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Production of Biosurfactant From *Bacillus Subtilis* MTCC 441 And Its IndustrialAnd Environmental Applications

Amod Anil Apte[1], Dr. Unnati Padalia[2]
[1]*Department of Microbiology, K.J. Somaiya College of Science and Commerce, Vidyavihar-77, Mumbai, India.*
[2]*Associate Professor, Department of Microbiology, K.J. Somaiya College of Science and Commerce, Vidyavihar-77, Mumbai, India.*
Corresponding Author:Amod Anil Apte1

Abstract: *Biosurfactants are amphiphilic biological compounds produced extracellularly as a part of cell membranes by a variety of yeast, bacteria and filamentous fungi. Specifically, lipopeptide biosurfactant are produced by Bacillus subtilis species and are classified into three types: surfactin, iturin and fengycins. The biochemical mechanism for their biosynthesis depends upon non-ribosomal peptide synthetases. Particularly, surfactin synthesis is dependent upon surfactin synthetase operon which is regulated by complex cascade of reactions. Biosurfactants play an extravagant role and have varied industrial and environmental applications. This study focuses upon production of surfactin and to demonstrate its activity against bacteria and biofilm formation. Its use is also demonstrated in water purification stain removal.*

Keywords:*Biosurfactant, surfactin, Bacillus subtilis, biofilm, water filtration, stain removal.*

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I. Introduction

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi from various substances including sugars, oils and wastes. The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a number of industrial operations. They reduce surface tension and interfacial tension in both aqueous solutions and hydrocarbon mixtures. Biosurfactants display a wide variety of chemical structures, including glycolipids, lipopeptides, phospholipids, fatty acids, or neutral lipids, among others. Biosurfactants have a special advantage over the chemical surfactants, such as lower toxicity, higher biodegradability, biocompatibility and digestibility, better environmental compatibility, higher foaming, high selectivity, effectiveness at extremes of pH, temperature, salinity and widespread applicability, and their unique structures which provide new properties that chemical surfactants may lack.

This study makes the use of the above mentioned properties of biosurfactant (surfactin) for its application in the prevention of biofilm formation, in water purification and stain removing activity.

Biosurfactant production from microorganisms is achieved by exposing them to stimulant such as a complex carbon source, oil, crude oil etc. The microorganisms in a process to utilize that sole source of carbon produce the biosurfactant.

Factors affecting biosurfactant production:

A) Carbon source: The type of carbon source to be used for production of biosurfactant depends upon the type of biosurfactant (lipopeptides, rhamnolipids, glycolipids etc.). It is very important to use a right carbon source in right amount for maximum biosurfactant production.
B) Nitrogen source: The nitrogen source in the medium also influences the production of biosurfactant. It is very important to use the right C:N (carbon : nitrogen) combination in a correct ratio for maximum biosurfactant yield. (It is favorable to keep the ratio approximately 7).
C) Other factors: These include pH, enrichment period, temperature and shaker or static. All these factors are to be standardized according to the type of biosurfactant that is produced. Lipopeptide Biosurfactants produced by *Bacillus species* require pH to be around 7, enrichment period is variable, and temperature is set at around 35°C to 37°C and requires shaking conditions.
D) Salt concentration: The type and amount of salts present in the medium affect the amount of biosurfactant produced by the microorganism. It was found that the presence of chelating agents favors the biosurfactant production.
E) Growth phase: The inoculation of the culture in the production medium must take place at the right time of the growth cycle of the microbe. This is because production of biosurfactant occurs at specific stages of the

growth in different organisms. (For example: *Bacillus subtilis* produces surfactin at its mid-log phase of its growth cycle).

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MEDIUM DESIGN:

The medium used for biosurfactant production must be designed based upon all the above mentioned factors. A production medium used for biosurfactant production typically comprises of two components:

1) The main ingredients.
2) The trace elements.

The trace element and main ingredients composition depends is variable and can be standardized for a particular medium. Till date various media composition have been quoted and each has its own advantages and disadvantages.

