Isolation, Screening And Production Of Biosurfactants From Normal Human Skin Flora

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Abstract : Most commercially available surfactants are produced from petroleum but concern about the environment has stimulated the search for microbially produced surfactants. Its medical application is as antimicrobial biocontrol agents and as an adjuvants. In present study for the isolation and screening of biosurfactants human skin is selected as a source. Isolates were characterized for colonial and cellular morphologies and analyzed through different screening methods like hemolytic assay, Mineral Salt Medium (2% oil as a substrate), Bacterial Adherence To Hydrocarbons [BATH], oil spreading activity. Confirmatory test was performed with Bap method. 24 isolates were obtained from which 18 were hemolytic and 6 were non hemolytic on blood agar plate. Primary screening of 24 isolates on MSM medium was carried out in which 8 bacterial isolates were found as potential biosurfactant producers in which 5 were hemolytic and 3 were non hemolytic bacteria. These isolates were further subjected for the secondary screening test : BATH assay and oil spreading activity to check for the highest biosurfactant producers. For confirmation Bap method was used in which 4 cationic biosurfactants were obtained. The production and extraction process of selected isolates were carried out. TLC and antagonistic activity of the crude biosurfactant were done in which the R_f value were calculated and antagonistic activity against the lab test organisms were performed. The FTIR of highly produced biosurfactant F4 was done and the biosurfactant produced was lipopeptide in nature.

IndexTerms **-Biosurfactants, Hemolysis, BATH, Bap, Antagonistic activity.**

I. INTRODUCTIONS

Human skin is considered to be a critical barrier between the human body and its outer environment. Normal skin flora has ability to produce secondary metabolites and prevents the growth of other microorganisms.

Biosurfactants are surface active biomolecules that are produced extracellular by a variety of microorganisms. They are ampliphilic secondary metabolites with both hydrophilic and hydrophobic moieties. Biosurfactants are considered to be "GREEN" as an alternative for the chemical surfactant that is widely used in different aspect of human activity, example: medicine, farming, industry.

Biosurfactants are mainly produced by *Bacillus* and *Pseudomonas* and yeast like *Candida*.

Biosurfactants are the broad group of molecules which include lipopeptides, lipoproteins, glycolipids, phospholipids, neutral lipids etc.

These compounds may be divided into two major classes of biosurfactants:

1. Low molecular weight consisting of lipopeptides, glycolipids and proteins.
2. High molecular weight compounds such as polysaccharides, liposaccharides, proteins and lipoproteins.

The low molecular weight compounds can decrease surface and interfacial tension and the high molecular weight molecules are amphiphilic and polyphilic polymers which normally stabilize emulsions of oil-in-water but are unable to effectively reduce surface and interfacial tension between two lipids.

Types of microbial lipopeptides:-

Lipopeptides are small sized lipidia peptides of 1.1 to 1.2kDa and of bacterial origin.

- **ITURINS:-**

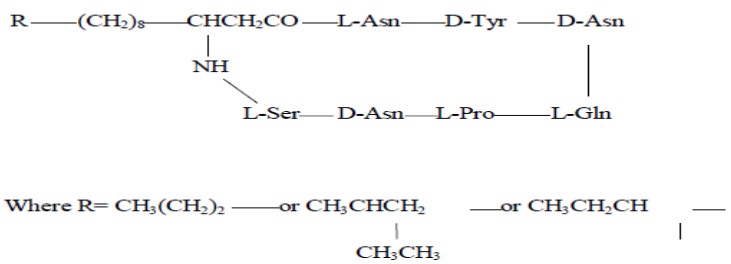
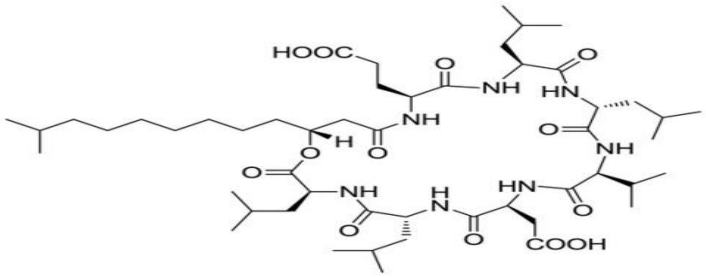


Figure 1: Structure of Iturin A.

