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ANTIBACTERIAL ACTIVITY OF BIOSURFACTANT DERIVED FROM *BACILLUS LICHENIFORMIS*

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ABSTRACT

Bacillus licheniformis produces lipopeptides biosurfactant that have a great potential for biopharmaceutical and biotechnological applications. Surfactin is the most important lipopeptide biosurfactant known. In this work, the lipopeptide biosurfactant produced by Indian marine bacteria, *Bacillus licheniformis* was analyzed. After 300 h cultivation in Zoball marine medium, a crude biosurfactant concentration of 2 g/L was produced. Biosurfactant product characterization have displayed high stability product in the wide range variation of pH and salinity. Evaluation of antimicrobial activity of biosurfactant against six human pathogenic microbes (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli, Streptococcus pnemoniae*, *Klebsiella pneumoniae*, and *Salmonella typhi*) demonstrated that biosurfactant produced by *B. licheniformis* have a specific antibacterial activity. Highest zone of inhibition was found against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Lowest zone of inhibition was found against *Escherichia coli, Streptococcus pnemoniae* and *Salmonella typhi.* Derived biosurfactant was concluded that the potential antibacterial agent against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Keywords*: Bacillus licheniformis, biosurfactant, antibacterial, Staphylococcus aureus, Pseudomonas aeruginosa*

INTRODUCTION

Surface-active amphipathic molecules produced by a number of microorganisms is known as Biosurfactant. They are derive in nature as a different group of molecules comparing of neutral lipids, lipopeptides, phospholipids, fatty acid, glycolipids, lipoproteins, polymeric and particulate biosurfactant **[1]**. At the air-water interfaces, the low-molecular-weight biosurfactants reduce the interfacial tension and the surface tension at oil-water interfaces and some of highmolecular-weight biosurfactant are more effective in stabilizing oil-in-water emulsions is known as bioemulsifiers **[2]**. Biosurfactant have been used to enhance bioremediation, oil recovery, and industrial emulsification, but in current years, biosurfactant have been found to possess many properties of biomedical and therapeutic importance, such as antibacterial, antiviral and antifungal properties **[3]**. The antibacterial, antiviral and antifungal activities of biosurfactants make them potentially applied in resisting many diseases and it could be used as therapeutic agents in medical industries. Lipopeptides can predominantly act as antitumor agents, antiviral, antibiotics, enzyme inhibitors and immunomodulators **[4]**.

So as to decrease or eradicate the effect of oil spillage on the living organisms and environment effort such as applications of skimming of the surface oils chemical dispersant. Application of inoculating the biological oil agents to the spilled area with relevant bacteria are the outcomes of interesting research **[4]**. The most hopeful of many research carried out to deal with large scale of oil spillage is the benefits of microorganisms to provide an effective alternative **[5]**. Surface-active components produced by microorganisms are of two main types, those that reduce the interfacial tension between solidliquid or immiscible liquids at the interface (bioemulsifiers) and those that reduce surface tension at the air-water interface (biosurfactants).

Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension **[6]**. Because of the presence of hydrophobic and hydrophilic groups, surfactants partition preferentially at the interface between fluid phase of different degrees of polarity and hydrogen bonding **[7]**. These amphiphilic compounds have functional properties like surface and interface activity, emulsification, wetting, foaming, detergency, phase dispersing, solubilization and density reduction of heavy hydrophobic compounds and find wide applications in industries **[8]**.

The total surfactant production has exceeded 2.5 million tons in 2010 for many purposes such as polymers, lubricants and solvents. From the total surfactants output, about 54% of them is consumed as household or laundry detergents, with only 32% destined for industrial use. The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection **[9]**.

Majority of surfactants produced today is of petrochemical origin beside of the renewable resources like fats and oils. Petroleum-related industries have been identified as one of the major source of pollution in all countries **[10]**. At present, biosurfactants plays an important application in petroleum-related industries which is use in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludge [**11]**.

MATERIALS AND METHODS

Microorganism

Microorganism used in this study, *Bacillus licheniformis*, is a Indian marine bacteria isolated from coastal area, Chennai, Tamilnadu, India. The strain was collected in research center for biotechnology, Indonesian Institute of Science (LIPI). The microbes that were used for antibacterial analysis, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli, Streptococcus pnemoniae*, *Klebsiella pneumoniae*, and *Salmonella typhi* are human pathogenic microbes collected by MTCC, Chandigarh.

