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Review Paper

Biosurfactants and their Screening Methods

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Abstract

Biosurfactant screening is a concept of primary methods which are easy and essential to detect surfactant properties of the molecules produced by variety of organisms, using carbon as a substrate. There are different direct/indirect screening methods including oil spreading, hemolysis activity, E_{24} index etc. which provides valuable information about surfactant properties of molecules. Biological molecules having screening properties are considered as biosurfactants. Biosurfactants are a large group of microbial synthesized surface active, amphiphilic molecule having many advantages over surfactants synthesized chemically. They are differentiated according to their molecular weight, structure and properties. Biosurfactants could be synthesised biologically on cell membrane or sometimes outside the cell membrane. There are number of properties of biosurfactants which makes it distinct and valuable from other molecules.

Keywords: Biosurfactants, Screening, Surfactant, Biologically, Chemically.

Introduction

Microorganisms like bacteria and yeasts synthesize broad range of surface-active compounds, known as biosurfactants[1]. Some biosurfactants are produced biologically from different substrates like oils, sugars, alkanes and wastage products. These are amphilic molecules having both hydrophobic and hydrophilic moieties making them to interfere at different interfaces between the fluids having unlike polarities like hydrocarbons and water. To produce biosurfactants, non-toxic biodegradable renewable resources with high surface activity and effectiveness are used[2-4]. *Pseudomonas aeruginosa* was used to produce first rhamnose with biosurfactant. Initially biosurfactants were only used in bioremediation and oil recovery processes[5]. Most of the biosurfactants are produced from hydrocarbons which are used as main carbon source and carbohydrates. They are secondary metabolites, which are created during the late logarithmic or stationary growth phase. *Bacillus* sp. and *pseudomonas* sp. synthesize most of the biosurfactants. To produce of such kind of biologically active molecules the spore forming property of *Bacillus* sp. is consider as microbial factory and the spores show enhanced level of resilience to dryness which is mandatory feature for stable product formation. *B.subtilis* strains are a rich source of antimicrobial peptides with a high potential for biological control applications. In soaps and detergents surfactants are major ingredients and are vary commonly used to separate a particular media from oily materials by reducing their surface tension at different media (air-water, oil-water) interfaces[6]. Because of persistent and recalcitrant nature of majority of surfactant compounds, they cause toxicity and cause environmental problems[7]. However, chemically synthesized surfactant compounds are usually of toxic nature and are non biodegradable; due to such problems the microbially synthesized surfactants gain more attention as they are eco friendly and biodegradable[8]. Environmental concerns and stringent regulations have pushed forward the development of natural bio-surfactants as an alternative to chemically synthesized products.

Classification of biosurfactants

Biosurfactants are mainly classified according to their molecular weight, physico-chemical properties and mode of action, as on the basis of physico-chemical properties they can be generally grouped either as lowest or higher molecular weight biosurfactants; the lower molecular weight consisting of glycolipids and lipopetides and the higher molecular weight are polysaccharide polymeric biosurfactants[9]. The best known glycolipids are rhamnolipids, sophorolipids and trehalolipids. Most of these compounds are either negatively charged or neutral only a few are positively charged that containing amine groups[6-7](Table-1).

Glycolipids: Sophorolipids are obtained from yeasts, rhamnolipids are obtained from *Pseudomonas* sp. and trehalose lipids obtained from *Mycobacterium* and related bacteria, are most effective glycolipids from view point of surface-active properties. Otto *et al*. describes a process using whey, (without protein content) concentrate as the substrate by a two-step process for production of sophorose lipids (SLs)[10]. Mannosylerythritol lipid (MEL) is a glycolipid biosurfactant and is derived from vegetable oils by *Candida* stains, shows several immunological, antimicrobial and neurological properties. Kitamoto *et al.* described that Gram-positive bacteria is particularly very sensitive to antimicrobial activity of MEL[11]. Isoda *et al.* researched upon biological activities of many surfactants including MEL A, MEL B and came up with analysis that polyol lipid, MEL A, MEL B, SL and STL1 and STL3 incurred cell differentiation in HL 60 (man promyelocytic leukaemia) whereas rhamnolipid participated in cell proliferation process[12]. Process activity in STL and MEL was observed as interaction with plasma membrane unlike in detergent-process effect.