Source: Google

Among the three types of lipopeptides iturins has molecular weight ~1.080kDa, made of up 2 parts – a hydrophobic tail of 11 to 12 carbons and a peptide part of 7 amino acid residue. It is a cyclic peptide. It exhibits strong antifungal property by forming ion conducting pores on fungal membrane.

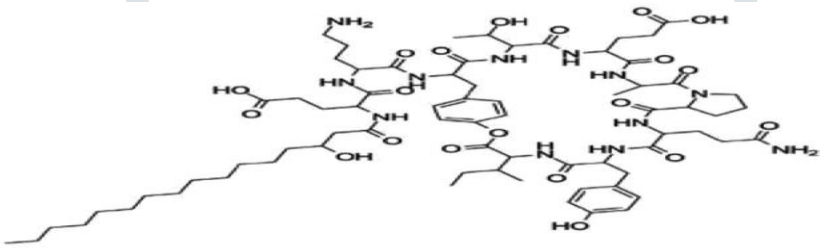
- **SURFACTINS:-**



surfactin A
Source:Google

It is amphipathic cyclic liopopeptide. The type of surfactin varies according to the amino acid order. Under the natural conditions mixtures of surfactin is produced.

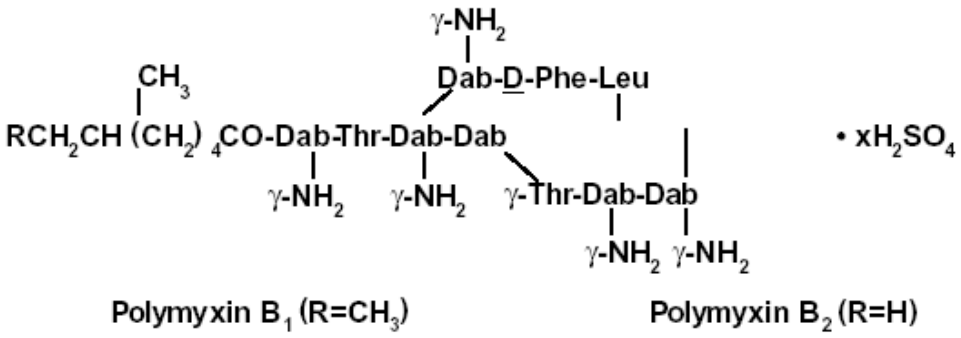
- **FENGYCINS:-**



Fengycin
(Molecular formula $C_{72}H_{110}N_{12}O_{20}$,
Molecular weight 1463.71 m/z)
Source:Google

It is a lipopeptide produced by many strains of *Bacillus subtilis*. It shows antifungal activity against filamentous fungi. It is 3rd family which represents lipopeptide after the surfactin and iturin. It is also called as plipastatin.

- **POLYMYXINS:-**



Source:Google

It is a family of lipopeptides which contains non proteinogenic amino acid. The 2 representatives, polymyxin B and polymyxin E are in clinical use. It shows little activity against gram-positive and anaerobic bacteria but shows the bactericidal activity towards many gram negative bacteria.
It has gained attention due to different properties like low toxicity, good biodegradability in comparison to synthetic surfactants, high biodiversity bacterial lipopeptides are getting more attention due to low irritancy and compatibility with human skin.

II MATERIALS AND METHODS

2.1 Sample collection

Six different body parts of women like vagina, underarms, under breast, forehead, elbow and lower back were selected as a source for isolation of the microorganisms from two different individuals. The age of women were between 20-24 years old.

2.2 Isolation

for the isolation of microorganisms, samples were collected from the six different body parts with sterile cotton swab and inoculated in the nutrient broth for enrichment at 37°C for 24 hours.

