CHAPTER 6
PRODUCTION AND CHARACTERIZATION OF
BIOSURFACTANTS FROM BACTERIAL ISOLATES

6.1 ABSTRACT

The production of biosurfactant was carried out using RT9 (*Pseudomonas aeruginosa*), RT19 (*Bacillus siamensis*) and RT21 (*Bacillus subtilis* subsp. *inaquosorum*). For extraction of biosurfactant, the isolate was cultured in MSM with diesel as the sole carbon source. The biosurfactant was extracted using the method of acid precipitation. The crude biosurfactant precipitate was extracted with chloroform-ethanol mixture and concentrated from the organic phase using a rotary evaporator. Solvents were evaporated leading to a viscose honey-brown colored biosurfactant. For studying the kinetics, *B.siamensis* was grown using powdered garlic peel, as the sole carbon source. It was observed that growth kinetics followed the Logistic Model. Biosurfactant production kinetics followed Logistic-incorporated Leudeking-Piret Model. *B.subtilis* subsp. *inaquosorum* was grown using powdered garlic peel, as the sole carbon source. It was observed that growth and biosurfactant production kinetics followed the Logistic Model and Logistic-incorporated Leudeking-Piret Model, respectively. Similar results were obtained when *P.aeruginosa* was grown using powdered garlic peel, as the sole carbon source.

The biosurfactant samples from the three isolates were subjected to thin layer chromatography. The plate was developed with a solvent system consisting of chloroform, methanol and water. To understand the overall chemical nature of the extracted biosurfactant, Fourier transform infrared spectroscopy was employed. The $^1$H NMR spectra of the samples taken in deuterochloroform were acquired on an NMR spectrometer. Stability studies were done using the 24-hr cell-free culture broth. The stability of the biosurfactant was inferred by
observing the change in emulsification index and surface tension, when three parameters were altered: temperature (between 4°C & 121°C), pH (between 2 & 12) and salinity (between 1% & 20% w/v).

6.2 INTRODUCTION

The production of biosurfactants by *Bacillus* sp. has been extensively studied and characterized by many researchers. One of the most well researched and reported biosurfactant is a cyclic lipopeptide, surfactin, which has most often been associated with *Bacillus subtilis*. Other *Bacillus* sp. that are known to be associated with biosurfactant production include *B.circulans*, *B.licheniformis*, *B.cereus*, *B.pumilus*, *B.sphaericus*, *B.brevis*, *B.polymyxa* and *B.megaterium*, amongst others. In the current study, one of the isolates, RT19, was identified as *B.siamensis* by 16S rRNA sequencing. To the best of the author’s knowledge, the strain has not been reported to produce a biosurfactant. This is the first report that discusses the production of a lipopeptide biosurfactant using this strain. Fermentation experiments are carried out using a novel and cheap carbon source. The extracted biosurfactant has been characterized and shown to be similar to the well-known surfactin. Kinetics of growth and biosurfactant production has also been discussed.

A cassava flour-processing effluent was evaluated as a substrate for biosurfactant production by two *B.subtilis* strains (Marcia Nitschke and Glaucia Maria Pastore, 2004). *B.subtilis* ATCC 21332 reduced the surface tension of the medium to 25.9 mN/m, producing a crude biosurfactant concentration of 2.2 g/L. The wild-type strain, *B.subtilis* LB5a, produced corresponding values of 26.6 mN/m and 3 g/L. The biosurfactant produced on cassava effluent medium by *B. subtilis* LB5a was similar to surfactin.

Biosurfactant production by some bacterial isolates using molasses, milk whey and cassava flour wastewater as substrates was evaluated and compared with the production in
conventional medium (Marcia Nitschke et al., 2004). Isolates growing in cassava effluent medium decreased the surface tension around 42%, the highest reduction among all the substrates tested that gave surface tension of about 26 mN/m were identified were Bacillus isolates. In a study by Freitas de Oliveira et al., 2013, B. subtilis LAMI005 was capable to grow and to produce biosurfactant in an alternative culture media, prepared by using clarified cashew apple juice, an agroindustrial by-product of the cashew industry, supplemented with ammonium sulfate.