Phospholipids, neutral lipids and Fatty acids

While undergoing surface activity on *n-alkanes* some yeasts and bacteria results into quantum of phospholipid bio-surfactants and fatty acids. In prematurely born children deficiency of phospholipid protein complex is found out to be a major cause of respiratory failure[13]. Medical application is one essential area where biosurfactants are used for cure. It is also assumed that fermentation process can also be carried out by using isolation and cloning of genes used in process of making bio-surfactants[14].

Polymeric biosurfactants

Liposan, Emulsan, alasan, lipomanan and other polysaccharide protein complexes are major polymeric surfactants. Emulsan is found in quantity concentrations of 0.001% to 0.01% in water and is used as emulsifier for different hydrocarbons[15-16]. Similarly another water soluble extracellular emulsifier is Liposan which contains 17% of proteins and 83% carbohydrates.

Lipopeptides biosurfactants

Due to their higher surface activeness lipopeptides attracts special attention among all biosurfactants. Lipopeptides can act as antifungal, antibacterial, antiviral and antitumor agents, immunomodulators or specific toxins and enzyme inhibitors. *Bacillus* cyclic lipopeptides are formed of three different categories called as fengycin families, iturin and surfactin. Surfactin is most important of all the lipopeptides. Structure of surfactin made up of 7 amino acid cyclic peptide connected to a C_{13}–C_{16} fatty acid[17] whereas, Iturin consists of 7 amino acids linked to C_{14}–C_{17} and fengycin is composed of 10 amino acids with fatty acid chain length of C_{14}–C_{18}[18].

The bacterial hydrophobicity of lipopeptides varies with strains[19]. Surfactin is found to be more effective then iturin in modifying the B[20]. Most of these surfactant compounds are either anionic or neutral but only a few among them are cationic those containing amine groups.

Table-1
Major types of biosurfactants and their applications

Biosurfactant family	Biosurfactant class	Microorganisms	Applications
Glycolipids	Rhamnolipids	*Pseudomonas* sp.	Pollution control
		P. chlororaphis	Biological pest control agent
		B. subtilis	Antimicrobial agent
		Renibacterium salmoninarum	Bioremediation
	Sophorolipids	*Candida* sp.	Emulsification , MEOR*,
	Trehalose lipids	*Rhodococcus* sp.	Environmental application
		Tsukamurella sp. *and Arthrobacter* sp.	Antifungal and antibacterial agent
	Mannosylerythritol lipids(MEL)	*Candida antartica*	Neuroreceptor opponent, antimicrobial Agent
		Kurtzmanomyces sp.	Biomedical and medicinal application
Lipopeptides	Surfactin	*Bacillus subtilis*	Antifungal and antibacterial agent, biomedical and medicinal application
	Lichenysin	*B. licheniformis*	Chelating and Hemolytic activity

