Research Article

Effect of Lipopeptide from Marine Sponge in Biofilm Degradation, Bioremediation and Emulsification

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Abstract

Lipopeptide represent a class of biosurfactant which posse's surface and biological activities due to the presence of hydrophilic head and hydrophobic tail, and are able to reduce the surface and interfacial tension by accumulating at the interface. *In vitro* biofilm degradation activity of lipopeptide isolated from marine sponge MMD32 strain was evaluated for pathogenic biofilm forming species *S. aureus* and it was found that lipopeptide degraded the biofilm formed by *S. aureus* under dynamic conditions and it was observed that as the concentration of lipopeptide increasing anti-biofilm activity also increasing. Lipopeptide also showed the activity to degrade polycyclic aromatic hydrocarbon (PAH) against phenanthrene and pyrene. The strain MMD32 uses phenanthrene and pyrene as the source of carbon and energy for their growth and removes them from environment. Moreover, lipopeptide also have the emulsification activity, which helps to improve the softness of the muffin and its stability after baking when compared with positive control. Results gathered in this work suggested that the lipopeptide recovered from MMD32 could be used as an alternative surfactant to the commercial chemical surfactants with the potential use in bioremediation, food formulation and drug designing.

Keywords: Lipopeptide, Marine Sponge, Anti-biofilm, Bioremediation, Emulsifier

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Introduction

Marine microorganisms exhibits unique physiological and metabolic activity, which conferring them to survive in extremely harsh conditions such as pH, temperature, salt and so on [1, 2]. Marine microorganism are reported to be a great source for relevant and novel functional compounds including biosurfactant, colorants, enzymes, antibiotics, antimicrobials, vitamins, amino acids etc [3, 4]. Recently, biosurfactant has gained so much attention over chemical biosurfactant due to reduced toxicity, good biodegradability and there stability in extreme environmental conditions such as pH, salt and temperature. Among all the marine microbial based biosurfactants, lipopeptide represents a class of surfactants which have remarkable biological and surface activities like anti-microbial, anti-viral, anti-tumour, anti-biofilm, anti-adhesive, surplus crude oil recovery and emulsification activity [5]. Lipopeptide are low-molecular weight biosurfactant having both hydrophilic and hydrophobic tail, are able to accumulate between the two fluid phases and helps to lower the surface and interfacial tensions [6, 7]. Lipopeptides are generally produced by *Bacillus and Pseudomonas* species. Lipopeptide have 7 to 10 amino acid long hydrophilic peptides linked to hydrophobic fatty acid part, they are cyclic in nature.

Lipopeptide posses' anti-biofilm activity that helps to inhibit or degrade the biofilm formed on the surfaces that are in contact with the food. Biofilms are the potential source of contamination and may lead to spoilage of food that ultimately results in health borne illnesses. The bacterial biofilm present in food industry surfaces are potential source of contamination, may lead to food spoilage and disease transmission. Therefore, controlling the adherence of microorganism or degrading the formed biofilm is an essential step in providing safe and quality products to the consumer [8].

Polycyclic aromatic hydrocarbons (PAH) are the largest group of organic compound made up of carbon and hydrogen atom combining with two or more benzene rings. PAH are hydrophobic structures and therefore found to be insoluble in polar solvents causing major problem of biodegradation processes. They are primarily produced during incomplete combustion of organic material and emitted into the environment due to natural (forest fire and volcanic eruption) and anthropogenic activity (incomplete fossil fuel combustion, diesel engine exhaust, cigarette smoke, industries) [9]. They found everywhere in the environment such as air, water, soil with harmful effect on flora, fauna and human beings due to their mutagenicity, carcinogenicity and teratogenicity [10]. Biosurfactant esp. Lipopeptide

are able to solubilise hydrophobic substrate by forming micellar system and provides the less stressful environment to the bacterial cells to degrade PAHs.

