

Biosurfactants: An Alternative to the Synthetic Surfactants and their Production by Bacteria Isolated from Solid Waste

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Biosurfactants are a structurally diverse group of amphiphilic, surface-active substances produced by microorganisms which can be used as an eco-friendly approach for environmental remediation. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents. An attempt was made to produce biosurfactant from solid waste bacteria. Organisms were isolated from solid waste. The isolated organisms were identified to be *Micrococcus luteus*, *Serratia liquefaciens*, *Bacillus macquariensis*, *Bacillus popilliae* and *Corynebacterium kutscheri* by using various biochemical tests. All these organisms were screened for biosurfactant production using penetration assay, microplate assay and blood haemolysis technique. The yield of biosurfactants was compared in different media with and without oil stimulant and the role of an oil stimulant in biosurfactant production was determined.

Keywords: Biosurfactants, amphiphilic, emulsifiers and microplate assay.

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly. They contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively. Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic portion can be a carbohydrate, amino acid, phosphate or cyclic peptide¹

Considerable attention has been given in the past to the production of surface-active molecules of biological origin because of their potential utilization in food processing, pharmacology, and oil industry. Although the type and amount of the microbial surfactants produced depend primarily

on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affect their production by the organism¹

Many microbial surfactants have been purified and their structures have been elucidated. The high molecular weight microbial surfactants are generally polyanionic heteropolysaccharides containing polysaccharides and proteins and the low molecular weight microbial surfactants are often glycolipids. The productivity of microbial surfactants varies with the nutritional environment of the growing microorganism¹

MATERIALS AND METHODS

Screening techniques [2] Penetration assay

The penetration assay was developed by Maczek *et al.* in 2007. This assay relies on the contacting of two insoluble phases which leads to a colour change. For this assay, the cavities of a 96 well microplate are filled with 150 μ l of a

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hydrophobic paste consisting of oil and silica gel. The paste is covered with 10 μ l of oil. Then, the supernatant of the culture is coloured by adding 10 μ l of a red staining solution to 90 μ l of the supernatant. The coloured supernatant is placed on the surface of the paste. If biosurfactant is present, the hydrophilic liquid breaks through the oil film barrier into the paste. The silica enters the hydrophilic phase and the upper phase changes from clear red to cloudy white within 15 minutes.

Microplate assay

The surface activity of individual strains can be determined qualitatively with the microplate assay developed and patented by Vaux and Cottingham. This assay is based on the change in optical distortion that is caused by surfactants in an aqueous solution. Pure water in a hydrophobic well has a flat surface. The presence of surfactants causes some wetting at the edge of the well and the fluid surface becomes concave and takes the shape of a diverging lens. For this assay, a 100 μ l sample of the supernatant of each strain is taken and put into a microwell of a 96-microwell plate. The plate is viewed using a backing sheet of paper with a grid. If biosurfactant is present, the concave surface distorts the image of the grid below. The optical distortion of the grid provides a qualitative assay for the presence of surfactants.

Blood haemolysis technique

Biosurfactants can cause lysis of erythrocytes. This principle is used for the haemolysis assay which was developed by Mulligan *et al.* Cultures are inoculated on sheep blood agar plates and incubated for 2 days at 25°C. Positive strains will cause lysis of the blood cells and exhibit a colourless, transparent ring around the colonies.

Production of biosurfactants

Production of biosurfactants was tried in four different media which include nutrient broth with hydrocarbon stimulant, nutrient broth without hydrocarbon stimulant, production medium with hydrocarbon stimulant and production medium without hydrocarbon stimulant.

Procedure

The bacteria are inoculated in 50 ml of the four different media. Incubate the media at 30°C for 3 days with 2 min of light shaking each day

Measurement of dry weight [4]

Instead of obtaining the dry weight of

the bacterial pellet obtained by centrifugation, the media was filtered and the dry weight of the bacterial residues on the filter paper was measured.

