Short Communication

Substrate dependent production of extracellular biosurfactant by a marine bacterium

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ABSTRACT

The potential of a marine microorganism to utilize different carbon substrates for the production of an extracellular biosurfactant was evaluated. Among the several carbon substrates tested for this purpose, production of the crude biosurfactant was found to be highest with glycerol (2.9 ± 0.11 g L^{-1}) followed by starch (2.5 ± 0.11 g L^{-1}), glucose (1.16 ± 0.11 g L^{-1}) and sucrose (0.94 ± 0.07 g L^{-1}). The crude biosurfactant obtained from glycerol, starch and sucrose media had significantly higher antimicrobial action than those obtained from glucose containing medium. RP-HPLC resolved the crude biosurfactants into several fractions one of which had significant antimicrobial action. The antimicrobial fraction was found in higher concentrations in biosurfactant obtained using glycerol, starch and sucrose as compared to the biosurfactants from glucose medium, thereby explaining higher antimicrobial activity. The carbon substrate was thus found to affect biosurfactant production both in a qualitative and quantitative manner.

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1. Introduction

Biosurfactants are microbially produced surface-active agents and occur in nature as chemical entities such as glycolipids, phospholipids and lipopeptides. These molecules have attracted considerable scientific attention due to lower toxicity, higher biodegradability, activity at extremes of temperature, pH and salinity and possibility of their production through fermentation using cheap agro-based substrates (Desai and Banat, 1997). Apart from their potential applications in environmental protection and management and crude oil recovery, these molecules have also emerged as potential agents in health care and food processing industries (Desai and Banat, 1997). In recent years, these molecules were found to posses several interesting properties of therapeutic and biomedical importance (Singh and Cameotra, 2004; Das et al., 2008a). In spite of their important biological activities, the main bottlenecks in their commercialization are their low productivities and high production cost. Different strategies including the use of inexpensive substrates have been suggested towards making their production economically viable (Mukherjee et al., 2006).

Carbon substrate is an important limiting factor affecting the production of microbial surfactants (Sen, 1997). The type of carbon substrate used for production has been reported to influence both the quality and quantity of biosurfactants (Robert et al., 1989; Panilaitis et al., 2007; Abouseoud et al., 2008). Microbes produce biosurfactants as a mixture of various isoforms. These isoforms vary in the carbohydrate and peptide part or in the chain length or branching of the lipid part of the molecule (Robert et al., 1989; Déziel et al., 1999; Vater et al., 2002; Mukherjee and Das, 2005; Costa et al., 2006; Perfumo et al., 2006). It was reported earlier that specific biosurfactant isoforms confer some kind of competitive advantage to the producer strains in their parent habitats such as utilization of hydrophobic substrates and/or antibiotic action against competing microorganisms (Mukherjee and Das, 2005). In the present study, potential capabilities of a marine strain of Bacillus circulans to uptake various carbon substrates for biosurfactant production was investigated. The results showed that carbon substrate affected the biosurfactant production process both qualitatively and quantitatively. The accumulation of the antimicrobial biosurfactant fraction in the culture broth was found to be dependent on the carbon substrates in terms of the levels of biomass and biosurfactant production, surface tension reduction, emulsification index and antimicrobial action.

2. Methods

2.1. Microorganisms, media composition and culture conditions

A marine microorganism isolated from a water sample of Andaman and Nicobar Islands, India was used in this study (Das et al., 2008a, b). Zobell marine broth (HiMedia, Mumbai, India) was used for culture maintenance and preparation of the inocula. For biosurfactant production, a slightly modified mineral salts medium was used (Das et al., 2008a, b). Apart from glucose, different other

carbon substrates like sucrose, lactose, starch, sodium gluconate, glycerol, sodium acetate, sodium carbonate, sodium oxalate, lactic acid, acetic acid, trisodium citrate, yeast extract, beef extract and oily substrates such as kerosene, hexadecane, mustard oil, sunflower oil and rice oil were evaluated for their potential to support growth and biosurfactant production. The amount of the carbon source added to the mineral salts medium was 2% (w/v or v/v). An uninoculated control flask was prepared for each carbon source for the measurement of the initial surface tension. The media were inoculated with a mid-log phase inoculum ($OD_{600} = 2.3–2.5$; 10^8 cfu ml^{-1}) at 1% (v/v). To monitor bacterial growth, absorbance of the culture broth was measured at 600 nm. Stable foaming coupled with the reduction in surface tension of the media was considered as a qualitative indicator of biosurfactant production. All the fermentations were performed under normal atmospheric conditions at 37 °C with shaking speed of 180 rpm.