Culture media

The *Bacillus licheniformis* was grown on liquid medium with composition (L^{-1}) 2 g sucrose, 6 g Na2HPO4, 3 g KH2PO4, 1.0 g NH4Cl, 0.5 g NaCl, 1 ml MgSO4 1 M, and 2.5 ml microelement that contained (L^{-1}) 53 mg MnCl4 H2O, 31 mg H3BO3, 36 mg CoCl2 6H2O, 10 mg CuCl2 2H2O, 30 mg Na2MoO4 2H2O and 50 mg ZnCl2. The pH of this medium was adjusted to 7.0 and the solution was sterilized in high pressure sterilizer for 20 min at 121ºC. The experimental flasks were inoculated with 2% (v/v) inoculum and incubated at 28°C on a rotary shaker (100 rpm).

Isolation of biosurfactant

The fermentation broth was centrifuged at 13,000 rpm for 30 minute to obtain a cell free broth. Supernatant was then dissolved in a 4N hydrochloric solution and allowed to stand overnight at 4°C, followed by the biosurfactant extraction step with a chloroform-methanol (2:1 v/v) solvent **[12]**.

Emulsification index

The visual method was applied to analyze emulsification activity. Centrifugation at 13,000 rpm to separate biosurfactant from microorganism cells yielding a cell free broth. A mixture of 1:1 between biosurfactant and kerosene is agitated for about 2 minute then stabilized for 24 hour. Emulsification activity was analyzed by comparing emulsion of biosurfactant product with the blank **[13]**.

Emulsification stability test

The aim of extreme ionic strength was to study the pH stability of biosurfactant product. Five millimetre of cell free broth was exposure with various of pH, the pH value was between 3 to 12 then the emulsification index were measured **[14]**.

Fourier transforms infrared spectroscopy (FTIR)

FTIR was used to determine the functional groups and the chemical bonds present in the biosurfactant compound. 0.3-0.5 mg sample was dispersed in 80 mg pellet of KBr.

Antibacterial activity of biosurfactant Bacillus licheniformis

The antibacterial activity of the biosurfactant was evaluated using the agar diffusion method. In order to produce an appropriate inoculum, the bacterial culture was cultivated for 18 hours in Muller Hinton Agar medium. This cellular concentration is about 10^7 CFU/mL, the optical density of cell suspension (OD600 nm) is about 0.8 - 0.9.

Two ml of standardized suspensions of the microorganisms were deposited in petridishes (diameter 90 mm) and 18 ml of Muller Hinton agar at 45^0C was added. Aliquots of 20 mL of the filtrate were applied to paper disks (5 mm in diameter, Whatman paper No. 1), which resulted in disks containing 15 µl of the product with concentration of 5000 ppm, and 15 µl of 5000 ppm amoxicillin was used as comparison standard. After evaporation of the loading solvent, each disk was placed at the center of the petridishes and incubated at 37ºC for 24 h. At the end of the incubation time, the diameter of microbial growth inhibition halo was measured in millimeters using a ruler **[15]**.

The evaluation of the antimicrobial activity of biosurfactant *Bacillus licheniformis* compound was carried out against six pathogenic bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli, Streptococcus pnemoniae*, *Klebsiella pneumoniae*, and *Salmonella typhi*).

RESULTS AND DISCUSSIONS

Production and isolation of biosurfactant

The *Bacillus licheniformis* produce biosurfactant when growth on minimum salt medium contains sucrose as single carbon source. **Figure 1** showed the growth of *Bacillus licheniformis* that is divided into 4 phases: adaption phase (0 - 24 h), growth phase (24 – 300 h), stationary phase (300 – 350 h), and death phase (after 350 h cultivation). Biosurfactant product has been produced during growth phase, and stationary phase. The existence of biosurfactantproduct could be identified by the reduction of surface tension and emulsification activity. The biosurfactant product was isolated from medium culture after 300 h incubation. The acid precipitation and biosurfactant extraction step has conducted by adding chloroform-methanol (2:1 v/v) solvent. The final concentration of crude biosurfactant was 2.0 g/L.