Microbial enhanced oil recovery (MEOR)*

Surfactin Biosurfactants

Surfactin is a major class of lipopeptide of ~ 1.36 kDa (Figure 1). Surfactin is cyclic in nature and possess good antimicrobial properties and is produced from *B. subtilis*[19]. It also interacts with enveloped viruses and other artificial biomembrane systems[21]. As per differences in the amino acids and chain sequences, surfactins can be classified into surfactin A, B and C. In addition to antibacterial and antifungal properties, it also has several biological activities, includes the inhibition of fibrinogen clot formation, the beginning of ion channel formation in lipid bilayer membranes, the inhibition of cyclic adenosine monophosphate, and the retardation of platelet formation, spleen cytosolic phospholipase A2 (PLA2), and can check antitumor activities and inflammatory responses. Cell proliferation rates found increased and also the morphology of mycoplasma infected mammalian cells found changed after treatment by surfactin. Surfactin is very effective against many different viruses like SFV (*Semliki Forest virus)*, HSV(*Herpes simplex virus)*, SHV (*Suid herpes virus*), VSV (*Vesicular stomatitis virus)*, SIV (*Simian immunodeficiency virus*), FC (*Feline calicivirus*) *and* MEV *(Murine encephalomyocarditis virus*) [22]. There is significant effect of surfactin on enveloped viruses then on non-enveloped viruses. It shows that interaction occurs between active membrane of biosurfactant and outer layer of enveloped virus due to change in permeability at larger concentrations and results in disintegration of mycoplasma membrane by surface-active effects[23]. In today's medical industry concerns are high over development of antimicrobial resistance for known surfactants, and it is playing a role in developing alternative prophylactic and therapeutic compounds. By genetic engineering a novel lipohexapeptide was created with changed antimicrobial resistance activity of the surfactin biosynthesis mechanism[24]. Thus, to improve the therapeutic applications, surfactin derivatives exhibits reduced toxicity against eukaryotic cells.

Iturin biosurfactants

Molecular mass of Iturin is ~ 1.1kDa, consists of 7 amino acids linked to C_{14}–C_{17}. The iturin group of surfactant is cyclic type lipoheptapeptides that contains a β amino fatty-acid in its extended chain (Figure 2). Lipopeptide profile and bacterial hydrophobicity vary with the strains, iturin A being the only lipopeptide type produced by all *B. subtilis* strains[19]. Iturin A is an effective antifungal compound for intense mycosis[25], other agents of the iturin like biosurfactant family have been used as alternative potent antifungal agents. Iturin shows anti bacterial activity against *micrococcus* and *sarcina* strains along with antifungal activity.

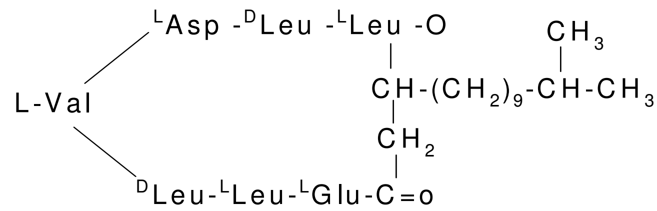

Figure-1
Heptapeptide cyclic structure of Surfactin, containing both hydrophobic and hydrophilic amino acids. This lipopeptide contains two *D*-amino acids (Leu and Leu) and five *L*-amino acids (Val, Asp, Leu, Glu and Leu) thus indicating its amphipathic nature

Screening methods for biosurfactants

Biosurfactants are biomolecules with different compositions. There are different types of biosurfactants are lipopolysaccharides, glycolipids, phospholipids or lipopeptides. The common method used for classification of biosurfactants forming strains is based on surface activity caused by them. Interaction of produced strains with hydrophobic membranes is also studies for classification purposes. Also, particular methods for classification of biosurfactants have also been developed for a limited family of agents for example Colorimetric and CTAB agar assay etc. These methods produce quantitative as well as qualitative analysis of agents under process. Some of these methods are explained below.

Surface/interfacial activity: Based on surface interaction with agents surfactants are classified into different categories. The basic method of classification includes direct measurement of surface interaction of agent. This method is very appropriate in preliminary classification of surfactants forming microbes. Various scientific methods are available to measure the surface tension of liquid.