2.2. Study of growth and biosurfactant production

The growth and biosurfactant production were studied in the screened carbon sources mineral salts media – glucose (GMSM), sucrose (SMSM), starch (STMSM) and glycerol (GLYMSM). These studies were carried out in 1000 ml flasks with 200 ml working volume. Fermentation broth samples were collected every alternate hour and checked for bacterial growth, surface tension and biosurfactant concentration. Biomass was measured by dry weight method and also by optical density of the fermentation broth at 600 nm ($OD_{600\ nm}$). $OD_{600\ nm}$ was measured with a UV–Visible spectrophotometer (Perkin-Elmer, MA, USA). In these experiments, timely variations in surface tension and critical micelle dilutions (CMD^{-1} and CMD^{-2}) were measured with the help of a DCAT-11 digital surface-tensiometer (Dataphysics, Instruments, Filderstadt, Germany). CMD^{-1} and CMD^{-2} were determined by diluting the free fermentation broth ten and hundred times, respectively, followed by surface tension measurements (Joshi et al., 2008). Emulsification was measured by the method reported earlier (Cooper and Goldenberg, 1987; Das et al., 2008b). Briefly, equal volumes of culture supernatant and hydrocarbons (kerosene, petrol, diesel or hexadecane) were mixed and vortexed at high speed for 5 min followed by incubation at 25 °C for 24 h. The emulsification index value (E_{24}) was then calculated using the formula:

$$E_{24} = (\text{Height of emulsion layer/Height of the total mixture}) \times 100$$

Biosurfactant concentration was measured by quantitative thin layer chromatographic analysis described later.

2.3. Isolation and partial purification of surface-active molecules

The extracellular surface-active molecules produced in each of the media were isolated chemically by acidification of the cell free broth and precipitation followed by solvent extraction of the acid precipitate as reported earlier (Das et al., 2008a). The methanol extracts were dissolved in a minimum quantity of water and lyophilized to obtain partially purified biosurfactants. These partially pure biosurfactants obtained from different carbon substrates were stored at −20 °C and were used in further experiments.

2.4. Biochemical characteristics

The chemical nature of the biosurfactants was determined with TLC. The biosurfactant was spotted in triplicate on readymade silica gel TLC plates (Merck, Darmstadt Germany). After development in the suitable solvent system described later, one of the plates was put into a jar saturated with iodine vapors to detect lipids. Another plate was sprayed with rhodamine B reagent (250 mg rhodamine B in 100 ml absolute alcohol) and visualized under UV to confirm the presence of lipids. The third plate was sprayed with ninhydrin reagent (0.2% ninhydrin solution in acetone) and dried. It was then heated at 120 °C for detection of peptides. The chemical nature of the biosurfactants was further confirmed by FTIR spectroscopy described later.

2.5. Antimicrobial action

Minimum inhibitory concentration (MIC) of the biosurfactants from all the viable substrates for the bacterial strains was done by broth microdilution assay (Das et al., 2008a). Overnight grown cultures of the bacterial test strains (*Escherichia coli*, *Micrococcus flavus*, *Proteus vulgaris* and *Staphylococcus aureus*) were used to inoculate 96-well microtiter plates containing diluted biosurfactants (1.0–0.01 mg ml^{-1}) in Mueller Hinton Broth (MHB; HiMedia, Mumbai, India). These tests were performed with negative control (only MHB), positive control (MHB + test organism) and sterility control (MHB + biosurfactant). Plates were incubated at 37 °C for 24 h. Bacterial growth was monitored by measuring $OD_{600\ nm}$ with the help of a microtiter plate reader (Technocorp, Multiskan, Waltham, MA, USA). The minimum concentrations of biosurfactant at which more than 90% growth was inhibited were considered as MICs expressed in mg ml^{-1}. MIC determination for fungal strains (*Aspergillus niger* and *Candida albicans*) was done on potato dextrose agar plates using the agar cup diffusion method. The plates were incubated at 28 °C for 72 h and then checked for growth inhibition. The minimum concentration of biosurfactants inhibiting the growth was considered as MICs.