In this study a complex medium was designed for maximum biosurfactant production from *Bacillus subtilis* MTCC 441. The production is checked in terms of Emulsification capacity Index.

II. Materialsand Methods

ACTIVATION OF CULTURE:

The lyophilized *Bacillus subtilis* MTCC 441 pellet was activated by inoculating a small part of it into 100ml sterile Nutrient broth flask. The flask was kept on shaker at 37°C for 24 hours.

STANDARDZING PRODUCTION MEDIUM:

(*A typical production medium used for biosurfactant production consists of two components:*
Main ingredients
Trace elements solution.)

A pre-defined Carvalho's medium (250 ml) was used at first as production medium. The culture was inoculated followed by 5-days enrichment period. (1)

Result: Qualitative tests (oil-drop spread assay and emulsification index test) results showed that the produced biosurfactant had lower activity.

Mukherjee medium: This medium had many salts which led to large amount of salt precipitation after autoclaving. But the trace element composition was remarkable and was further used for designing the production medium. (5)

Based upon literature survey, 3 media were designed (250 ml) with varying carbon and nitrogen sources. Also, each of the three media had elevated levels of iron and magnesium. The ratio of carbon to nitrogen was maintained 7 (approximately). (2,5).Carbon sources used: Molasses, Palm oil and Glucose. (All three act as stimulants for biosurfactant synthesis *Bacillus subtilis* and are sole source of carbon in the three media respectively)Nitrogen sources used: Urea, sodium nitrate and ammonium sulphate respectively.Each of these 3 media was inoculated with a pre-activated culture followed by 5-days enrichment period.

Result: On comparison (after 5 days) of the three media in termsof their emulsification index (EI) capacities, it was observed that:

[Emulsification Capacity
Two (2ml) of kerosene was added to 2ml of the culture supernatant and the mixture was vortexed at high speed for 2 minutes. The mixture was then left for 24 h; the height of the stable emulsion layer was measured. The emulsification capacity of biosurfactant was developed by Cooper and Goldenberg (1987) and the emulsion index E24 was calculated as the ratio of the height of the emulsion layer and the total height of liquid.
E24 = *Hemulsion/ htotal*X 100%]. (8)

Medium with molasses as the carbon source: EI = 65%.
Medium with palm oil as carbon source: EI = 48%.
Medium with glucose as carbon source: (No emulsification was seen).

Conclusion: Since emulsification index of medium with molasses was higher it was used as the production medium. The final production medium design had the following composition: (grams or ml)/ liter.

A	Quantity/liter	B	Quantity/liter
Molasses	25.5ml	$ZnSO_4.7H_2O$	2.32g
Urea	4.104g	$MnSO_4.4H_2O$	1.78g
NaCl	0.1g	H_3BO_3	0.56g
Na_2HPO_4	0.14g	$CuSO_4.5H_2O$	1.0g
$MgSO_4$	1.0g	$Na_2MoO_4.2H_2O$	0.39g
KH_2PO_4	1.4g	$CoCl_2.6H_2O$	0.42g
$CaCl_2$	0.04g	Na_2-EDTA	1.0g
$FeSO_4$	0.08g	$NiCl_2.6H_2O$	0.004g
		KI	0.66g

TABLE 1: MEDIUM COMPOSITION

The previously activated culture of *Bacillus subtilis* MTCC 441 was inoculated into the production medium (1 liter) followed by 5 days enrichment period.

After 5 days enrichment, qualitative tests were performed namely the oil drop spread assay and Emulsification Index test (EI test). HPLC (High-Performance Liquid Chromatography) was performed as qualitative test to further confirm biosurfactant presence.