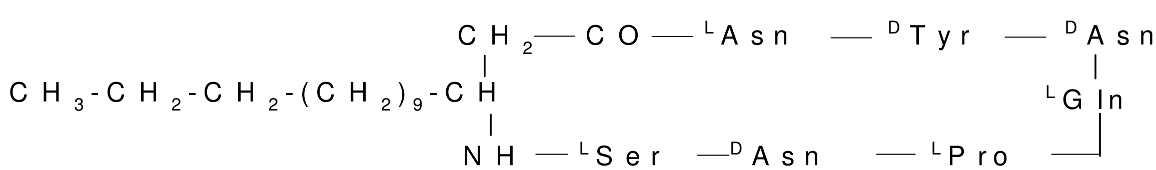

Figure-2
Cyclic structure of the lipopeptide Iturin, containing 7 amino acids as well as 13-carbon long chain that indicate its amphipathic nature. The amino acids involved in this structure are three *D*-amino acids (Tyr, Asn, and Asn) and four *L*-amino acids (Pro, Ser, Asn and Gln)

Du-nouy-ring method: Force required to detach a wire loop or ring from surface of an agents is measured and is used as basis for classification of different agents. A scientific equipment Tensiometer is used to measure this force which is usually proportionate to interfacial surface tension. Platinum or Wilhelmy rings are used in this method[26]. These loops are flamed prior to use to remove any contamination present. This particular method is known as Du-nouy-ring method.

Emulsification activity: Goldenberg and cooper put forwarded a formula for classification of bio-surfactants based on emulsification attributes of agents[27]. Different levels of emulsification activity of agents is quantified and used as basis for classification of surfactants. In water media a particular amount of hydrocarbons are added and media is vortexed at high speeds for two minutes of time. The media is then kept for twenty four hours and depth of emulsified layer is measured and E_{24} is calculated.

E24= Height of emulsion layer X 100/ Total height of the solution

Oil spreading method: Morikawa developed a technique to classify surfactants on the basis of oil spreading[28]. The size of clear loop of oil is measured in petri plate after adding 10 µL of sampling agents on 100 µL crude oil base layered upon 50mL of distilled water. The diameter of oil bubble defines the spreading activity of oil in comparison to negative control.

Surface tension measurements: Direct measurement of surface tension also used for classification of bio-surfactants. Surface tension of the agent is measured by Tensiometer. At defined temperature of 24 degrees Celsius force is measured to pull out the platinum loop through liquid-air interface in free culture broth/ tested sample. These values are used for screening of bio-surfactants[29-31].

Oil displacement assay: In a petri plate1mL of crude oil is added over 30mL of distilled water. After that petri plate is topped with 20µl of bio-surfactant sample on crude oil bubble. The increase in diameter of oil is measured due to activity caused by surfactant[32].

Drop collapse test: This drop-collapse procedure is very easy and fast to perform. It does not require any special equipments but only a small sample volume. Crude oil in small drop is placed on the slide and adds 10µl of surfactant by piercing the drop using micropipette without disturbing the dome shaped of the oil. The drop collapsed within 1min will be considered to be favorable for the drop collapse test[32].

Conclusion

Bio-surfactants are surface-active, amphilic compounds formed by some bacteria and fungi[33]. In aqueous media with hydrocarbons, these compounds reduce the surface activity and surface, interfacial tension. These biosurfactants reduce surface and interfacial tension in aqueous solutions and hydrocarbon mixtures. These bio-surfactants are used in oil industry for oil extraction and for enhancement is quality of the oils. Interest in bio-surfactants due to its wide characteristics has paved the way for evolution of new procedures for the classification of bio-surfactant producing strains/microorganisms. A mixture of number of various methods is better strategy to do a successful screening of bio-surfactants. Among those some of the screening procedures can also be carried out automatically and some can be used for HTS. These new screening methods would lead to development of various new bio-surfactants forming strains or surfactants. In future with the availability of new production strains number of new bio-surfactants will also increases with many more properties and then the economic obstacle like, expensive downstream processing of bio-surfactants may also be overcome soon as bio-surfactants are non toxic, biodegradable and renewable as compared to chemically synthesised surfactants.

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