Lipopeptide also have the ability to reduce surface and interfacial tension and confer wide range of application in food industry such as bioemulsifier, thickener, and solubilisers. These lipopetides can be widely used as food additive or ingredient in emulsion based formulation due to good emulsification property [11]**.** The ability of surface active compound to modify the rheological properties of the food system explains the need of their usage in dairy, bakery and meat processing industries. There is very less information on the application of lipopeptide biosurfactant from marine environment. Marine based biosurfactants are not much explored, mainly due to the complexity of growing marine microorganisms under laboratory conditions. This paper studies the application of lipopeptide produced by marine microorganism MMD32 strain in biofilm degradation, bioremediation and as a food emulsifier.

Materials and Methods

Media components and other analytical grade chemicals that used in this study were procured from HiMedia (Mumbai, India). MMD32 strain was isolated from the marine sponge *F. cavernosa* and the isolated bacterial strain were then used to screen for biosurfactant production. After that biosurfactant was extracted from MMD32 and then characterized using FTIR, NMR and were found to be lipopeptide, was then purified using thin layer chromatography (TLC). Purified lipopeptide was being used to check for its antibiofilm activity, hydrocarbon degradation activity and as an emulsifier to improve the sensory characteristics of muffin.

Antibiofilm activity- Microtitre Plate Assay

Antibiofilm activity of the lipopeptide biosurfactant produced by MMD32 strain was studied as per [12] with little modification. *S. aureus* is a biofilm former and a multi drug resistant strain which was collected from Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry. 10 µl of *S. aureus* overnight culture diluted was added to the sterile 96-well microtitre plate and kept for incubation at 37° for 72hrs, for biofilm formation and wells were washed with phosphate buffer (pH 7) to remove the non-adherent bacterial cells. Lipopeptide was then added in the biofilm formed 96-well microtitre plate in varying concentration (50µg - 200µg/ml) and kept for 24hr to allow the disruption of biofilm and then lipopeptide extract was washed off and dried in inverted position. 95% ethanol was then added to the microtitre plate to fix the biofilm and was stained using 1% crystal violet (w/v) for 6 minutes and dye was washed off using sterile distilled water. The wells were allowed to air dry and then 200µl dimethyl sulfoxide (DMSO) was added to solubilise crystal violet. Enzyme-linked immunosorbent assay reader (Labnics) was used to quantify biofilm at 595 nm.

Biodegradation of phenanthrene and pyrene by Lipopeptide biosurfactant

Lipopeptide obtained from MMD32 strain was used to check the bioremediation property as per [13] method. Bacterial culture of MMD32 was inoculates in the nutrient broth and incubated for 24hr at 37°C. Take two test tubes containing 50 ml of nutrient broth supplemented with 100 mg/l phenanthrene and pyrene (polycyclic aromatic hydrocarbon) respectively, to this add 100 µl of previously cultured bacterial colonies and incubate for seven days. On seventh day, the culture was centrifuged at 6000 rpm for 10 min at 4°C to separate the bacterial isolates. Bacterial isolates were re-suspended in 2 ml nutrient broth, from this 500 µl was transferred to 5 ml nutrient broth supplemented with 100 mg/l phenanthrene and pyrene respectively in two sets and was kept in shaker incubator at 37°C with 180 rpm speed. After that extraction was done on first, third, fifth and seventh day by adding equal volume of n-hexane. After the addition of n-hexane it was vortexed for 5 min followed by centrifugation in the cooling centrifuge (4°C) at 6000 rpm for 10 min and supernatant is collected and O.D was taken at 292 nm and 303 nm for phenanthrene and pyrene respectively.

Effect of lipopeptide on the quality parameter of muffins

In order to study the effect of muffin on the textural characteristics of muffin, the muffins were incorporated with lipopeptide at 1% level. The muffin preparation was carried out as per [14] with little modification. The ingredients used for muffin preparation are 28g wheat flour, 24g margarine, 24g sugar, 15g whole egg, 0.45g baking powder and 20ml water (for positive control muffin formulation). To prepare positive control sugar and margarine were creamed together for 2-3 min using hand blender, to this whole egg was added and mixed for 4-5 min. The wheat flour, baking powder and water were added and mixed till a ribbon like consistency reached and then transfer the batter into a greased and floured tray. In test sample 1% lipopeptide was used instead of egg. In negative control mixture it neither

contains egg nor lipopeptide. Muffins were baked at 180°C for 20 min in a preheated oven, muffins were removed from the tray and cooled for 45 min and to avoid drying they were packed in laminate.