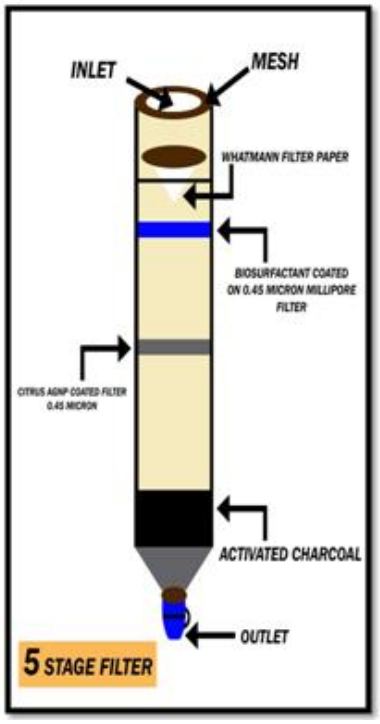
APPLICATIONS:

1) Synergistic activity of biosurfactant with anti-bacterial antibiotics against *Staphylococcus aureus.*

A bore well assay was carried out. Standard solutions of anti-bacterial antibiotics (of defined concentrations) chloramphenicol (30 μg) and streptomycin (10 μg) were prepared. A defined amount of biosurfactant solution was mixed with defined amount of antibiotic solutions respectively. (1).

2) A 5-stage filter was designed and constructed. The 5 stages of filter were as follows:

- Mesh
- Whatmann filter paper
- Biosurfactant coated on 0.45 micron Millipore filter.
- Citrus silver nanoparticle coated on 0.45 micron Millipore filter.
- Activated charcoal.



APPRATUS DESIGN

ACTUAL APPRATUS

UNCTIONS OF FILTER COMPONENTS:

- Mesh: To remove debris from the contaminated water.
- Whatmann filter paper: To remove soil particles.
- Biosurfactant coated on 0.45 micron Millipore filter: To remove oil from the contaminated water.
- Citrus silver nanoparticle coated on 0.45 micron Millipore filter: For Anti-microbial action.
- Activated charcoal: For final water purification.

3) Activity of Biosurfactant against Biofilm formation by *Pseudomonas species:*

Slides were immersed in two plates containing trypticase soy broth. One plate also had biosurfactant in it mixed with the broth. The other plate contained only the broth. Each plate was then inoculated with *Pseudomonas species* culture suspension and incubated at 37°C 24-48 hours. The biofilm formation on each slide was observed under microscope with an objective of 100X magnification the oil immersion lens).

4) Stain removal activity of biosurfactant:

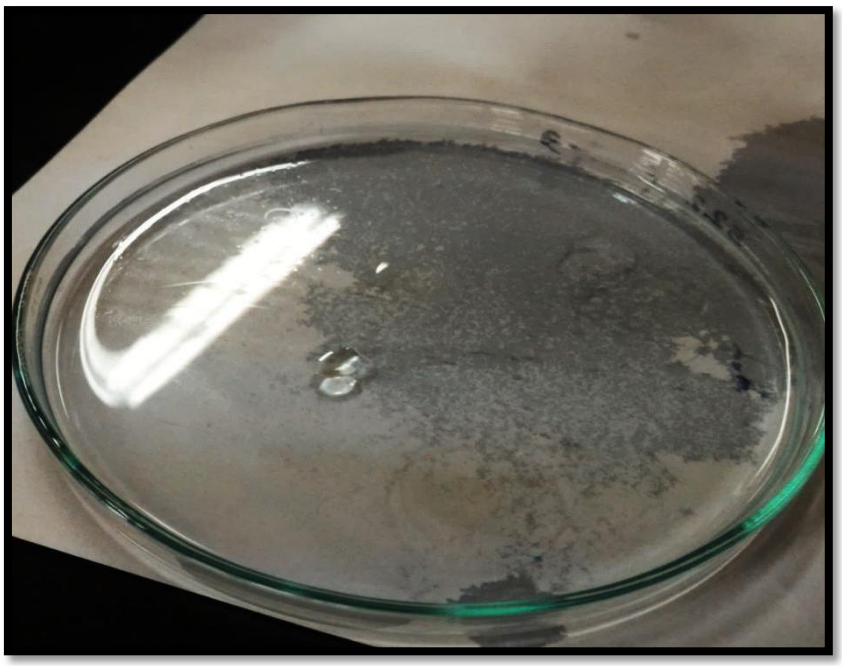
Three cloth pieces stained with sauce were used. One was treated solely with biosurfactant, one was treated with water and the third was control cloth.