*Pseudomonas aeruginosa* PACL strain was isolated from oil-contaminated soil (de Lima et al., 2009) and was used to investigate the efficiency and magnitude of biosurfactant production, using different waste frying soybean oils. At optimum levels of aeration rate and agitation speed, a maximum rhamnose concentration of 3.3 g/L, an emulsification index of 100%, and a minimum surface tension of 26 mN/m were achieved. Waste soybean oils resulted in biosurfactant production of 75–90% of the maximum value (achieved using unused soybean oil). The kinetic behavior and the modeling of the rhamnose production, nutrient consumption, and cellular growth were established.

Thavasi et al., 2011, dealt with the production and characterization of biosurfactant from renewable resources by *Pseudomonas aeruginosa*. Biosurfactant production was carried out using waste motor lubricant oil and peanut oil cake. Maximum biomass (11.6 mg/mL) and biosurfactant production (8.6 mg/mL) occurred with peanut oil cake at 120 and 132 h respectively. Characterization of the biosurfactant revealed that, it is a lipopeptide. Results obtained in the present study showed the possibility of biosurfactant production using renewable, relatively inexpensive and easily available resources.
Considering the regional significance, easy availability and the economic advantages, waste frying coconut oil was used as a cost-effective carbon source in the fermentative production of biosurfactants (Seba George and Jayachandran, 2012). A novel \( P.\) aeruginosa strain, isolated from soil collected from the premises of a coconut oil mill, was used in the production of biosurfactant. The new isolate gave the highest rhamnolipid production (3.55 g/L) and emulsifying activity (71.7%). The final surface tension of culture broth reached from a value of 53 mN/m to a level up to 24 mN/m, translating to a 54.68% reduction.

6.3 PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT FROM BACTERIAL ISOLATES

The production of biosurfactant was carried out using RT9 (\( Pseudomonas \) aeruginosa), RT19 (\( Bacillus \) siamensis) and RT21 (\( Bacillus \) subtilis subsp. inaquosorum). For extraction of biosurfactant, the isolates were cultured in MSM with the selected cheaper carbon substrate as the sole carbon source, as outlined in Section 3.8. The biosurfactant was extracted using the method of acid precipitation, as described in Section 3.9. The biosurfactant sample was subjected to thin layer chromatography, as outlined in Section 3.10.1. To understand the overall chemical nature of the extracted biosurfactant, Fourier transform infrared spectroscopy was employed, as described in Section 3.10.2. The \(^1\)H NMR spectra of the biosurfactant samples taken in deuterochloroform were acquired on a Bruker 400 MHz spectrometer (with tetramethylsilane as internal standard), as explained in Section 3.10.3. Stability studies were done using the 24-hr cell-free culture broth, as mentioned in Section 3.11. The stability of the biosurfactant was inferred by observing the change in emulsification index and surface tension, when temperature
(between 4°C and 121°C), pH (between 2 and 12) and salinity (between 1% and 20% w/v) were altered.

6.4 RESULTS AND DISCUSSION

6.4.1 Production and characterization of biosurfactant from *Bacillus siamensis*

6.4.1.1 Extraction of Biosurfactant

The biosurfactant, extracted from the fermentation broth appeared as an oily brown colored material.

6.4.1.2 Kinetics of growth and Biosurfactant Production

The time profile of biomass and biosurfactant production is shown in Figure 6.1.

![Figure 6.1: Kinetics of *B.siamensis* growth and biosurfactant production](image)
Growth kinetics was studied using Logistic Model represented by Eqns. 6.1 and 6.2. The Logistic equation reflects the impact of biomass on growth rate.

\[
\frac{dX}{dt} = \mu_m \left(1 - \frac{X}{X_m}\right)X
\]  
(Eqn. 6.1)

which, upon integrating gives,

\[
X = \frac{X_0 e^{\mu_m t}}{1 - \frac{X_0}{X_m} \left(1 - e^{\mu_m t}\right)}
\]  
(Eqn. 6.2)

where, \(\mu_m\): Coefficient of carrying capacity, \(X_0\): initial biomass concentration, \(X_m\): maximum biomass concentration, \(t\): time

Using the data obtained from the study, the estimated values of the parameters were: \(\mu_m = 0.0882\) /hr, \(X_0 = 0.149\) g/L and \(X_m = 3.0794\) g/L.

