There are two factors influencing the production which are medium compositions and fermentation conditions.

2.7.1 Medium compositions

The major component for the production of biosurfactant is carbon substrates. Primarily, hydrocarbons have been the substrates of interest for the production of biosurfactants. However, the predicament of employing hydrocarbons as the substrate is they are insoluble in the water. Thus, carbohydrates are preferred since they are not just water-soluble but also cheap and acceptable for many applications such as in food, cosmetics and pharmaceutical industries as well (Makkar and Cameotra, 1999; Fox and Bala, 2000).

The influence of carbon substrates on the production of biosurfactant by various types of bacteria have been reported by Abushady et al. (2005). They have conducted a study on the evaluation of various carbon sources for growth and surfactin production by *B. subtilis*. In the study, two type of *B.subtilis* were cultured into various kinds of vegetable oils and carbohydrates. It is reported that surfactin concentration between 100-1500 mg/L was achieved using vegetable oils, while concentrations of 2000-2750 mg/L surfactin was obtained from carbohydrate substrates. Among the carbohydrate substrates, supplementation of glucose into the media enhanced the production of surfactin where the highest yield was achieved upon addition up to 30 g/L.

Another important component associated with biosurfactant production by microorganisms is nitrogen source supplemented into the nutrient media. As the matter of the fact, a study was conducted by Abushady et al. (2005) managed to compare the efficiency of using organic and inorganic nitrogen source in the media toward surfactin production. Based on the report, inorganic nitrogen sources are preferred choice due to enhancement of surfactin production observed after completion of fermentation process. Among inorganic nitrogen, however, NH_4NO_3 and $NaNO_3$ showed the highest surfactin concentration procurement with 2230 and 1950 mg/L respectively. Meanwhile, a report by Onwosi and Odibo (2012) showed similar finding that support efficiency of inorganic nitrogen source on biosurfactant production. In the study, rhamnolipid production has been reported to increase when $NaNO_3$ as the inorganic nitrogen source was used compared to urea and yeast extract.

Nonetheless, biosurfactant production also could possibly be strongly affected by availability of various nutrients in the medium. In fact, the concentration and availability of nutrients such as iron and manganese in the medium formulation have significantly enhanced surfactin production and the growth of *B. subtilis* as the producer microorganism (Makkar and Cameotra, 2002).

Iron is a general cofactor for microbial and enzymes development, as well as an essential mineral to microorganisms. Bacteria utilize host iron compounds to reduce ferric to ferrous ions with consecutive transport of the ferrous ions for their imminent benefit (Wei et al., 2003). Meanwhile, The presence of manganese in the medium is crucial since it supports cell growth by affecting nitrogen utilization and K^+ ions uptake of *B. subtilis* cells (Wei and Chu, 2002).This behavior stimulates *B. subtilis* to yield high production of surfactin provided that the appropriate amount of manganese is supplied into the media.

There are several studies that evaluated the effects of iron and manganese cations on surfactin production. Wei and Chu (2002) reported that both the both biomass and surfactin concentration increased 5-fold when 0.0013 M $FeSO_4$ was added to the culture broth, while only the surfactin concentration increased when 4.0 μM manganese was added to the culture broth. On the other hand, Abushady et al. (2005) evaluated the effect of $FeSO_4$ and $MnSO_4$ concentrations on surfactin production and found that at concentrations of 152 and 50 mg/L respectively resulted in maximum surfactin concentrations of 2450 and 2500 mg/L respectively.

2.7.2 Fermentation conditions

Environmental conditions during fermentation process also plays important role that influence both cell growth and biosurfactant production. Thus, various types of conditions such as pH, temperature, agitation and aeration must all be taken into consideration, so maximum production could be achieved.

In fermentation process, appropriate temperature will provide convenient condition for any microorganisms to survive in the media. Extreme temperature might cause severe distressed not only on microorganisms but also against their own growth activity as well. A report by Abushady et al. (2005) showed the effect of temperature on surfactin production where the temperature value below or above 30 °C reduced its yield.