Procedure

The initial weight of the Whatmann filter paper is taken. The media is filtered using Whatmann filter paper (Fig 2.1) and the weight of filter is taken at 0 min after filtration. The weights are taken for every 5 min until constant weight is obtained (Fig 2.2). This gives the dry weight of bacteria

Extraction of biosurfactants [3]

Extraction of biosurfactants is done using solvent extraction where the solvent system is in 2:1 ratio (Fig 3).

Procedure

50 ml of the culture filtrate is taken and its pH is adjusted to 2 using 1N HCl. It is then taken in a separating funnel. To this, Chloroform and Methanol is added in 2:1 ratio. The bottom solvent layer will contain biosurfactants and this is carefully collected in a initially weighed beaker. The upper aqueous layer is reextracted with the solvent mixture. The biosurfactant is then collected by evaporating the solvent. The increase in weight of the beaker indicates the amount of biosurfactants obtained. The biosurfactant is stored by diluting it as 1 g/l of water.

Confirmation of biosurfactants

Oil spreading technique [5]

The oil spreading assay was developed by Morikawa *et al.* 30 ml of distilled water is taken in petri plate. 1 ml of coconut oil and gingely oil is added to the centre of these plates. 20 μ l of cell free supernatant is then added to centre. The biosurfactant producing organism can displace the oil and spread in water.

Thin layer chromatography [6]

10 cm long and 1 cm wide TLC strips are cut. The test solution is spotted on the strips using capillary tubes. The strips were dipped in a solvent mixture of chloroform: methanol: water (70:10:0.5). The solvent front is marked. Then the TLC plate is exposed to iodine vapour, biosurfactant if present will appear as bright yellow coloured spots.

Emulsification capacity assay [7]

Emulsification capacity assay of biosurfactants was developed by Cooper and Goldenberg. Kerosene or any other hydrophobic

compound is added to an equal volume of aqueous sample. The mixture is vortexed at high speed for 2 minutes. After 24 hours, the height of the stable emulsion layer is measured. The emulsification index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid. E_{24} correlates to the surfactant concentration.

$$E_{24} = \frac{h_{\text{emulsion}}}{h_{\text{total}}} \times 100 \%$$



Fig. 1. Filtration



Fig. 2. Dry weight

RESULTS AND DISCUSSION

Screening of organisms for biosurfactant production

Penetration assay

The culture supernatants of all the organisms showed colour change from clear red (Fig 3.70) to cloudy white within 15 minutes (Fig 1) indicating that they are biosurfactant producing organisms.

Microplate assay

The culture supernatants of all the organisms showed optical distortion of the grid indicating that they are biosurfactant producing organisms. (Fig 2)

Blood Haemolysis technique

All the organisms showed haemolysis of sheep blood and a colourless, transparent zone (Fig 3) and greenish zone (Fig 4) was formed around the colonies indicating that they are biosurfactant producing organisms.

Production of biosurfactants

Confirmation of the obtained biosurfactants

Oil spreading technique

All the extracted samples showed displacement of the oil (Fig 5) and spread in water



Fig. 3. Extraction

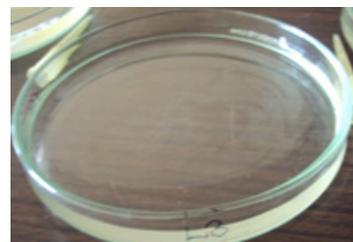
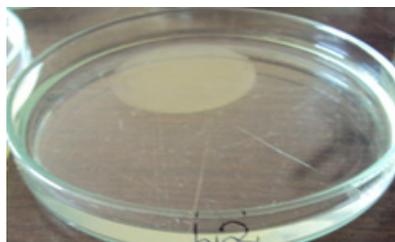


Fig. 4. Oil spreading technique

confirming that they are biosurfactants.

Thin layer chromatography

Yellow spots developed on the silica plates on exposure to iodine vapour indicating that all the extracted samples are biosurfactants. (Fig 6)

Emulsification capacity assay

The emulsion index E_{24} (Fig 7) was calculated for all the extracted samples taking SDS as control.