Characterization of biosurfactant

In order to investigate the quality of biosurfactant product for industrial and environmental application, the characteristic of biosurfactant was analyzed. These analyses consist of emulsification activity of biosurfactant and itsstability in the influence of extreme salinity and extreme ionic strength. The influences of pH of biosurfactant produced by *Bacillus licheniformis* was showed in **Figure 2**. These results indicated that biosurfactant from *Bacillus licheniformis* had high stability in the wide range of pH, furthermore it could be promote to the both industrial and environmental application.

The hydrophilic moiety of biosurfactant that consist of polypeptide compounds was showed the absorbance at 3300-2800 cm^{-1}, 3400-3250 cm^{-1}, and 1700-1670 cm^{-1} which are indicating the presence of O-H stretching, N-H stretching, and C=O stretching of secondary amides, respectively. The hydrophobic moiety of biosurfactant was showed the absorbance at 2960-2850 cm^{-1} that indicating the presence of aliphatic C-H stretching. The functional groups present in biosurfactant produced by *Bacillus licheniformis* are summarized in **Table 1** and it showed that biosurfactant produced by *Bacillus licheniformis* contains aliphatic and peptide-like moieties.

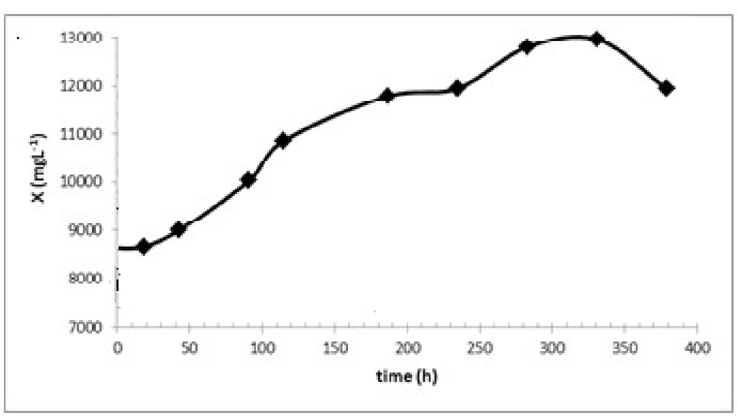

Figure 1: Growth of *Bacillus licheniformis*

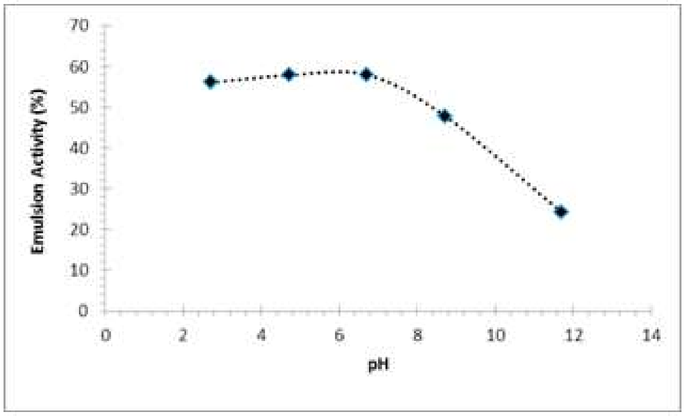

Figure 2: Effect of pH on the emulsification activity of biosurfactant *Bacillus licheniformis* Initial structural characterization

Table 1: FTIR Absorbance of biosurfactant produced by *Bacillus licheniformis*

Absorbance	Wavenumber, cm^{-1}	Remarks
O-H stretch	2958	O-H stretch of carboxylic acid
N-H stretch	3327	N-H stretching mode
C-H stretch	2852, 2922	aliphatic C-H stretch suggest the presence of an aliphatic chain
C=O stretch	1483, 1400,1681	stretching mode of the CO-N bonding

CONCLUSIONS

The *Bacillus licheniformis* was able to produce lipopeptide biosurfactant and it had high stability in variation pH and salinity; therefore, it could be applied in many industrial applications, including pharmaceutical industries. The biosurfactant produced by *Bacillus licheniformis* have a specific antibacterial activity, it could inhibit *Staphylococcus aureus* and *Pseudomonas aeruginosa* therefore, it could be more effective for *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection inhibition.

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