Biosurfactant production kinetics was studied using Leudeking-Piret Model, represented by Eqn. 6.3.

\[
\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X
\]  
(Eqn. 6.3)

where, \(P\): biosurfactant concentration at time, \(t\) (g/L), \(\alpha\): growth-associated constant (g/g), \(\beta\): non-growth-associated constant or specific productivity rate (g/g hr). The Logistic-incorporated Leudeking-Piret Model is given by Eqn. 6.4.

\[
P = P_0 + \alpha (X - X_0) + \beta \frac{X_m}{\mu_m} \ln \left\{ 1 - \left(\frac{X_0}{X_m}\right) \cdot (1 - e^{\mu_m t}) \right\}
\]  
(Eqn. 6.4)

Using the data obtained from the study, the estimated values of the parameters were: \(\alpha = 0.8988\) g / g and \(\beta = 0.2322\) g / g hr.
6.4.1.3 Characterization of Biosurfactant

6.4.1.3.1 Thin Layer Chromatography

The biosurfactant fraction was seen as a single spot on TLC (Figure 6.2). This fraction showed positive reaction with ninhydrin reagent and iodine vapour indicating the presence of peptide and lipid moieties in the molecule. The result was in agreement with Das et al., 2008, who obtained similar spots for a lipopeptide biosurfactant, derived from a marine *Bacillus circulans*.

Figure 6.2: TLC chromatograms of biosurfactants viewed under ordinary lamp (left) and long UV light (right)
6.4.1.3.2 Fourier Transform Infrared Spectroscopy

The FTIR spectrum obtained for the biosurfactant sample was analyzed. As a result of C–H stretching vibrations and N–H stretching vibrations, a broad absorbance peak (centred around 3433 /cm) with wave numbers ranging from 3600 /cm to 3100 /cm was observed (Figure 6.3). This is typical of carbon-containing compounds with amino groups. Sharp absorbance peaks are observed at 1463 /cm, 1379 /cm, 2955 /cm and 2854 /cm are indicative of aliphatic chains (–CH₃ and –CH₂–). These peaks reflect the presence of alkyl chains in the compound. A strong band was also observed at 1741 /cm, 1726 /cm & 1713 /cm. This is due to a carbonyl group. The presence of C=O bonds causing C=O stretching vibrations leads to absorbance peaks in these regions. The FTIR spectrum confirms the production of a lipopeptide biosurfactant. This was compared against the FTIR spectrum obtained for commercially available purified surfactin, as reported by Das et al., 2008. The peaks were found to be identical in both the cases.

Figure 6.3: FTIR Spectrum of biosurfactant produced by *B.siamensis*
6.4.1.3.3 $^1$H-Nuclear Magnetic Resonance Spectroscopy.

The assignment of the signals, as obtained from H-NMR spectrum, was done as per information from the literature survey (Franqoise Peypoux et al., 1991, Yu-Hong Wei and I-Ming Chu, 1998, Orathai Pornsunthorntawee et al., 2008, Xiang-Yang Liu et al., 2009 and Jorge Pereira et al., 2013). The resonance signals, shown in Figure 6.4, were interpreted. The presence of a long aliphatic hydrocarbon chain (CH$_2$) was highlighted by the resonance signals between 1.4 and 0.7 ppm. The signals between 8.00 - 7.24 ppm and 4.8 - 4.2 ppm show the presence of NH and CH. The occurrence of peptide backbone in the sample is thus confirmed. The presence of an ester group (which may be a part of lactone ring) is realized by the signal at 5.204 ppm. The presence of a methoxy (-CH$_3$O) group is shown by the signal at 3.393 ppm. The presence of $\alpha$-carbon protons is portrayed by the bands between 3.350 and 3.393 ppm. The terminal proton of the fatty acid chain is indicated by the signal at 5.247 ppm.
Figure 6.4: NMR Spectrum of biosurfactant produced by *B.siamensis*
The spectrum obtained for the biosurfactant sample was compared against that obtained for commercially available purified surfactin. The observed resonance signals for the standard matched with most of the signals obtained for the biosurfactant sample, except for extra peaks in the sample, that might have shown up due to the relatively crude nature of the *B. siamensis* biosurfactant. The NMR resonance signals for the standard surfactin are shown in Figure 6.5.