Total height = 37 mm

Table 1. Yield of biosurfactants in production medium without hexane

Organism	Dry weight of organism (g)	Weight of biosurfactant (g)	Yield
1a	0.098	0.017	0.173
1b	0.105	0.005	0.048
1c	0.0489	0.005	0.102
2a	0.0718	0.003	0.042
2b	0.0681	0.010	0.147
3c	0.0441	0.009	0.204
4a	0.0517	0.004	0.077
4b	0.0561	0.005	0.089
5a	0.076	0.014	0.184
5c	0.073	0.024	0.329
6a	0.067	0.005	0.075
6b	0.075	0.002	0.027
6c	0.076	0.024	0.316
7c	0.059	0.007	0.119

Table 3. Yield of biosurfactants in nutrient broth without hexane

Organism	Dry weight of organism (g)	Weight of biosurfactant (g)	Yield
1a	0.071	0.019	0.268
1b	0.0595	0.042	0.706
1c	0.0144	0.070	4.861
2a	0.069	0.061	0.884
2b	0.079	0.093	0.177
3c	0.027	0.021	0.777
4a	0.051	0.025	0.490
4b	0.032	0.044	1.375
5a	0.0137	0.024	1.752
5c	0.061	0.010	0.164
6a	0.063	0.097	1.539
6b	0.1249	0.138	1.105
6c	0.0273	0.133	4.872
7c	0.077	0.032	0.416

The bacteria screened using Penetration assay, Microplate assay and Blood haemolysis test proved to be positive for biosurfactant production.

For the production of biosurfactants from different bacteria, production medium and nutrient broth with and without hydrocarbon stimulant (hexane) were used. For most of the bacteria, yield of the biosurfactants was found to be higher in the production medium in the presence of hexane and

Table 2. Yield of biosurfactants in production medium with hexane

Organism	Dry weight of organism (g)	Weight of biosurfactant (g)	Yield
1a	0.050	0.011	0.22
1b	0.051	0.004	0.078
1c	0.055	0.010	0.182
2a	0.050	0.005	0.100
2b	0.0152	0.003	0.197
3c	0.013	0.020	1.538
4a	0.004	0.004	1.000
4b	0.059	0.008	0.136
5a	0.015	0.003	0.200
5c	0.0466	0.004	0.086
6a	0.0426	0.005	0.117
6b	0.0404	0.003	0.074
6c	0.0422	0.009	0.213
7c	0.034	0.011	0.324

Table 4. Yield of biosurfactants in nutrient broth with hexane

Organism	Dry weight of organism (g)	Weight of biosurfactant (g)	Yield
1a	0.0921	0.028	0.304
1b	0.0155	0.007	0.452
1c	0.042	0.014	0.333
2a	0.0548	0.039	0.712
2b	0.0396	0.018	0.455
3c	0.1394	0.059	0.423
4a	0.1125	0.055	0.489
4b	0.1037	0.062	0.598
5a	0.091	0.013	0.143
5c	0.071	0.024	0.338
6a	0.100	0.038	0.380
6b	0.087	0.023	0.264
6c	0.065	0.032	0.492
7c	0.085	0.010	0.118



Fig. 5. Emulsification capacity assay

Table 5. Oil displacement zones

Sample	Diameter of Oil displacement zone (mm)
SDS (Control)	35
1a	2
1b	4
1c	7
2a	4
2b	4
3c	5
4a	2
4b	8
5a	4
5c	2
6a	30
6b	60
6c	4
7c	5

in the nutrient broth the yield was higher in the absence of hexane.

Micrococcus luteus (1a), *Serratia liquefaciens* (1b), *Bacillus popilliae* (1c), *Aeromonas* spp. (2a), *Bacillus macquariensis* (2b), *Bacillus azotoformans* (3c), *Staphylococcus* spp. (4a, 4b), *Corynebacterium xerosis* (5a), *Corynebacterium kutscheri* (6a), *Streptococcus mitis* (6b) and *Staphylococcus saprophyticus* (7c) showed higher yield in production medium with hexane. Hexane being an insoluble substrate and the sole carbon source, bacteria produce biosurfactants to facilitate their diffusion into the cell. [1] (Fig 1)

