

VOLUME 9

ISSUE 1

2023

ISSN 2454 – 3055



**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

*Forum for Biological and
Environmental Sciences*

Published by Saran Publications, India



Quantitative Analysis of Bio-Surfactants Produced from Bacterial Strains

Chetan D.M.¹, Raghavendra H.L.², Narendra Kumar S.³, Ravikumar Patil H.S.⁴, Ramesh S. Bhat⁵, Venkatesh Kamath H.^{1*} and Venkatesh⁴

¹Department of Biotechnology, NMAM Institute of Technology-Affiliated to NITTE (Deemed to be University), Nitte, Karnataka 574110, India

²Center for Biotechnology, Pravara Institute of Medical Sciences (Deemed to be University), Loni-413736, Rahata Taluk, Ahmednagar District, Maharashtra, India

³Department of Biotechnology R. V. College of Engineering, Bangalore, Karnataka, India

⁴Department of Studies in Food Technology, Davangere University, Shivagangotri Campus, Davanagere, Karnataka 577007, India

⁵Department of Chemistry, NMAM Institute of Technology-Affiliated to NITTE (Deemed to be University), Nitte, Karnataka 574110, India

**Corresponding Author*

Received: 21st February, 2023; **Accepted:** 18th April, 2023; **Published online:** 3rd May, 2023

<https://doi.org/10.33745/ijzi.2023.v09i01.091>

Abstract: Synthetic surfactants are now known to have negative effects on the environment, necessitating technical advancement to eliminate them. It has been determined that the use of bio-surfactants for this purpose is both an environmentally acceptable method and an alternative to a traditional intricate cleanup system. Many different surface-active substances, or "bio-surfactants," are produced by microorganisms. Due to their potential advantages over their synthetic counterparts in numerous domains, including environmental, food, biomedical, and other industrial uses, bio-surfactants are garnering a lot of attention. Seven bacterial strains were obtained from industrial wastewater for this investigation. Two strains were chosen from a group of seven based on their capacity to produce bio-surfactants, colony form and features, and froth production. In two different low salt media, a comparison between the carbon sources sucrose and hexane was conducted.

Keywords: Bio-surfactants, Waste water, Bacteria, Colony Morphology

Citation: Chetan D.M., Raghavendra H.L., Narendra Kumar S., Ravikumar Patil H.S., Ramesh S. Bhat, Venkatesh Kamath H. and Venkatesh: Quantitative analysis of bio-surfactants produced from bacterial strains. Intern. J. Zool. Invest. 9(1): 818-823, 2023.

<https://doi.org/10.33745/ijzi.2023.v09i01.091>



This is an Open Access Article licensed under a Creative Commons License: Attribution 4.0 International (CC-BY). It allows unrestricted use of articles in any medium, reproduction and distribution by providing adequate credit to the author (s) and the source of publication.

Introduction

Surface tension of the aqueous cleaning solution is reduced by synthetic detergents, which are chemicals employed as cleaning agents. Soaps and

detergents fall within the category of substances known as surfactants. Surfactants are compounds that lower the surface tension (or interfacial

tension) and act as wetting agents, emulsifiers, foaming agents, and dispersants (Ana *et al.*, 2017). These substances are amphiphilic compounds which contain both hydrophobic (their *tails*) and hydrophilic groups (their *heads*) and form aggregate structures such as micelles. They have the ability to alter surface and interfacial properties. Reducing the surface tension allows the water to spread out, influencing the degradation of oil droplets or other pollutants (Laurier *et al.*, 2003). Despite all the advantages of surfactants, its release into the environment can be a potential danger due to high usage. Synthetic surfactants are toxic in nature and non-biodegradable.

Biosurfactants are biological compounds; an alternative for synthetic surfactants, which are secondary metabolites synthesised by microbial cells (bacteria, fungi and yeast) (Thando *et al.*, 2017). Its major function is increasing the surface area of hydrophobic substrates which in turn increases the bioavailability of the substrate through solubilisation/desorption. Similar to synthetic surfactants they possess both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities (Vijayakumar and Saravanan, 2015). They control how microbes adhere to surfaces and are cleared away. Microbial Surface-Active Agents, or biosurfactants, have lately grown in importance as a biotechnology product for use in both industrial and medical settings.