2.6. Analytical procedures

2.6.1. High performance thin layer chromatography

Different hour fermentation samples were spotted on TLC plates (Merck, Darmstadt Germany) and were developed in a solvent system containing chloroform, methanol and water in ratio 65:25:4, respectively. After development, a densitometric scan was performed for detection and quantification of the biosurfactant (Das et al., 2008a, b).

2.6.2. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used for the molecular characterization of the biosurfactant. Lyophilized samples were analyzed by KBr pellet method (Das et al., 2008a).

2.6.3. High performance liquid chromatography

Reverse phase high performance liquid chromatography (RP-HPLC) was performed with a Zorbax Eclipse reverse phase column [C_{18}, 5 μm, 4.6 (ID) × 250 mm (L)] on an Agilent 1100 series HPLC instrument equipped with a diode array detector (DAD) system. The elution was done with a 5–95% gradient of acetonitrile (0.1% TFA) and water for 60 min at a flow rate of 0.2 ml min^{-1}.

3. Results

3.1. Screening of carbon substrates

Different water miscible and immiscible carbon substrates (Table 1) were screened for their capacity to support growth and biosurfactant production by the strain of *B. circulans* used in this work. Water-soluble substrates like glucose, sucrose, starch and glycerol were found to support both growth and biosurfactant production. Other substrates such as sodium gluconate, trisodium citrate, yeast extract and beef extract supported growth whereas biosurfactant

Table 1
Screening of carbon substrates as supporter of growth and biosurfactant production

Carbon substrate	Biomass (gm L^{-1})	Biosurfactant (gm L^{-1})
Glucose	3.56 ± 0.20	1.16 ± 0.11
Sucrose	2.50 ± 0.10	0.94 ± 0.07
Starch	3.40 ± 0.05	2.5 ± 0.11
Glycerol	5.07 ± 0.06	2.9 ± 0.11
Sodium gluconate	2.76 ± 0.30	0.25 ± 0.05
Tri-sodium citrate	0.46 ± 0.15	ND
Yeast extract	1.03 ± 0.15	0.12 ± 0.02
Beef extract	0.83 ± 0.05	0.11 ± 0.02

ND, not detected.

production was insignificant or absent. Carbon substrates such as lactose, sodium acetate, sodium carbonate and sodium oxalate neither supported growth nor the biosurfactant production. Similarly, hydrocarbon and oily substrates such as kerosene, hexadecane, mustard oil, sunflower oil and rice oil did not support growth or biosurfactant production.

3.2. Time course of growth and biosurfactant production

The growth and biosurfactant production by this marine microorganism was monitored in Glucose mineral salts medium (GMSM), Sucrose mineral salts medium (SMSM), Starch mineral salts medium (STMSM) and Glycerol mineral salts medium (GLYMSM). The microorganism actively produced biosurfactants in the exponential growth phase in all these media. The exponential phase of this microorganism was completed within 36 h in glucose and sucrose containing media, while the same extended to more than 60 h in media containing glycerol and starch as carbon substrates (Fig. 1). Although biosurfactant production began as early as 12 h in both GMSM and SMSM as seen from the reduction in surface tension, significant concentration of biosurfactant was achieved only after about 14–16 h of fermentation which continued up to the 26–28 h. On the other hand, the biosurfactant production in GLYMSM and STMSM began around 20–24 h but continued almost up to 60 h (Fig. 1). The least surface tension values recorded for all the media at the end of fermentation was 27 mN m^{-1} (Table 2). Starting with the same percentage of carbon substrate, comparatively higher cell densities were achieved in medium containing starch and glycerol (STMSM and GLYMSM) than in media containing glucose and sucrose (GMSM and SMSM). CMD^{-1} and CMD^{-2} values at the end of the growth phase were also lower for the cell free spent media containing glycerol and

Table 2
Comparison of various physicochemical properties of biosurfactants obtained using different carbon substrates