Serratia liquefaciens (1b), *Bacillus popilliae* (1c), *Aeromonas* spp. (2a), *Bacillus azotoformans* (3c), *Staphylococcus* spp. (4a, 4b), *Corynebacterium xerosis* (5a), *Corynebacterium kutscheri* (6a), *Streptococcus mitis* (6b), *Bacillus*

Table 6. Emulsification index of the extracted samples

Sample	Height of the emulsion(mm)			
	Hexane	Kerosene	Hexane	Kerosene
SDS (Control)	2	4	5.405	10.811
1a	2	2	5.405	5.405
1b	1	1	2.703	2.703
1c	2	1	5.405	2.703
2a	2	2	5.405	5.405
2b	2	3	5.405	8.108
3c	1	2	2.703	5.405
4a	2	2	5.405	5.405
4b	2	2	5.405	5.405
5a	4	3	10.811	8.108
5c	2	2	5.405	5.405
6a	1	2	2.703	5.405
6b	3	1	8.108	2.703
6c	2	10	5.405	27.027
7c	2	2	5.405	5.405

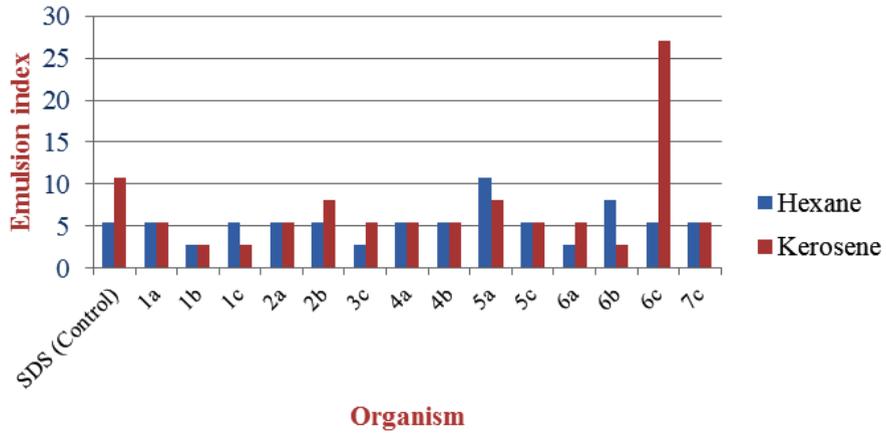


Fig. 7. Emulsification Capacity Assay

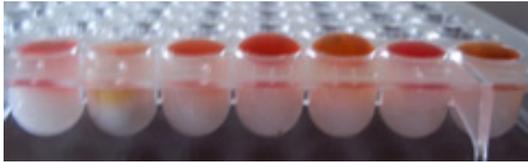


Fig. 8. Penetration assay- 0 min

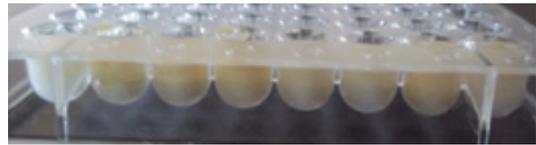


Fig. 9. Penetration assay- 15 min

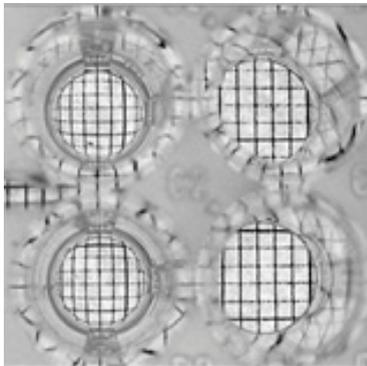


Fig. 10. Microplate assay

pantothenicus (6c) and *Staphylococcus saprophyticus* (7c) showed higher yield in nutrient broth without hexane. This medium consists of complex, soluble carbon and nitrogen sources that can be easily used by the bacteria for biosurfactant production. [25] When hexane is added to the medium, there is secondary metabolite production causing alteration of pH, thereby limiting the biosurfactant production. [9]. (Fig 1)