III. Result

A) QUALITATIVE TESTS:

1) Oil drop spread assay:

The main function of biosurfactant is to alter the surface tension of the medium in which it is mixed (usually the medium is water). As seen in the image, the circumference of the oil drop on water is not altered. This is because biosurfactant has been mixed with water which has altered the surface tension and hence prevents the oil drop from spreading onto the water surface.



PETRIDISH CONTAINING BIOSURFACTANT MIXED WITH WATER + Oil drop

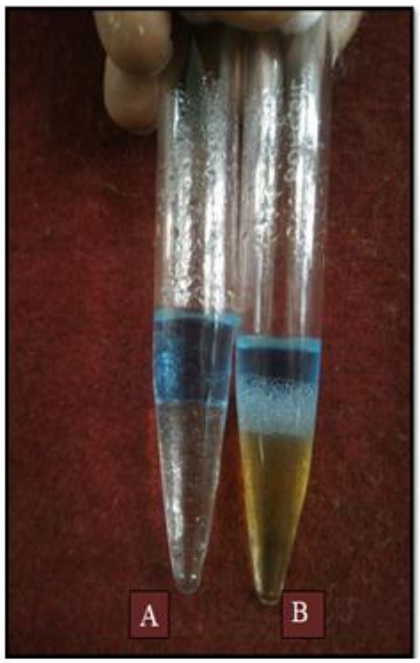
2) Emulsification index test (EI)/ Emulsification Capacity test:

Two (2ml) of kerosene was added to 2ml of the culture supernatant and the mixture was vortexed at high speed for 2 minutes. The mixture was then left for 24 hrs. The height of the stable emulsion layer was measured. The emulsification capacity of biosurfactant was developed by Cooper and Goldenberg (1987) and the emulsion index E24 was calculated as the ratio of the height of the emulsion layer and the total height of liquid**.**

E_{24} = *Height of emulsion/Total height* X 100%.

EI test results were as depicted below. The emulsification capacity of the produced biosurfactant was found to be 65%. A tube is control (Kerosene only, no biosurfactant) and B tube is test (2ml of kerosene + 2ml of biosurfactant). Both tubes were kept for 24hours at R.T and E.I for 24 hours was calculated with the formula mentioned above.

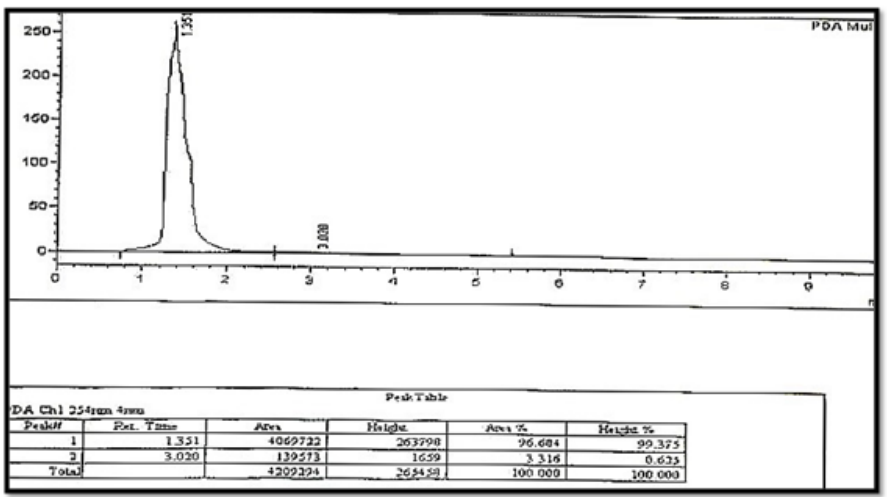
$E.I_{24}$ = 2.6/4x% = 65%



EMULSIFICATION CAPACITY INDEX TEST

3) HPLC (High – Performance Liquid Chromatography):