2.2.1 Hemolytic activity

The hemolytic activity was performed for the isolation of biosurfactant producing microorganisms. Different samples from enriched nutrient broth were grown on blood agar plates (5% freshly collected blood) and incubated for 48 hours at 37°C. the plates were visually inspected for checking their hemolytic or non hemolytic activity at every 24 hours. the isolates were further taken for primary screening.

2.3 Primary screening

For primary screening the isolates obtained on blood agar plates were grown on sterile MSM(Mineral Salt Agar Medium) plates (2% engine oil as a substrate) incubated at 37 °C for 48 hours. The potential isolates obtained on MSM agar plates were further selected for secondary screening.

2.4 Secondary screening

For secondary screening the isolates that were grown on MSM agar plates were subjected for BATH assay and oil spreading activity to check their biosurfactant activity.

2.4.1 Bacterial Adherence To Hydrocarbon

BATH assay is similar to the method given by Rosenberg *et al.* Bacterial cells were washed twice with the equal volume of buffer salt solution (K_2HPO_4, KH_2PO_4), and then were resuspended in the same buffer salt solution and OD taken at 620nm. crude oil (100µL) was added and vortex it for 3 minutes in test tubes. After vortex-shaking the crude oil and aqueous phase were allowed to separate for 2 hours. OD of aqueous phase was then again measured at 620nm in spectrophotometer. Percentage of cell adherence to crude oil was calculated as follows:

{1 - (OD of aqueous phase ÷ OD of initial cell suspension)} * 100

2.4.2 Oil Spreading Activity

50 µL of crude oil was added to petriplate containing 25ml of distilled water. The isolates that were grown in LB medium for 72 hours at 37 °C after centrifugation 25 µL of cell free supernatant was added to the surface of oil. If the oil layer is evacuated with the clearing zone then the cell free supernatant exhibited biosurfactant activity.

2.5 Confirmatory Test

2.5.1 Bap Method

Blue agar plate method was used to identify the cationic biosurfactant producing microorganism by adding SDS (0.5mg/ml) and methylene blue (0.2mg/ml). 30 µL of cell free supernatent was loaded in each well and incubated at 37°C for 72 hours. The dark blue halo zone was observed and was considered as positive for cationic biosurfactant.

2.6 Production Of Biosurfactant

The production of biosurfactant was performed in Erlenmeyer flask of 500ml in which 100ml of MSM medium (2% oil as a substrate) was prepared (pH 7.0) and the isolate capable of producing biosurfactants were inoculated in the MSM medium and kept on rotatory shaker for 7 days at 37°C.

2.7 Extraction Of Biosurfactant

The production medium was centrifuged at 7000rpm for 10 minutes and the supernatant was collected in the sterile test tubes. 0.5ml of 6N HCL and organic solvent chloroform and methanol (2:1v/v) was added to the supernatant and kept at room temperature for 30 minutes. Again after centrifugation the supernatant was collected in the sterile flask and placed on the evaporator to obtain the dried crude biosurfactant.

2.7.1 Antagonistic Activity

The antagonistic activity of biosurfactants were performed against the lab test organisms like *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* and the zone of inhibition were observed.

2.8 Characterization Of Crude Biosurfactant

2.8.1 Thin Layer Chromatography.

TLC was performed in the plates prepared with silica gel. The following solvent system were used: chloroform/methanol/acetic acid/water (25:15:4:2) volume. The sports were revealed with standard vapour spraying reagent iodine and were observed under the U.V irradiation as yellow spots. R_f value of the spots were calculated.

2.8.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of unknown mixture. It is rapid and intensive method. FTIR is a physiochemical method based on measuring the vibrations of a molecule excited by IR radiation at specific wavelength range.

III RESULTS AND DISCUSSION

3.1 Results of isolation

Table 3.1 of isolation

Sr No.	Sample Collection	Isolates Obtained
1	Forehead	5
2	Elbow	5
3	Underarms	3
4	Under breast	4
5	Lower back	4
6	Vagina	3

Table 3.1 shows that Total 24 isolates were obtained from different parts of skin from 2 different women in which 18 were hemolytic organism and 6 were nonhemolyitc organisms.