Bacterial biosurfactants: Microorganisms involving only bacteria synthesise bio-surfactant by making use of a wide range of carbon source and energy for their growth and development. Some of the bacteria excrete ionic surfactants which emulsify the hydrocarbon substance in the growth medium. Rhamnolipids commonly produced by *Pseudomonas aeruginosa*, cyclic lipopeptide produced by *Bacillus* spp. such as surfactin and subtilisin, that are produced by *Bacillus subtilis*.

Fungal biosurfactants: If fungi are involved for the production of bio-surfactants, which is relatively

less explored than bacterial bio-surfactants. Among fungi, *Candida lipolytica* produces cell wall-bound lipopolysaccharides when grown on n-alkanes. whereas, *Aspergillus* spp. produces large quantities of fatty acid and phospholipids during growth on n-alkanes.

In this study seven bacterial strains were obtained from industrial wastewater. Two strains were chosen from a group of seven based on their capacity to produce bio-surfactants, colony form and features, and froth production. In two different low salt media, a comparison between the carbon sources sucrose and hexane was conducted.

Materials and Methods

To separate the cells after incubation, the production media was centrifuged at 7,000 rpm for 12 min. The supernatant was collected, pH-acidified with 6N hydrochloric acid to 2, and incubated for an overnight period at 4°C. The desired product precipitates as a result, and the following day it is separated by centrifuging at 10,000 rpm for 20 min. The pellets are then put back into 0.1M sodium bicarbonate suspension. After dissolving, the solution is transferred to a separate funnel where it is extracted with an equal volume of a 2:1 chloroform and ethanol combination. Two further times of re-extraction were carried out. Separate collections of the lower organic and higher aqueous phases are made, and they are both stored for later evaporation at 65°C in a water bath. The ultimate result of the evaporation has been produced.

Estimation Methods:

Sample preparation:

Biosurfactant estimation was done using qualitative and quantitative tests. Sample dissolved in chloroform is evaporated initially and then used for testing.

Quantitative tests:

(a) Anthrone Test (for total sugar estimation):

Starches are dried out with focused H₂SO₄ to

Table 1: Anthrone reagent method (Total Sugars estimation)

TUBE NO.	VOL. OF MALTOSE (ml)	VOL. OF DISTILLED WATER (ml)	ANTHRONE (ml)	Incubation in boiling water bath at 100°C for 10 min	OD 600 nm	CONC. (mg/ml)
1	0.0	1.0	3.0		0.0	0.0
2	0.02	0.98	3.0		0.21	0.031
3	0.04	0.96	3.0		0.29	0.043
4	0.06	0.94	3.0		0.39	0.059
5	0.08	0.92	3.0		0.50	0.075
6	0.10	0.90	3.0		1.12	0.169

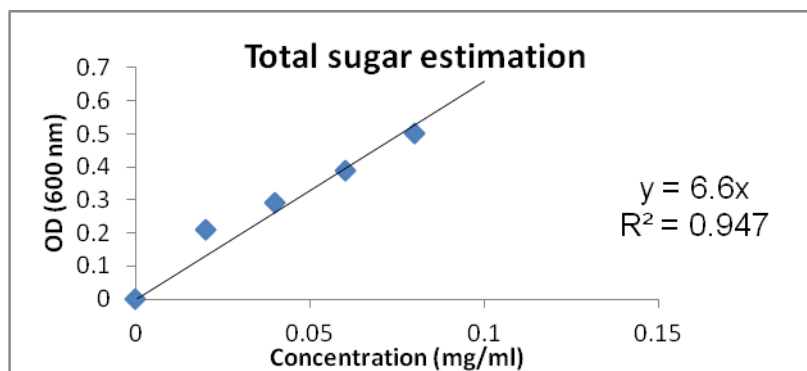


Fig 1: Standard graph for Total sugar estimation.

frame "Furfural", which gathers with anthrone to form an inexperienced shading complex that can be measured colorimetrically at 620 nm. In check tubes, 0.0, 0.02, 0.04, 0.06, 0.08, and 0.1 ml of maltose were added and made up to 1 ml with distilled water. To the above solution 3 ml of Anthrone reagent (Anthrone dissolved in focused H_2SO_4) added (Table 1). Samples were incubated at 100°C for 10 min. After incubation optical density was measured at 620 nm.