Figure 6.5: NMR Spectrum of standard Surfactin
6.4.1.4 Stability Analysis of Biosurfactant

The effect of temperature, pH and salinity on surface activity and emulsification index has been shown in Figures 6.6, 6.7 and 6.8, respectively. It was observed that over a wide range of temperatures (0°C to 121°C), the EI and surface tension values did not change much. Several studies confirm the stability of biosurfactants under extreme conditions of temperature. Joshi et al., 2008, reported that the biosurfactant produced by four different Bacilli isolates, were stable for nine days at 80°C. Desai and Banat, 1997 observed that heat treatment on some biosurfactants caused no appreciable change in their properties even after autoclaving at 120°C for 15 min. The biosurfactant showed high stability in pH values ranging from 2 to 12.

Figure 6.6: Effect of Temperature on surface tension and emulsification index
Several reports confirm the stability of biosurfactant at different pH values mostly in the alkaline medium (Batista et al., 2005; Ghojavand et al., 2008; Joshi et al., 2008, Gudina et al., 2010). It was observed that salinity did not affect the surface activity of the biosurfactant even upto 20% sodium chloride. Oil reservoirs are one of the harsh environments, where temperature can range from 20 to 90°C, normal salinity to hypersalinity and pH over a wide range. So, the suitable candidate for EOR studies has to be stable over those criteria. The biosurfactant produced by B.siamensis meets those criteria and showed stability over a wide range of environmental factors, while retaining its surface activity.

Figure 6.7: Effect of pH on surface tension and emulsification index
6.4.2 Production and characterization of biosurfactant from *B. subtilis* subsp. *inaquosorum*

6.4.2.1 Extraction of Biosurfactant

The biosurfactant, extracted from the fermentation broth appeared as an oily brown colored material.

6.4.2.2 Kinetics of growth and Biosurfactant Production

The time profile of biomass and biosurfactant production is shown in Figure 6.9. As described in Section 6.4.1.2, growth kinetics was studied using Logistic Model represented by Eqns. 6.1 and 6.2. Using the data obtained from the study, the estimated values of the parameters were: coefficient of carrying capacity (*μ_m*) = 0.0645 /hr, initial biomass concentration (*X_0*) =
0.2343 g/L and maximum biomass concentration (X_m) = 5.298 g/L. Similarly, biosurfactant production kinetics was studied using Logistic-incorporated Leudeking-Piret Model, represented by Eqn. 6.3. Using the data obtained from the study, the estimated values of the parameters were: α = 1.0034 g / g and β = 0.3565 g / g. hr.

![Figure 6.9: Kinetics of B. subtilis subsp. inaquosorum growth and biosurfactant production](image)

6.4.2.3 Characterization of Biosurfactant

6.4.2.3.1 Thin Layer Chromatography

Preliminary characterization of biosurfactant using TLC revealed a single spot (Figure 6.10). As with the previous isolate, this fraction showed positive reaction with ninhydrin reagent and iodine vapour indicating the presence of peptide and lipid moieties in the molecule. The result was in agreement with Das et al., 2008, who obtained similar spots for a lipopeptide biosurfactant, derived from a marine *Bacillus circulans*. 
6.4.2.3.2 Fourier Transform Infrared Spectroscopy

The FTIR spectrum obtained for the biosurfactant sample was analyzed. Spectral analysis suggested the lipopeptidic nature of the extracted biosurfactant (Fig. 6.11). The spectrum showed a broad absorbance peak centred around 3444 /cm, ranging from 3100 /cm to 3600 /cm. This is a
A typical feature of compounds containing carbon and amino groups and is caused due to stretching vibrations of C-H and N-H bonds, present in the compound. The absorbance peak at 2926/cm indicates the presence of alkyl chains (−CH2 and −CH3). The absorbance peak at 1706/cm is attributed the stretching vibrations of C=O bonds. The absorbance peak at 1653/cm implies that peptide groups are present in the sample. A weak absorbance signal at 1456/cm is due to bending vibrations of C-H bonds associated with alkyl chains. Another absorption peak at 1227/cm is due to C-O stretching vibrations related to esters. The FTIR spectrum confirms the production of a lipopeptide biosurfactant. This was compared against the FTIR spectrum obtained for commercially available purified surfactin, as reported by Das et al, 2008. The peaks were found to be identical in both the cases.