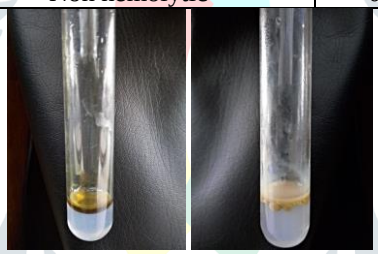
3.2 Results on MSM agar plates

Primary screening was done on MSM agar plates, growth of 8 isolates were observed from 24 isolates in which 3 were nonhemolytic organism and 5 was hemolytic organism.

3.3 Results of BATH assay and oil spreading activity

Table 3.3.1 Optical Density of BATH assay

Sr No.	Bacterial isolates	Characteristic of organism	Initial OD at 620nm	OD of aqueous phase
1	E2	Hemolytic	0.45	0.39
2	UB1	Hemolytic	0.45	0.35
3	UA1	Hemolytic	0.48	0.36
4	UA4	Hemolytic	0.44	0.39
5	LB2	Non hemolytic	0.46	0.38
6	LB5	Non hemolytic	0.5	0.46
7	F4	Hemolytic	0.52	0.40
8	V1	Non hemolytic	0.5	0.24



Control Test

Figure 3.3.1BATH assay

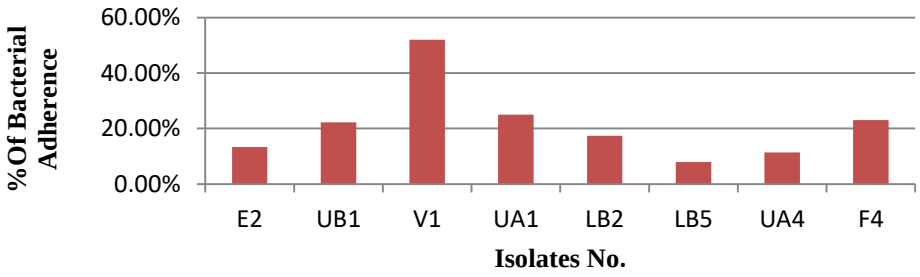
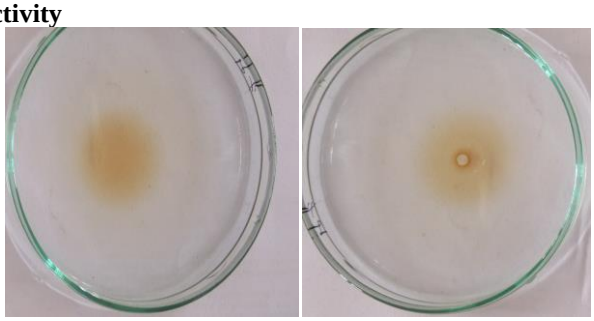


Figure 3.3.1 graph of BATH assay.

As shown in the above given graph the V1 organism obtained from vaginal sample has got the highest % of bacterial adherence to hydrocarbon that is 52% other organisms are as follows: E2-13.33% from elbow, UB1-22.22% from under breast, UA4-11.36% and UA1-25% from underarms, LB2-17.39% and LB5-% from lower back and F4-23.07% from forehead.

3.3.2 Results of oil spreading activity



Control Test

Figure 3.3.2 oil spreading activity

The results of oil spreading test were positive for all the 8 bacterial isolates. These results show the presence of biosurfactant in the cell free supernatant of the isolates.

3.4 Results of Bap method

Table 3.4 size of zone on blue agar plate

Bacterial isolates	Blue agar plate zone(mm)
E2	18mm
V1	18mm (non hemolytic organism)
UA1	19mm
UA4	17mm
LB2	18mm (non hemolytic organism)
LB5	18mm (non hemolytic organism)
F4	20mm
UB1	16mm

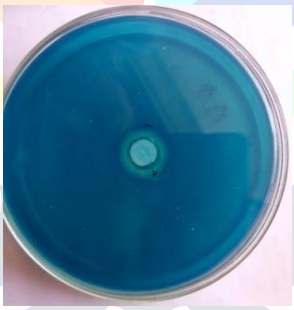


Figure 3.4 zone on blue agar plate

from the above observation table the bacterial isolates showing the blue halo zone indicated that the biosurfactant produced by organisms were considered as positive for cationic biosurfactants.