Texture analysis of muffin

Hardness of the muffin was analysed using Texture profile analyser (TA-HD plus, stable Micro systems, UK). The analyser pre-tested speed was set as 1.0 mm/s, post-speed at 10.0 mm/s and test speed of 3.0 mm/s at a distance of 10 mm with 12.5 pps data acquisition rate and 50kg load cell. For texture profile analyser (TPA), P75 probe was used to study the hardness and maximum force was recorded as the hardness of the muffin. Other parameters like Chewiness, gumminess, springiness and cohesiveness was also studied.

Color analysis of muffin

Hunter Lab Colorimeter (D-25, Hunter Associated Laboratory, USA) was used to study the crust color of muffin and expressed its results in terms of L*, a* and b*. The +L* represent the lightness and -L* value represent the darkness in the sample whereas a* represent redness (+a*) to greenness (-a*) and b* represents yellowness (+b*) to blueness (-b*). The readings were recorded in duplicate and mean was calculated.

Statistical analysis

The data for muffin was taken in triplicates and were analysed using IBM SPSS v. 20.0 software (SPSS, New York). Data obtained after texture and color analysis was subjected to analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) ($p \leq 0.05$) posthoc test was used to analyse the significant different the mean values of samples at 95 % level of confidence limit.

Results and Discussion

Antibiofilm activity- Microtitre Plate Assay

The ability of the lipopeptide to inhibit/degrade biofilm was determined by 96-well microtitre plate assay and 90% of the biofilm formed by *S. aureus* was degraded by lipopeptide at the concentration of 175µg/ml when compared with control. It was found that biofilm disruption was significantly increased as the concentration of lipopeptide MMD32 increases (**Figure 1**). Lipopeptides are one of the largest group of biosurfactant that have the capability to inhibit or degrade the biofilm. The inhibition of biofilm is one of the effective means of controlling infection. A study concluded that the lipopeptide from MSA31 strain was effectively reduce 90% of biofilm of *S. aureus* at a concentration of 125 µg/ml [15]. They supported the statement that as the concentration of lipopeptide MSA31 increases the antibiofilm activity also increases. Recently many researchers reported that lipopeptide from *Paenibacillus* sp. and *Bacillus* sp. was known to inhibit or degrade the biofilm [16-18].