![FTIR Spectrum of biosurfactant produced by B. subtilis subsp. inaquosorum](image)

**Figure 6.11: FTIR Spectrum of biosurfactant produced by B. subtilis subsp. inaquosorum**

### 6.4.2.4 Stability Analysis of Biosurfactant

The effect of temperature, pH and salinity on surface activity and emulsification index has been shown in Figures 6.12, 6.13 and 6.14, respectively. The results were comparable to that obtained for *B.siamensis*. The surface activity of the biosurfactant remained stable over the range
of temperature, pH and salinity, studied. With respect to pH, the least surface tension was obtained from neutral pH, onwards. Such extreme stability was also reported by Abdel-Mawgoud et al., 2009 and Kiran et al. 2009, for the Pseudomonas aeruginosa strain and Brevibacterium aureum MSA13 respectively. This suggests that the biosurfactant isolated may be used in microbial enhanced oil recovery processes where high temperatures prevail.

Figure 6.12: Effect of Temperature on surface tension and emulsification index
Figure 6.13: Effect of pH on surface tension and emulsification index

Figure 6.14: Effect of salinity on surface tension and emulsification index
The effect of salinity on the biosurfactant activity was tested. A steady surface tension of average 28 mN/m was noted even when the salinity value was increased to 20% sodium chloride concentration. However, at 20% concentration, emulsification capacity slightly dropped to 65% and surface tension activity slightly increased as well upto 30 mN/m. The results are in agreement with Aparna et al., 2012.

The findings indicate the potential application of the biosurfactant over a wide range of temperature, pH and salinity. This finds application in situations where extreme conditions of temperature, pH and salinity prevail such as bioremediation of soil as well as marine environments. It can also be used for enhanced oil recovery operations.

6.4.3 Production and characterization of biosurfactant from *Pseudomonas aeruginosa*

6.4.3.1 Extraction of Biosurfactant

The biosurfactant, extracted from the fermentation broth appeared as a viscose brown colored material.

6.4.3.2 Kinetics of growth and Biosurfactant Production

The time profile of biomass and biosurfactant production is shown in Figure 6.15. As described in Section 6.4.1.2, growth kinetics was studied using Logistic Model represented by Eqns. 6.1 and 6.2. Using the data obtained from the study, the estimated values of the parameters were: coefficient of carrying capacity (\(\mu_m\)) = 0.0792 /hr, initial biomass concentration (\(X_0\)) = 0.1299 g/L and maximum biomass concentration (\(X_m\)) = 3.0025 g/L. Similarly, biosurfactant production kinetics was studied using Logistic-incorporated Leudeking-Piret Model, represented by Eqn. 6.3. Using the data obtained from the study, the estimated values of the parameters were: \(\alpha = 0.9045 \text{ g / g} \) and \(\beta = 0.0033 \text{ g / g. hr} \).
Characterization of Biosurfactant

6.4.3.3 Thin Layer Chromatography

TLC analysis showed that there was no red spot on silica gel plates when using ninhydrin as color developing reagent, suggesting that no lipopeptide was found in this biosurfactant. Meanwhile, the appearance of yellow spot proved the presence of glycolipid. Accordingly, the biosurfactant secreted by the strain was glycolipid in nature (Hua Yin et al, 2009). The chromatogram has been shown in Figure 6.16.
Figure 6.16: TLC chromatograms of biosurfactants viewed under long UV light

6.4.3.3.2 Fourier Transform Infrared Spectroscopy

The FTIR spectrum obtained for the biosurfactant (Fig. 6.17) sample was analyzed. The broad band between 3300 and 3400 cm$^{-1}$ indicates the presence of hydroxyl bonds. Absorption at around 2900 cm$^{-1}$ is assigned to the symmetric stretch of CH$_2$ and CH$_3$ groups of aliphatic
chains. The absorption peak located at 1700 cm$^{-1}$ indicates the presence of ester carbonyl groups (carbonyl bond in COOH). The ester carbonyl group was shown from the band at around 1200 cm$^{-1}$ that is due to C=O deformation vibrations. The absorption peak at around 1000 cm$^{-1}$ indicates the presence of polysaccharide-like substances in the biosurfactant. The absorption peak between 650 and 700 cm$^{-1}$ indicates the presence of -CH$_2$ group. The assignment of wave numbers and chemical groups was similar to the results obtained by Aparna et al., 2012. The information obtained implied the rhamnolipid nature of the extracted biosurfactant.