Exceptions were *Bacillus pantothenicus* (6c) and *Staphylococcus* spp. (5c) which showed higher yield in production medium without hexane and *Micrococcus luteus* (1a), *Bacillus*



Fig 11. α-Haemolysis



Fig. 12. α-Haemolysis

macquariensis (2b) and *Staphylococcus* spp. (5c) which showed higher yield in nutrient broth with hexane. This may be because yield of biosurfactants is also dependent on the organism, type of biosurfactant and the environmental conditions. [10]. The extracted samples were confirmed to be biosurfactants by using oil spreading assay, thin layer chromatography and emulsification capacity assay. The effectiveness of the extracted biosurfactants was estimated using oil spreading assay and emulsification capacity assay. In oil spreading assay, all the biosurfactants showed oil displacement zones. Out of these, the ones from *Corynebacterium kutscheri* (6a), *Streptococcus mitis* (6b) gave displacements of 30 and 60 mm which are considerable with 35 mm displacement given by the SDS control. (Fig 2) Based on emulsification capacity assay, *Corynebacterium xerosis* (5a) and *Bacillus pantothenicus* (6c) showed higher emulsification indices of 10.811 with hexane and 27.027 with kerosene respectively when compared to 5.405 with hexane and 10.811 with kerosene given by the SDS control. (Fig 3)

CONCLUSION

Isolated organisms proved positive for biosurfactant production by using penetration assay, microplate assay and blood haemolysis technique. The biosurfactant yield was compared in production medium and nutrient broth with and without hexane. The yield was found to be higher in production medium with hexane and nutrient broth without hexane for most of the bacteria. The samples extracted were confirmed to be biosurfactants by using thin layer chromatography and measuring their effectiveness in terms of oil displacement zones formed and emulsification indices obtained with respect to that of SDS control.

REFERENCES

1. N. Karanth, P. Deo, and N. Veenanadig, "Microbial production of biosurfactants and their importance," *Current Science*, 1999; **77**(1), pp. 116–126.
2. R. Sen, "Biosurfactants," in *Advances in Experimental Medicine and Biology*, 2010, pp. 3-11.
3. K. B. Cheng, Z. Jian, and D. Z. Wang, "Emulsification properties of bacterial biosurfactants native to the Yellow River Delta on Hexadecane and Diesel Oil," *Biotechnology*, 2008; **7**(2): pp. 360-370.
4. G. Bratbak and I. Dundas, "Bacterial dry matter content and biomass estimations.," *Applied and environmental microbiology*, 1984; **48**(4), pp. 755-757.
5. R. Thavasi, S. Sharma, and S. Jayalakshmi, "Evaluation of Screening Methods for the Isolation of Biosurfactant Producing Marine Bacteria," *Petroleum & Environmental Biotechnology*, 2011; pp. 1-6.
6. R. A. Bayourni, B. M. Haroun, E. A. Ghazal, and Y. A. Maher, "Structural Analysis and Characteristics of Biosurfactants Produced by Some Crude Oil Utilizing Bacterial Strains," *Australian Journal of Basic and Applied Sciences*, 2010; **4**(8): pp. 3484-3498.
7. N. Mathiyazhagan, K. Danashekar, and D. Natarajan, "Amplification of Biosurfactant Producing Gene (rhlB) from *Pseudomonas aeruginosa* isolated from oil contaminated soil," *International Journal of Pharma and Bio Sciences*, **2**(1): pp. 497-504.
8. F. Nunizawati Daud, A. Yahya, M. Md Salleh, N. Suhaimi, and C. Hui Xuan, "Production of Biosurfactant by Locally Isolated Thermophilic Facultatively Anaerobic Bacteria strain B160," *Journal of Kustem*, 2010; pp. 1-5.
9. M. Abouseoud, R. Maachi, A. Amrane, and S. Boudergua, "Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*," *Desalination*, 2008; **223**: pp. 143-151.
10. L. Al-araji, R. N. Z. R. A. Rahman, M. Basri, and A. B. Salleh, "Microbial Surfactant," *Asia Pacific Journal of Molecular Biology and Biotechnology*, 2007; **15**(3), pp. 99-105.