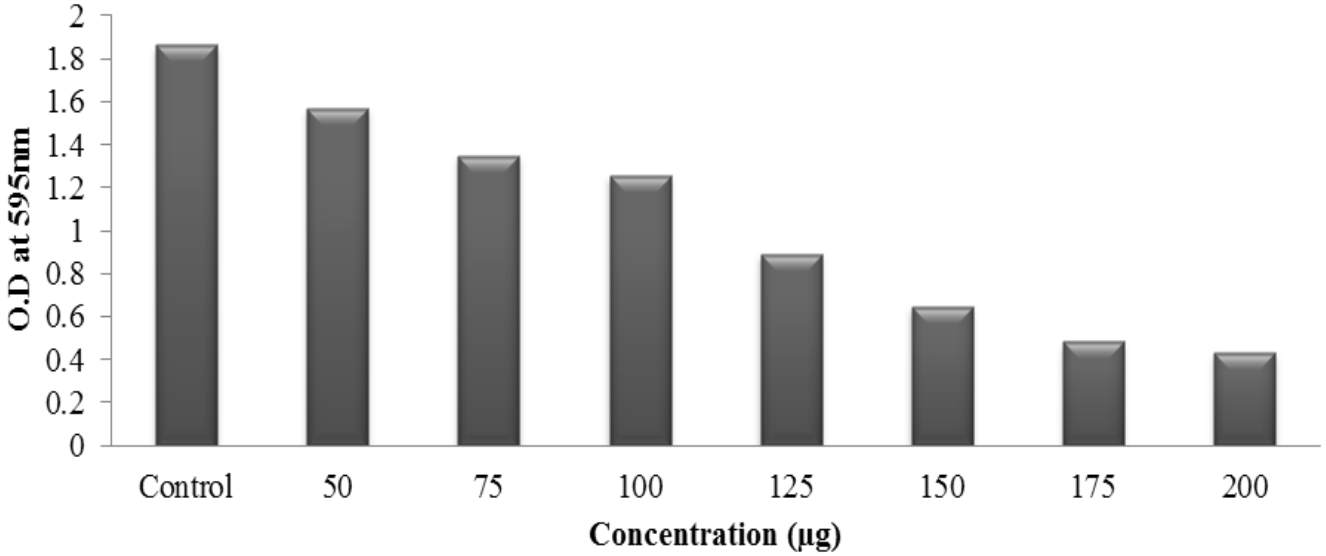

Figure 1 Antibiofilm activity of Lipopeptide MMD32 against *S. aureus*

Biodegradation of phenanthrene and pyrene by Lipopeptide biosurfactant

Phenanthrene and pyrene were added as a supplement in the culture medium of MMD32 strain at a concentration of 100mg/l. Absorbance was taken for 4days- 1st day, 3rd day, 5th day, and 7th day by extracting it with n-Hexane at 292 nm and 303 nm for phenanthrene and pyrene respectively. It was found that MMD32 strain has high efficiency in degrading pyrene from 100 mg to 8.425 mg in 5 days when compared to phenanthrene, but MMD32 also posses good results for phenanthrene as well (**Figure 2**). MMD32 efficiently utilises phenanthrene and pyrene as a source of carbon that results in degradation of these aromatic compounds and they were also reported to degrade other PAHs like 1-hydroxy-2-naphthoic acid and naphthalene. Our results are in confirmation with other findings [19]. The ability to degrade wide range of PAHs was related to the unique gene present in MMD32 strain. The gene of MMD 32 is vaguely comparable to *Pseudomonas* and other genera reported so far in sequence homology and gene organization [19]. The lipopeptide can enhance hydrocarbon degradation by two mechanisms: firstly it increases the substrate bioavailability for microorganism and secondly it increases the interaction of cell with surface that further enhances the association between hydrophobic substrate with bacterial cell by improving the hydrophobicity of the surface of molecule [20]. Generally both mechanisms take place but the dominance of one or the other depends on the strain.