Property	GMSM	SMSM	STMSM	GLYMSM
Initial surface tension	67.12 ± 0.03	64.31 ± 0.02	56.34 ± 0.01	64.13 ± 0.01
Final surface tension	28.10 ± 0.03[a]	29.31 ± 0.01[a]	27.72 ± 0.02[b]	27.10 ± 0.02[b]
CMD^{-1}	29.33 ± 0.02[a]	31.32 ± 0.01[a]	28.80 ± 0.02[b]	28.00 ± 0.01[b]
CMD^{-2}	35.13 ± 0.02[a]	41.23 ± 0.03[a]	32.76 ± 0.02[b]	30.02 ± 0.01[b]
Final biomass (g L^{-1})	3.56 ± 0.20[a]	2.5 ± 0.1[a]	3.4 ± 0.05[b]	5.07 ± 0.06[b]
Biosurfactant (g L^{-1})	1.16 ± 0.11[a]	0.94 ± 0.07[a]	2.5 ± 0.11[b]	2.9 ± 0.11[b]
Productivity (g L^{-1} h^{-1})	0.032[a]	0.026[a]	0.024[a]	0.028[a]
E_{24} values kerosene	65	70	80	85
Petrol	50	45	65	70
Diesel	70	70	75	80
Hexadecane	70	75	75	80

GMSM, glucose mineral salts medium; SMSM, sucrose mineral salts medium; STMSM, starch mineral salts medium; GLYMSM, glycerol mineral salts medium.
[a] At the end of 36 h of fermentation.
[b] At the end of 60 h of fermentation.

starch in comparison to spent media containing glucose and sucrose as carbon source (Table 2). This indicated a higher concentration of biosurfactant being accumulated in media containing glycerol and starch as carbon substrates. Emulsification power of the biosurfactant obtained from glycerol and starch was higher than that of the biosurfactant obtained from glucose and sucrose evident from E_{24} values (Table 2). This was also due to the higher concentration of the biosurfactant in the former. Emulsification index has been reported to be proportional to the surfactant concentration till the CMC (critical micellar concentration) value was reached thereby supporting this observation (Cooper and Goldenberg, 1987; Chen et al., 2007). In spite of being produced in lower amounts, emulsification activity of the biosurfactant produced in SMSM was higher than biosurfactants produced in GMSM. As the production of biosurfactant in this strain is growth associated, the higher biosurfactant concentration in starch and glycerol media can be explained by the extended growth phase in these media. The crude biosurfactants showed significant antimicrobial action against the Gram positive and Gram negative bacteria and fungal strains tested. As observed from the MIC values of the biosurfactants against the test organisms, the antimicrobial action was more pronounced and significantly higher in biosurfactants from glycerol, starch and sucrose as carbon substrate in comparison to media containing glucose as the carbon substrate (Table 3). It has been reported that there might be less correlation between biosurfactant yield and its activity (Youssef et al., 2005).
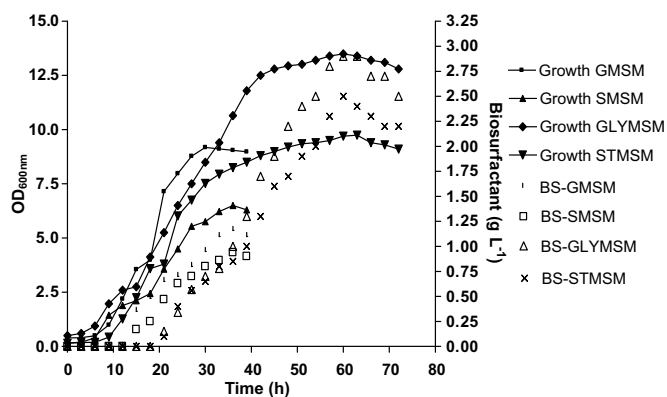

Fig. 1. Growth profiles of *B. circulans* in mineral salts medium containing the carbon sources that supported good growth and biosurfactant production. The same figure also shows biosurfactant production with time.