3.5 Result of extracted biosurfactant

Table 3.5 dry biosurfactant weight

Bacterial isolates	Extracted dry biosurfactant (g/100ml)	Color of dry biosurfactant
LB5	0.45g	Black
V1	0.21g	Black
F4	0.70g	Grey
E2	0.57g	White

Table 3.5 shows the highest dry biosurfactant was produced and extracted from the F4 bacterial isolate.

3.5.1 Results of antagonistic activity

Table 3.5.1 antagonistic activity against test organisms

	Zone of inhibition (mm)			
Bacterial isolates	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhi*
F4	30mm	27mm	23mm	22mm

E2	26mm	24mm	26mm	24mm
V1	20mm	15mm	18mm	14mm
LB5	20mm	17mm	1 9mm	16mm

Table 3.5.1 inhibitory zone were measured against different test organisms. Zone of inhibition indicates the antimicrobial activity of biosurfactants.

3.6 Results of thin layer chromatography

Table 3.6 results of TLC

Bacterial isolates	R_f
F4	Mono-lipopeptides : 0.66 Di-liopopeptide : 0.2
V1	Mono-lipopeptides : 0.66 Di-liopopeptide : 0.53
E2	Mono-lipopeptide : 0.62 Di-lipopeptide : 0.18
LB5	Mono-lipopeptide : 0.6

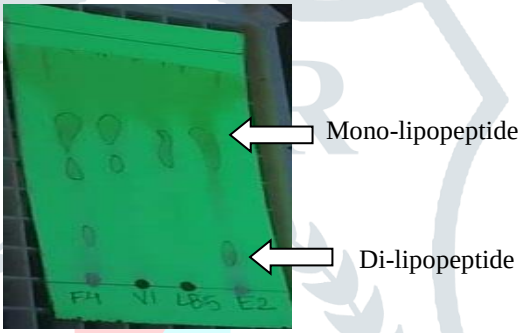


Figure 3.6 results on TLC

table 3.6 indicates the bands of the lipopeptide were obtained on tlc from which different molecular weight of lipopeptide were seperated and were visualized in u.v light. different R_f values of the mono-lipopeptide and di-lipopeptide were calculated.