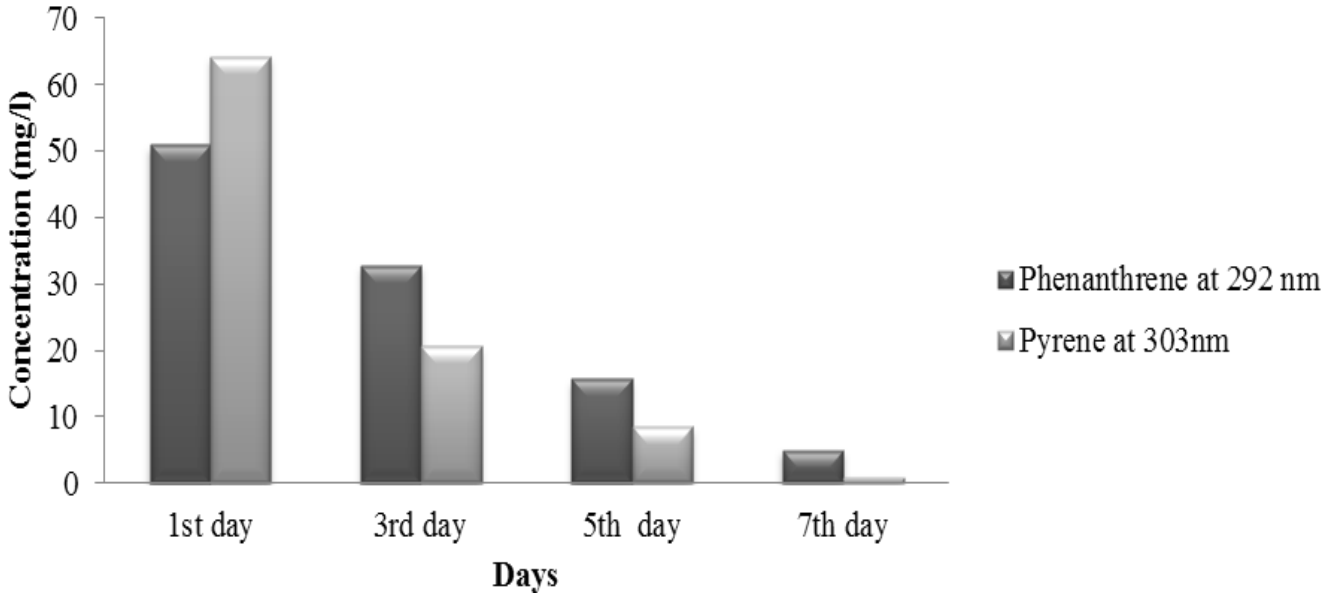

Figure 2 MMD32 showing Pyrene and Phenanthrene degradation

Effect of lipopeptide on the textural properties of muffin

Eating quality of any food product directly relates with the textural properties of that particular food product. Textural properties are important parameter to measure the sensory and quality characteristics of the muffin. The addition of emulsifiers is of special value for low-fat products [21] as it improves the texture and volume of the baked product. The lipopeptide produced by the isolated marine bacteria MMD32 was used in muffin batter as an emulsifier to study its activity in it and then it was compared with one which doesn't have any emulsifier as negative control (N-C) and muffin with egg as positive control (P-C). By using the texture analyzer, textural properties like hardness, chewiness, gumminess, springiness and cohesiveness was studied. Hardness, chewiness and gumminess were found to be decreased at 1% lipopeptide incorporation in muffin when compared with N-C and P-C (**Table 1**). This is consistent with the results of [15], in which hardness, chewiness and gumminess were found to be decreased whereas springiness and cohesiveness were increased at 0.75 % level of incorporation. Lipopeptide containing muffin has high value for springiness which relates it to the high quality and freshness of muffins [22]. Therefore, muffin with lipopeptide biosurfactant was of good quality and posses good sensory characteristics. Cohesiveness is the energy which is required to break the food in a manner so that it is ready for swallowing. In our study lipopeptide incorporated muffin required less force in breaking the food for swallowing due to high value of cohesiveness which indirectly relates with the softness of muffin. N-C showed highest value of hardness, chewiness and gumminess whereas lower values of springiness and cohesiveness, that indicates the hard texture of muffin. Overall study showed decrease in the hardness, chewiness and gumminess and increase in the value of springiness and cohesiveness, results in muffin with good quality and sensory characteristics.

Table 1 Shows the effect of lipopeptide on the textural properties of muffin. The data is presented as mean ± standard deviation

Sample	Hardness	Chewiness	Gumminess	Springiness	Cohesiveness
N-C	2593.02±0.040[a]	2098.27±0.015[a]	1882.80±0.010[a]	0.85±0.005[c]	0.59±0.010[c]
P-C	1813.31±0.025[b]	1223.92±0.027[b]	1186.18±0.016[b]	1.01±0.025[b]	0.67±0.015[b]
LM	780.14±0.015[c]	634.34±0.035[c]	509.43±0.025[c]	1.34±0.015[a]	0.73±0.010[a]