Table 3
Antimicrobial actions of biosurfactant from different carbon substrates on few bacterial and fungal strains

Test organism	Minimum inhibitory concentrations (µg/ml)			
	BS-GMSM	BS-SMSM	BS-STMSM	BS-GLYMSM
Bacterial strains[a]				
E. coli	200	100	50	50
M. flavus	400	300	350	300
P. vulgaris	200	100	50	50
S. aureus	200	100	50	50
Fungal strains[b]				
A. niger	300	200	250	200
C. albicans	450	250	100	100

BS-GMSM, biosurfactant from glucose mineral salts medium; BS-SMSM, biosurfactant from sucrose mineral salts medium; BS-STMSM, biosurfactant from starch mineral salts medium; BS-GLYMSM, biosurfactant from glycerol mineral salts medium.
[a] MIC determined by broth microdilution assay.
[b] MIC determined by agar diffusion method.

In the present study, although the crude yield of the biosurfactants in the sucrose medium is lowest it has higher emulsification and antimicrobial action than the biosurfactant from the glucose medium.

3.3. Biosurfactant purification and biochemical analysis

The chemical nature of the biosurfactant was revealed with the help of post-chromatographic detection after TLC. After spotting and development in the suitable solvent system (Das et al., 2008a), the plate kept into the jar saturated with iodine vapors showed yellow spots denoting the presence of lipids. On rhodamine-B solution treatment, the same spots showed fluorescence under UV confirming the presence of lipids. Violet spots showing amino acid residues were developed at the same locations after treatment with 0.2% ninhydrin solution and subsequent heating at 120 °C. These results thereby indicated that the surface-active compounds were lipopeptide in nature. The comparison of FTIR

spectra of the biosurfactant obtained using four different carbon substrates indicated that the biosurfactant produced in all these media were lipopeptide in nature (Das et al., 2008a).

3.4. Enhanced production of the active biosurfactant fraction

The solvent extracted biosurfactant obtained from various carbon sources were further resolved using RP-HPLC. The chromatograms showed major six biosurfactant fractions eluting approximately at retention times: 9.94, 15.1, 36.28, 37.9, 38.4 and 40.7 min, respectively, in all the samples (Fig. 2). All these fractions had surfactant property as they reduced the surface tension of water to a lesser or greater extent. Among these fractions, fraction 6 (retention time: 40.7 min) showed the highest surface tension reduction activity and reduced the surface tension of pure water to ~28 mN m^{-1}. Other biosurfactant fractions i.e., fractions 1–5 showed the surface tension values of 33, 50, 39, 35 and 37 mN m^{-1}, respectively. Interestingly, fraction 6 was the only ma-
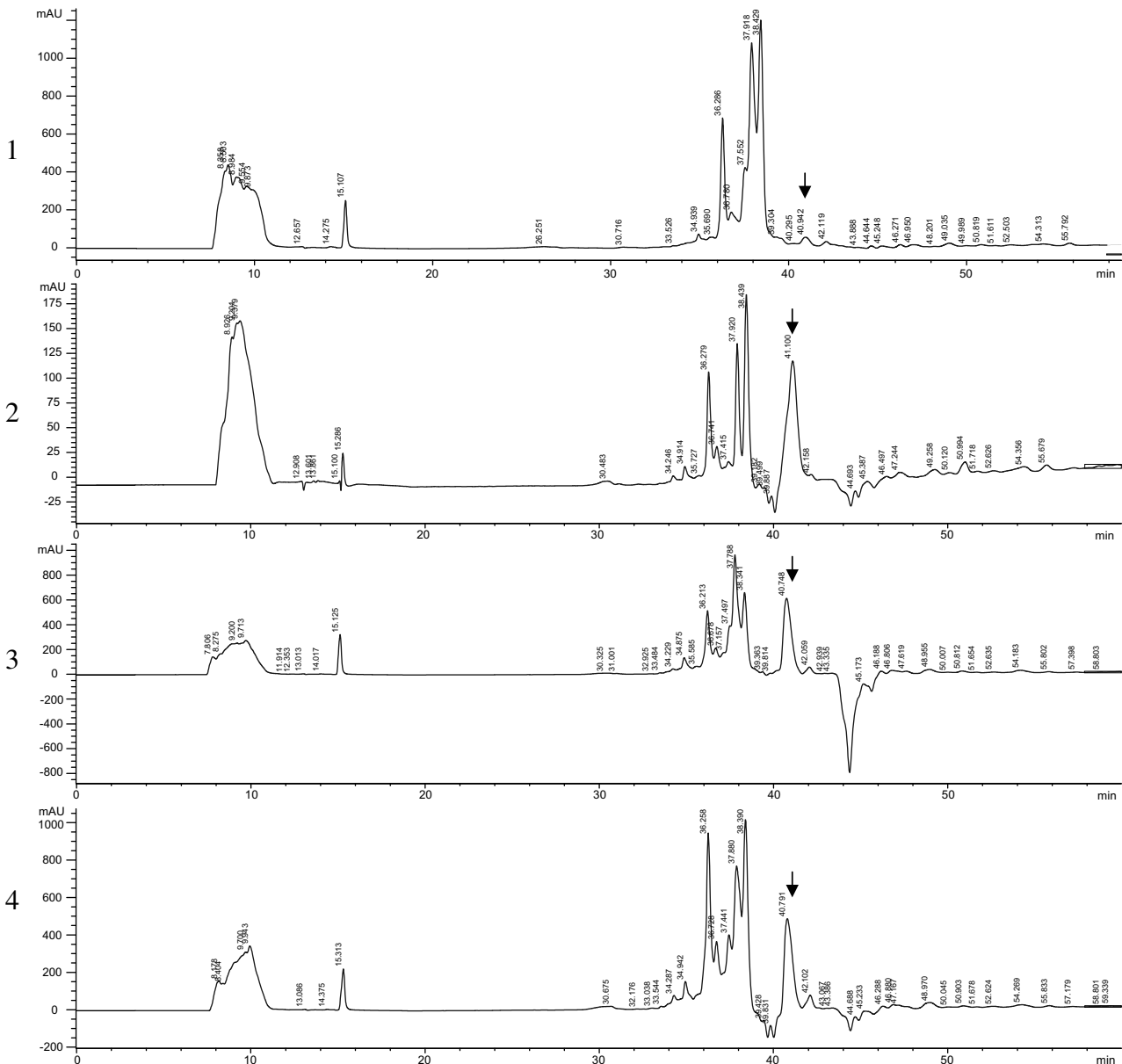