Inoculum or seed culture is a necessary factor input to a fermentation routine. From the commercial and industrial perspective, density and age of inoculum could influence the fermentation process since they might affect the duration of the lag phase, biomass yield, specific growth rate, sporulation, quality of the final product and the production cost (Sen and Swaminathan, 2004). In any fermentation processes, lag phase is a crucial factor since it is corresponded to the period needed for the cells to adjust to the existing physical and chemical environment. So, it is common that the medium for inoculum should be identical as the fermentation medium to reduce the time of adaptation. With that regard, age and density of inoculum should be well-managed to achieve appropriate lag phase during fermentation process (Zhang et al., 2003).

In a well-developed fermentation system, aeration and agitation are among substantial functions that could improve the yield of the product. Basically, the present of aeration and agitation in the fermentation system are to deploy and transfer oxygen throughout the medium culture as well as for mass transfer. However, aeration and agitation rate should be sustained at certain level since vigorous actions may lead to severe foaming thus, causing unstable and inefficient fermentor operation (Yeh et al., 2006).

2.8 Optimization technique

Production cost is the major bottleneck in biosurfactant production as in most cases of any biotechnological processes. Despite those advantages as mentioned at the previous sub-chapter, large scale production of biosurfactant has not been realized because of its low yields in production processes as well as high costs during recovery and purification period (Sen and Swaminathan, 2005). Hence, the production economy of biosurfactant as well as any microbial metabolite is administered by three basic aspects which were utilized worldwide in order to develop more cost-competitive process:

i. Reduce initial raw material costs by substitution to waste or cheaper substrates;

ii. Application of convenient and economic production and recovery methods by development of efficient bioprocesses, including optimization of the culture conditions and cost-effective separation processes to maximize biosurfactant production and recovery; and

iii. The product yield of the producer microorganisms by development and selection of overproducing mutant or recombinant strains to enhance yielding of biosurfactant (Mukherjee et al., 2006)

For this study, optimization technique has been employed to optimize the production of surfactin. The term optimization is described as a technique to improve the performance of a system, a process, or a product in order to obtain the maximum benefit from it. Many researchers used this technique as means of determining the conditions at which to apply a procedure that produces the best outcomes (Ayed et al., 2010; Huang et al., 2010).

Optimization technique has been carried out traditionally in analytical chemistry by monitoring the influence of one factor at a time on an experimental outcome. However, the technique is too old-fashioned and has a lot of shortcomings. Consequently, various multivariate statistic techniques have been developed to overcome the problem.

2.8.1 One-factor-at-a-time (OFAT) optimization

The old-fashioned strategy of medium optimization involves varying one variable at a time, with all other variables held constant. This approach is usually termed as one-factor-at-a-time (OFAT) technique. Currently, most researchers favor multivariate optimization, which offers efficient technique for experimentation that conclusively solves complex problem with many variables (Frey et al., 2003).

However, OFAT is favored by researchers and can be more effective than multivariate optimization under certain situation, for instance, whenever the primary goal is to gain improvements in the system and only limited number of runs available for experimentation. Furthermore, in the circumstances restricted by time and scarce of resources, free experimentation such as OFAT can be a possibility (Jamal et al., 2012). Although OFAT method requires a considerable amount of time and work, it is possible to utilize the technique to determine the desirable ranges of experiment including maximum and minimum settings.

2.8.2 Response surface methodology (RSM)

Conventional and classical methods of OFAT technique however, have many crucial disadvantages and less efficient compared to the statistical multivariate analysis (Wang et al., 2012). Conducting research by using OFAT technique leads to expand in the number of experiments and hence, more time and expenses required during the process as well as increase in the consumption of reagents and materials. Nevertheless, the major drawback of this technique is that it does not include and depict the interactive effects among the variables studied on the response (Bezerra et al., 2008; Desai et al., 2008).

Response surface methodology is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with objective of making statistical previsions (Bas and Boyaci, 2007). It is a statistically designed experimental protocol that can be well applied when a response or a set of responses of interest are influenced by several variables. The term was first introduced by Box and collaborators in the 50s and originated from the graphical perspective generated after fitness of the mathematical model (Kalil et al., 2000).

RSM used statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design. Through RSM, an equation representing the approximate relationship between a single response and control factors can be obtained based on experimental data (Choudhury et al., 2012; Sen and Swaminathan, 2004). Henceforth, linear or square polynomial functions are employed to describe the system studied. A contour plot or three dimensional modeling is usually utilized to characterize the response surface graphically and determine the optimal parameter-setting (Song et al., 2014).