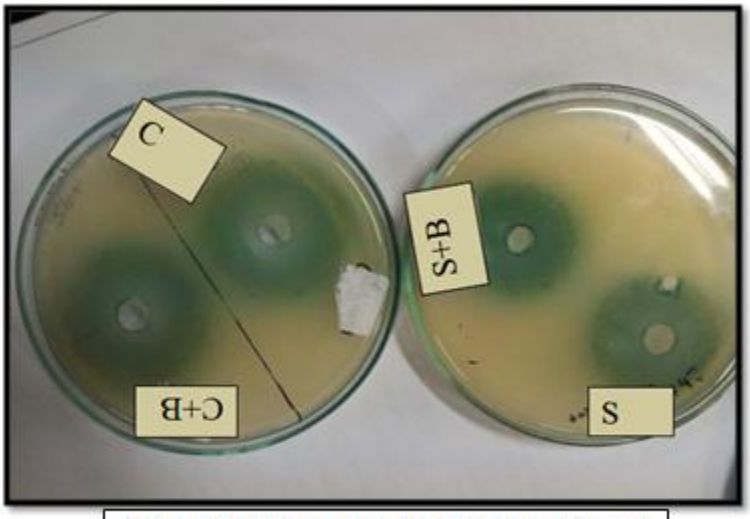
HPLC of the biosurfactant (surfactin) sample was performed with Acetonitrile (ACN) + Water as mobile phase in the ratio 80:20. C-18 column was used as the stationary phase. The obtained retention time readings were compared with a research paper having standard surfactin HPLC readings. The peak matched with the standard surfactin peak. (5)



APPLICATION RESULTS:

1) Synergistic activity of biosurfactant with anti-bacterial antibiotics against *Staphylococcus aureus.*

As depicted below, the individual zone sizes of antibiotics chloramphenicol and streptomycin were same as zone sizes of biosurfactant in synergism with the above mentioned antibiotics. Hence, it was concluded that the produced biosurfactant (surfactin) lacks anti-bacterial activity against *Staphylococcus aureus* that is it lacks antimicrobial activity. Lower potency of the produced biosurfactant can be the reason behind lack of antimicrobial activity**.**



ANTI-BACTERIAL ACTIVITY OF SURFACTIN AGAINST *Staphylococcus aureus*

2) 5-stage filter was designed and constructed:

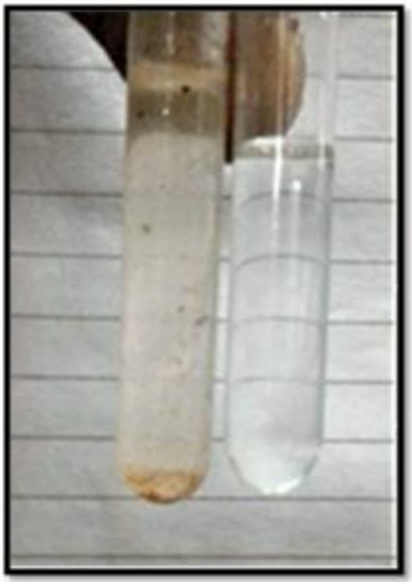
- A water sample contaminated with oil and soil was passed through the designed

5-stage filter and its purity was checked in terms of optical density at a wavelength 530nm using a colorimeter.

- The same water sample was passed through the filter but this time the stage of Biosurfactant coated on 0.45 micron Millipore filter was removed.
- Both the results are tabulated below.

FILTRATION STATUS	O.D at 530nm	O.D at 530 nm (without biosurfactant filter)
Before filtration	0.20	0.20
After filtration	0.01	0.06