Fig. 2. Reverse phase high performance liquid chromatography (RP-HPLC) of the rude biosurfactant from four carbon sources compared. Biosurfactants from (1) GMSM; (2) SMSM; (3) STMSM; (4) GLYMSM. The antimicrobial fraction has been indicated by an arrow.

jor biosurfactant fraction (Retention time: 40.7 min) showing significant antimicrobial action. A comparative analysis of the HPLC chromatograms showed that higher concentration of this biologically active fraction was produced in media using glycerol and starch as carbon substrates. This was also reflected in the antibiotic activity tests (Table 3). However it was comparatively less in sucrose medium followed by glucose medium, which contained the least amount of this fraction. Hence, the influence of carbon substrate was both qualitative and quantitative in nature. Although the crude biosurfactant yield was least using sucrose as carbon source, the concentration of the bioactive fraction was greater than glucose. The strong antimicrobial action of the biosurfactants from glycerol, starch and sucrose medium compared to glucose medium can thus be explained as a result of higher concentration of the biologically active fraction in these media.

The carbon substrates that enhanced the production of the antimicrobial fraction can be obtained from cheaper alternatives. Glycerol is a by product of the upcoming biodiesel industry, while starch is found in many agro-industrial waste products. Similarly, sucrose can be obtained from the molasses and effluent of the sugar processing industry. Thus, these wastes can be diverted for economic production of biosurfactants with therapeutic properties. Production of these valuable and new antimicrobial molecules can be an answer to the gradually uprising problem of microbial infections and antimicrobial resistance among pathogenic organisms.

4. Conclusion

The qualitative and quantitative effect of the carbon substrate on biosurfactant production by the marine *B. circulans* was evident from the difference in the yield and the antimicrobial action of the biosurfactants. The differential level of production of the biologically active fraction using these carbon substrates was evident from HPLC studies. Cheaper alternatives and agro-based materials containing these substrates which promote the production of the biologically active fraction may be preferred for the economical production of these valuable molecules for therapeutic purposes.

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