(b) Phospho Vanillin test (for lipids estimation):

Strong sulfuric acid and strong phosphoric acid react with a variety of unsaturated lipids to form colored and stable salts. The reaction between lipids and phosphoric acid results in a color change for vanillin. Strong carbon ionic reagents like phosphoric acid increase the reactivity of the carbonyl groups. The carbon cation reacts with these carbonyl groups to the stock solution; protocol (Hatha *et al.*, 2007) was applied. Since the standard solution was used at different

concentrations ranging 0.02 M, Infinity dilution was used for determination. The tests were made 0.02 M oleic acid solution which provided 16.8 $\mu\text{g/ml}$ oleic acid. Then, 2 ml standard solution was diluted and 0.5 ml solution was added into known test tubes. 2 ml of H_2SO_4 was then added to each solution. 2 ml of phospho-vanillin solution was added and kept into the water bath at 37°C for 15 min then optical density was measured.

(c) Confirmatory Test for Glycolipid:

To confirm the presence of glycolipid, 1 ml of the lower layer from the extraction layers of all the samples was collected and 3-4 drops of acetone was added. If there is precipitation, presence of glycolipids is confirmed.

Quantitative Tests:

Anthrone Reagent Method (Total Sugars estimation):

Potentiometric titration is the measurement of the equivalence – the amount of solution needed to

Table 2: Concentration of total sugars in biosurfactants produced by C5 and C7

Sample	Concentration (mg/ml)		
	Sucrose	Coconut oil	Waste cooking oil
C5	0.056	0.061	0.033
C7	0.065	0.086	0.054

Table 3: Phospho vanillin method (Lipid estimation)

TUBE NO.	VOL. OF STD. (ml)	C ₂ H ₅ OH (ml)	H ₂ SO ₄ (ml)	Incubation at room temp. for 10 min	PHOSPHO-VANILLIN (ml)	Water bath for 15 min	OD (540 nm)	CONC. (mg/ml)
1	0.0	2.0	2.0		2.0		0.0	0.0
2	0.2	1.8	2.0		2.0		0.35	0.330
3	0.4	1.6	2.0		2.0		0.52	0.490
4	0.6	1.4	2.0		2.0		0.65	0.613
5	0.8	1.2	2.0		2.0		0.88	0.830
6	1.0	1.0	2.0		2.0		0.96	0.905

saturate the solution. The linear relationship graph was obtained by plotting the measured concentration of the sample on the x-axis, and the measured absorbance on the y-axis. The regression coefficient of 0.947 was obtained (Fig. 1).

Phospho Vanillin Method (Lipid Estimation):

The concentration of lipid was estimated by using Phospho-Vanillin method with the olive oil (1 mg/ml) as stock solution. The regression coefficient of 0.937 was obtained on plotting the graph by taking a concentration in the x-axis and OD in y-axis (Fig. 2).

Results and Discussion

From the qualitative and quantitative tests performed it was inferred that the biosurfactant molecule produced and extracted from the

bacterial strains C5 and C7 is a glycolipid.

An experimentation was done by the addition of 0.3% (v/v) coconut oil and 0.3% (v/v) waste cooking oil separately into the MSM containing 0.4% (w/v) sucrose. The addition of these oils induced the C5 and C7 bacteria to produce more amount of biosurfactants (Table 2). The drop collapse test conducted showed the best result for the sample C7 inoculated in 0.3% (v/v) coconut oil. BATH assay result showed maximum hydrophobicity in the sample C7 in 0.4% (w/v) sucrose. Highest emulsification index value was obtained for C7 in 0.3% (w/v) coconut oil. These results suggest that the biosurfactant production was increased with the addition of an inducer and that C7 has a better biosurfactant production capacity than C5 in the specified medium and conditions.