TABLE 2: FILTRATION READINGS



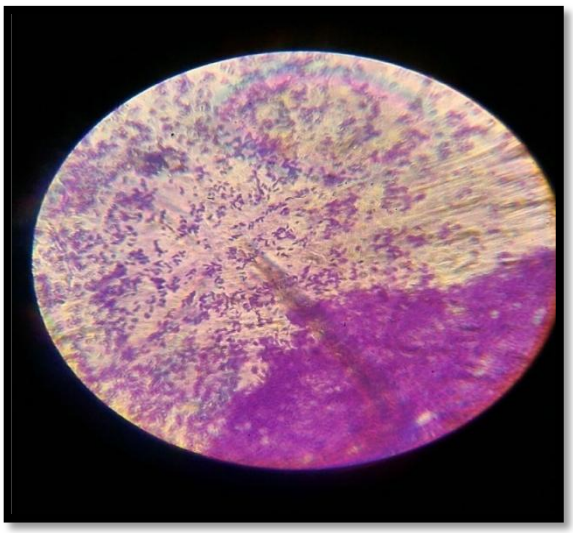
LEFT TUBE: BEFORE FILTRATION

RIGHT TUBE: AFTER FILTRATION

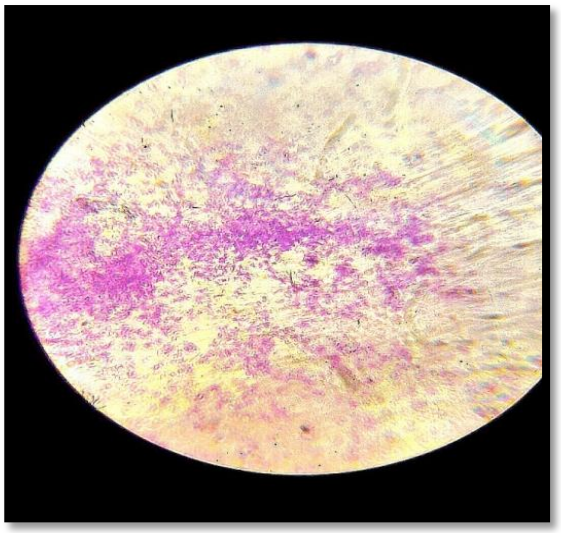
3) Activity of Biosurfactant against Biofilm formation by *Pseudomonas species:*

Slides were immersed in two plates containing trypticase soy broth. One plate also had biosurfactant in it mixed with the broth. The other plate contained only the broth. Each plate was then inoculated with *Pseudomonas species* culture suspension and incubated at 37°C 24-48 hours. The biofilm formation on each slide was observed under microscope with an objective of 100X magnification the oil immersion lens).

Result: There was a decrease in biofilm formation on the slide which was treated with biosurfactant. This is because alteration in the surface tension of the medium leading to lesser adsorption of *Pseudomonas species* on slide leading to decrease in biofilm formation. (The first step necessary for successful biofilm formation on a surface is proper and firm adsorption). The results are depicted below.



Biofilm observed under 100X magnification without biosurfactant treatment



Reduction in biofilm formation observed under 100X magnification with biosurfactant treatment

4) Stain removal activity of biosurfactant:

Three cloth pieces stained with sauce were used. One was treated solely with biosurfactant, one was treated with water and the third was control cloth.

Results: The cloth stain which was treated with biosurfactant only had no traces of stain left (I) whereas the cloth that was only washed with water had the stain traces (II). The control cloth shows the original stain (III). This confirmed the surfactant activity of the produced biosurfactant. The results are depicted below.

(I) (II) (III)

IV. Conclusion

Biosurfactant production was carried out using *Bacillus subtilis* MTCC 441 in a complex medium designed to increase its production. The production level in medium was analyzed by using the Emulsification capacity Index test (E.I test). The medium having molasses as a sole carbon source gave a higher emulsification index (65%) when compared to other two media and hence was chosen as the final production medium. HPLC was performed to confirm the presence of surfactin. This was done by comparing the HPLC result with a research paper depicting standard surfactin results. Quantification of biosurfactant was not carried out due to lack of surfactin standard. All the applications of the produced surfactin showed positive results except for anti-bacterial application. The lack of anti-bacterial activity may be due to the low potency of produced surfactin. A scale-up is needed to overcome this problem.The other three applications successfully showed the environmental and industrial use of the produced biosurfactant.

V. Future Prospects

1) To check microbial load of filtered water.
2) To check biosurfactant activity against swarming activity of *Proteus* species**.**

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