![FTIR Spectrum of biosurfactant produced by P.aeruginosa](image)

**Figure 6.17: FTIR Spectrum of biosurfactant produced by P.aeruginosa**

### 6.4.3.4 Stability Analysis of Biosurfactant

The effect of temperature, pH and salinity on surface activity and emulsification index has been shown in Figures 6.18, 6.19 and 6.20, respectively.
The results were in agreement with many papers that reported thermal stability of rhamnolipid biosurfactants. Bordoloi and Konwar, 2008 exposed the biosurfactant produced by *Pseudomonas aeruginosa* strains to temperature of 100°C and found that the biosurfactant activity remained unaffected with respect to surface activity. The effect of salinity on the biosurfactant activity was tested. A steady surface tension of average 30 mN/m was noted even when the salinity value was increased to 20% sodium chloride concentration. However, at 20% concentration, emulsification capacity slightly dropped to 70% and surface tension activity slightly increased as well upto 32 mN/m. The results are in agreement with Helvaci *et al.*, 2004.

It was concluded that electrolytes directly affect the carboxylate groups of the rhamnolipids. The solution/air interface has a net negative charge due to the ionized carboxylic acid groups
at alkaline pH with strong repulsive electrostatic forces between the rhamnolipid molecules. This negative charge is shielded by the Na\(^+\) ions in an electrical double layer in the presence of sodium chloride, causing the formation of a close-packed monolayer and consequently a decrease in surface tension values.

Figure 6.19: Effect of pH on surface tension and emulsification index
Figure 6.20: Effect of salinity on surface tension and emulsification index

6.5 CONCLUSION

*B. siamensis* was grown using powdered garlic peel as the sole carbon source. The maximum amount of biomass of 3.09 g/L and biosurfactant of 0.64 g/L was attained after 96 hr. Using the Logistic Model, the estimated values of the parameters were: coefficient of carrying capacity ($\mu_m$) = 0.0882 /hr, initial biomass concentration ($X_0$) = 0.149 g/L and maximum biomass concentration ($X_m$) = 3.0794 g/L. Using Leudeking-Piret Model for biosurfactant production kinetics, the estimated values of the parameters were: $\alpha = 0.8988$ g / g and $\beta = 0.2322$
Characterization of the biosurfactant by TLC, FTIR spectroscopy and NMR spectroscopy, led to its identification as the lipopeptide biosurfactant, surfactin.

*B. subtilis* subsp. *inaquosorum* was also grown using powdered garlic peel as the sole carbon source. The maximum amount of biomass of 5.57 g/L and biosurfactant of 1.18 g/L was attained after 120 hr. Using the Logistic Model, the estimated values of the parameters were: \( \mu_m = 0.0645 \text{ /hr} \), \( X_0 = 0.2343 \text{ g/L} \) and \( X_m = 5.298 \text{ g/L} \). Biosurfactant production kinetics was studied using Leudeking-Piret Model. The estimated values of the parameters were: \( \alpha = 1.0034 \text{ g / g} \) and \( \beta = 0.3565 \text{ g / g hr} \). Similar characterization of the biosurfactant identified the sample as the lipopeptide biosurfactant, surfactin.

*P. aeruginosa* was grown utilizing used engine oil as the sole carbon source. The maximum amount of biomass of 4.63 g/L was attained after 108 hr. The maximum amount of biosurfactant yield of 2.14 g/L was attained after 120 hr. Using the Logistic Model, the estimated values of the parameters were: \( \mu_m = 0.0792 \text{ /hr} \), \( X_0 = 0.1299 \text{ g/L} \) and \( X_m = 3.0025 \text{ g/L} \). Biosurfactant production kinetics was studied using Leudeking-Piret Model. The estimated values of the parameters were: \( \alpha = 0.9045 \text{ g / g} \) and \( \beta = 0.0033 \text{ g / g hr} \). The biosurfactant was characterized to be a rhamnolipid.

When the biosurfactants obtained from the three isolates were subjected to stability tests in terms of temperature, pH and salinity, it was observed that the surface tension and the emulsification index remained nearly constant. Hence, the biosurfactants were stable enough for industrial and MEOR applications.