3.7 Results of FTIR

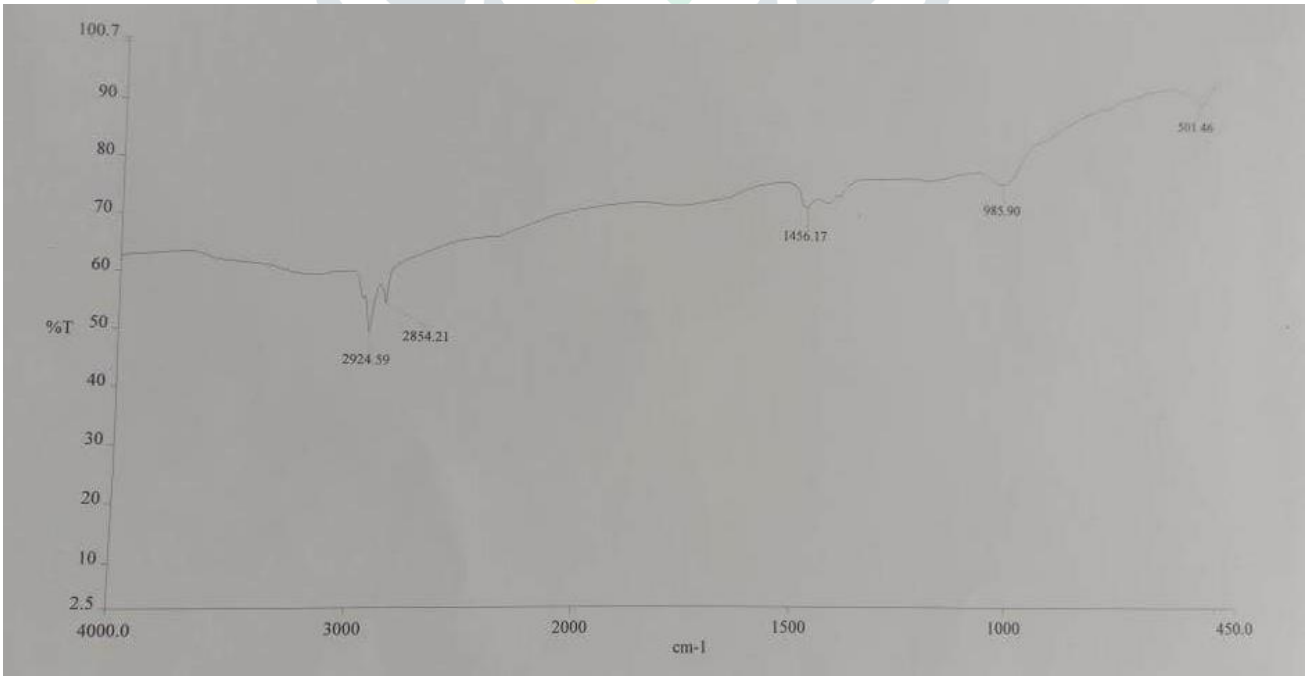


Figure 3.7 graph of biosurfactant produced F4 isolate

Table 3.7 showing respective stretch and bends at different IR of corresponding bonds in treated sample of F4 biosurfactant

Functional group	Range (cm^{-1})	Intensity	F4 sample
Aliphatic C-H	3000-2850 cm^{-1}	Strong	2924.59
Alkane C-H	3000-2850 cm^{-1}	Variable	2854.21
Typical band for the C⎓C ring stretching of benzene	1600-1400 cm^{-1}	Medium/weak	1456.17
Primary aliphatic compound C-O	995-985 cm^{-1}	Strong	985.90
Aromatic compound	Below 600cm^{-1}	Strong	501.46

The FTIR analysis of biosurfactant isolated from F4 organism revealed that the most important bands were located at 2924.59cm^{-1} (for the C-H aliphatic stretching), 2854.21cm^{-1} (for the C-H alkane), 1456.17cm^{-1} (for the C⎓C ring stretching of benzene) which confirmed the presence of polysaccharide, 985.90cm^{-1} (for the C-O primary aliphatic) which confirmed the presence of compound containing split of phenolic hydroxyl, 501.46cm^{-1} (for the aromatic compound). The region below 600 cm^{-1} in the fingerprint portion is often called as aromatic region. It provides the substitution of pattern of benzene, that supports the result of presence peak of 1456.17cm^{-1}. The above information from the respective wave number confirmed the lipopeptide nature of biosurfactant

The above nature of biosurfactant produced in the present study was similar to the results obtained in earlier work done by Thavasi Renga *et.al* (January 2010). The bands were similar to 2929cm^{-1} which indicated the C-H aliphatic stretching, 2858cm^{-1} indicates the presence of C-H alkane, 1456cm^{-1} indicates the C-H bond of alkane, 1066 cm^{-1} indicates the presence of C-O bond. From the entire result table it can be concluded that the diversified biosurfactant produced from the forehead of skin (F4 isolate), was lipopeptide in nature and structurally resembles to iturin lipopeptide.

IV ACKNOWLEDGEMENT

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[20]https://www.google.com/search?q=surfactin+molecular+structure&rlz=1c1skpl_enus440&source=lnms&tbm=isch&sa=x&ved=0ahukewjcpdnn_9pgahvofh0khycjbc4q_auidigb&biw=911&bih=405#imgrc=nauy20vhqb3bam

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