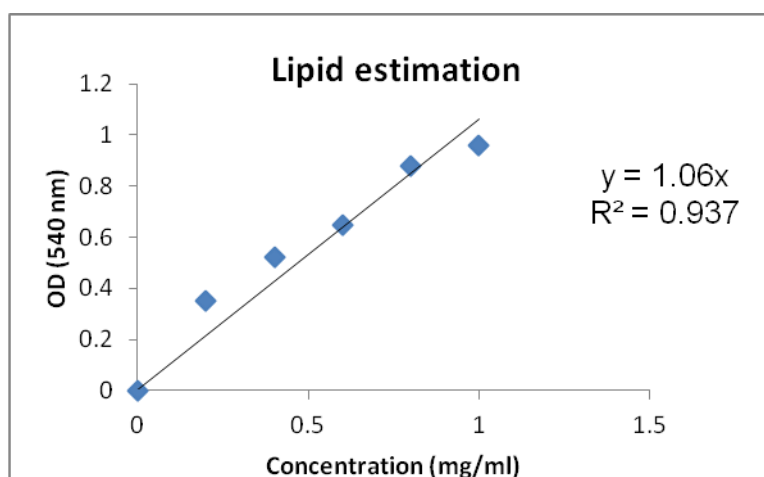


Fig. 2: Standard graph for lipid estimation.

Table 4: Concentration of lipids in biosurfactants produced by C5 and C7

Sample	Concentration (mg/ml)		
	Sucrose	Coconut oil	Waste cooking oil
C5	0.194	0.242	0.09
C7	0.271	0.465	0.11



Fig. 3: Glycolipid precipitated at the bottom.

A comparative study was conducted by adding 0.4% (w/v) hexane to the MSM, as the carbon source for C5 and C7. Hexane proved to be an inhibitor to the bacterial strains and hence no biosurfactant was extracted (Table 4).

The type of biosurfactant produced by C5 and C7 in 0.4% (w/v) sucrose-containing MSM, 0.3% (w/v) coconut oil as inducer in 0.4% (w/v) sucrose-containing MSM and 0.3% (w/v) waste cooking oil as inducer in 0.4% (w/v) sucrose-containing MSM was determined by Qualitative and Quantitative tests. Molisch test and Sudan Black test gave positive results indicating that the biosurfactant is a glycolipid (Table 3). This was verified by estimation of total sugars and estimation of lipids by Anthrone reagent method and Phospho vanillin method respectively. Further confirmation of the produced biosurfactant to be a glycolipid was done by the precipitation of glycolipid on the addition of acetone (Fig. 3).

Conclusion

Emulsification index assay results proved C7 showing a maximum percentage of 13.33 in MSM containing coconut oil as an inducer. On conducting qualitative and quantitative tests it was proven that the biosurfactants produced from both C5 and C7 is a glycolipid. Further, it was proven that MSM contained sucrose with 0.3% (w/v). Coconut oil is a better inducer for biosurfactant production than MSM containing sucrose with 0.3% (w/v) waste vegetable oil.

References

- Ana CK, Beatriz GC, Leandro GDC and Hérica RNS. (2017) Synthetic detergents: 100 years of history. Saudi Pharmaceut J. 25(6): 934-938.
- Asselineau C and Asselineau J. (1978) Trehalose - containing glycolipids. Prog Chem Fats Lipids 16: 59-99.
- Edwards JR and Hayashi JA. (1965) Structure of a rhamnolipid from *Pseudomonas aeruginosa*. Arch Biochem Biophys. 111: 415-421.
- Gautam KK and Tyagi VK. (2006) Microbial surfactants: A review. J Oleo Sci. 55: 155-166.
- Hatha AAM, Edward G and Rahman KSMP. (2007) Microbial biosurfactants-review. J Mar Atmos Res. 3: 1-17.
- Jarvis FG and Johnson MJ. (1949) A glycolipid produced by *Pseudomonas aeruginosa*. J Am Chem Soc. 71: 4124-4126.
- Laurier LS, Elaine NS and Gerrard DM. (2003) Surfactants and their applications. Annu Rep Prog Chem. 99: 3-48.
- Marcia N, Cristina F and Gláucia MP. (2004) Selection of microorganisms for biosurfactant production using agroindustrial wastes. Brazilian J Microbiol. 35(1-2): 81-85.
- Rosenberg E and Ron EZ. (1999) High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol. 52: 154-162.
- Thando N, Marina R, Johann AV, Sehaam K and Wesaal K. (2017) Characterisation and antimicrobial activity of biosurfactant extracts produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* isolated from a wastewater treatment plant. AMB Express 7: 108.
- Vijayakumar S and Saravanan V. (2015) Biosurfactants-types, sources and application. Res J Microbiol. 10(5): 181-192.