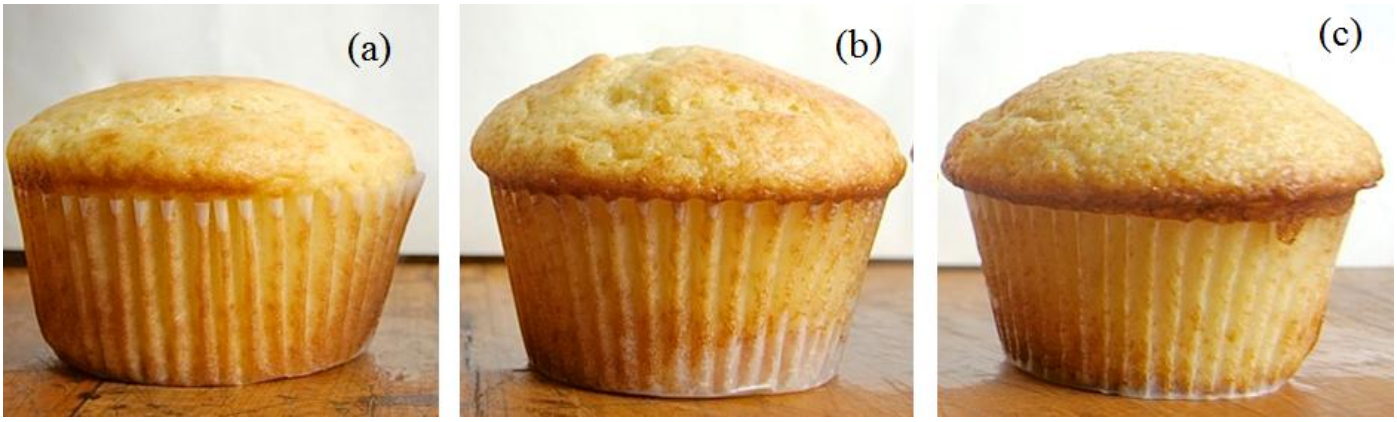

Figure 3 Showing the Muffin a) Negative Control, b) Positive Control, c) Lipopeptide muffin

Effect of lipopeptide on the color of muffin

Color is one of the important quality attribute both for raw material and finished product and is directly related to the acceptability of the food product [23]. Substituting emulsifier in the formulation significantly affects the surface or the crust color of muffin (**Table 2**). The L* value of N-C was found to be higher when compared with lipopeptide muffin followed by P-C, this shows the lightness of the sample whereas a* was found to be higher for P-C followed by Lipopeptide containing muffin and N-C, indicating redness of the sample. The muffin prepared using lipopeptide as an emulsifier shows higher b* value i.e. 37.19 when compared with negative and positive control, 31.03 and 34.61 respectively. This higher value of b* represents the yellowness of the sample. These results were equivalent to that observed by [15]. Baking promotes the color of muffin mainly due to the interaction between carbonyl group of sugar and amino group of protein. Dextrinization and starch caramelization accelerates by dry heating which affects the baked muffin color **[**24]. Muffin color obtained by incorporation lipopeptide at 1% was found to be optimum in appearance.

Table 2 Shows the color analysis of muffin. The data is presented as mean ± standard deviation

Sample	L*	a*	b*
N-C	68.57±0.015[a]	4.85±0.025[c]	31.03±0.035[c]
P-C	48.91±0.010[c]	17.45±0.035[a]	34.61±0.025[b]
LM	59.81±0.020[b]	13.71±0.025[b]	37.19±0.03[a]

Conclusion

MMD32 strain derived from marine sponge *F. cavernosa* is a lipopeptide producing strain. Lipopeptide is an amphipathic molecule that accumulates between the fluid phases and reduces the surface and interfacial tension in the food emulsion. Lipopeptide posses antibiofilm activity against *S. aureus* that is a pathogenic strain and help to remove the biofilm from the surfaces of industrial pipes and can be used clinically. They also have the ability to degrade polyaromatic hydrocarbon which are very harmful to human beings. Lipoppeptide was also found to be a good emulsifier and improves the texture and color of the muffin that are important quality attribute in food products, lipopeptide muffin are almost same as that of positive control (egg). In future, lipopeptide from MMD32 could be used potentially as an alternative to commercial chemical surfactants with potential use in bioremediation, food formulation and drug designing.

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