Chapter 4

Biochemical and physical characterization of bioemulsifier AM1 and its emulsions

Meaning:
Simple and beautiful object does not need processing.
A freed pearl doesn not need to be polished again.
-Drushtanta kalika (49)
Chapter 4

Biochemical and physical characterization of bioemulsifier AM1 and its emulsions

4A. Biochemical characterization of bioemulsifier AM1

4A.1. Introduction

As introduced in the chapter 1, emulsifiers are a subclass of surfactants that stabilize dispersions of one liquid in another like oil-in-water (oil-in-water emulsions). They are high molecular weight polymers that bind tightly to surfaces. Microbial emulsifiers or bioemulsifiers are found to have wide spectrum of activity which is reflection of their complex chemistry as well as the diversity of bacterial genera producing them (Osterreicher-Ravid, et al., 2001, Ron & Rosenberg, 2001, Abdel-Mawgoud, et al., 2010, Satpute, et al., 2010a, Satpute, et al., 2010b).

Elucidation of the structure and chemical composition of a biomolecule is essential, as these characteristics dictate its function and interactions. Bioemulsifiers with various chemical compositions from eubacteria (including actinomycetes), archaea, algae, cyanobacteria, fungi and yeasts have previously been reported. Polysaccharides and their derivatives have been reported as bioemulsifiers in Acinetobacter (Kaplan & Rosenberg, 1982), Klebsiella (Bryan, et al., 1986), Candida species (Cirigliano & Carman, 1984), Pseudomonas (Bonilla, et al., 2005) and Sphingomonas species (Ashtaputre & Shah, 1995). Many marine bacteria isolated from Indian coast with emulsification activity have been reported previously and detailed analyses of these bacteria were also discussed in many reviews (Iyer, et al., 2005, Kokare, et al., 2007, Kumar, et al., 2007, Nerurkar, et al., 2009, Satpute, et al., 2010b). Many of these bioemulsifiers reported were found to be derivatives of polysaccharides (Iyer, et al., 2005, Kokare, et al., 2007, Kumar, et al., 2007). Carbohydrates conjugated with proteins or other hydrophobic moieties, such as lipids and fatty acids, that are capable of emulsification have been reported in Bacillus sp.(Gurjar, et al., 2008, Suthar, et al., 2009) and fungi such as Trichosporon sp.(de Souza Monteiro, et al., 2012). Carbohydrates, lipids and proteins in different combinations from Geobacillus sp.(Zheng, et al., 2011) and Penicillium sp.(Luna-Velasco, et al., 2007) have been studied for their emulsification properties. Many
other emulsifying polymers, such as lipo-peptides, are found in Bacillus (Liu, et al., 2010) and Streptococcus sp. (Jenkinson, 1992).

The bioemulsifiers reported from Bacillus strains FE-1 and FE-2, both isolated from fenthion contaminated soil produced a high molecular weight, lysozyme sensitive and thermostable glycolipopeptide bioemulsifier and another consisting of carbohydrate, lipid, and peptide respectively (Patel & Gopinathan, 1986). Cooper and Goldenberg (1987) isolated Bacillus strains IAF 343 and IAF 346 where the former produced a neutral lipid bioemulsifier, latter produced a polysaccharide bioemulsifier. A proteinaceous bioemulsifier with minor carbohydrate and lipid content was reported from Bacillus stearothermophilus VR-8 (Gurjar et al., 1995) and a Bacillus sp. CP912 was reported for homopolysaccharide bioemulsifier (Yun and Park 2000).

Bacillus amyloliquifaciens LP03 was produced antifungal lipopeptide surface active agent with high emulsification activity (Lee, et al., 2007). In two separate reports, Bacillus licheniformis strains ACO1 and K125 were shown to be producing polysaccharide rich (Dastgheib, et al., 2008) and polysaccharide-protein-lipid (Suthar, et al., 2008) bioemulsifiers respectively. Kumar et al. (Kumar, et al., 2007) reported a lipoprotein bioemulsifier from Bacillus sp. DHT resistant to high range of salinity and temperatures. Many reports where bioemulsifier producing Bacillus strain isolates were from contaminated soils (Toledo, et al., 2008, Sathe, et al., 2012, Klawech, et al., 2013) and marine waters are available (Pavitran, et al., 2006).

A few reports of proteinaceous bioemulsifiers exist those studied include a multi-protein complex bioemulsifier from Methylobacterium sp. (Joe, et al., 2013) and Methanobacterium thermoautotrophicum (Trebbau de Acevedo & McLnerney, 1996). Moreover, the studies on glycoprotein bioemulsifiers are rare, and the only reports are from Pseudoalteromonas sp. (Gutierrez, et al., 2008) and Antarctobacter sp. (Gutiérrez, et al., 2007).

In the present chapter in section A, the results of purification and physicochemical characterization of the bioemulsifier produced by the selected novel isolate Solibacillus silvestris AMI are discussed. Additionally, a comparison of the emulsion formed by bioemulsifier AMI with Emulsan from Acinetobacter calcoaceticus RAG-1 with varied solvents was characterized with respect to its stability. Further, in the second section B, the characters of the emulsion produced by bioemulsifier AMI was studied.
Interesting observations regarding bioemulsifier AMI showing amyloid nature aggregating into fibrils and noted in this work. Amyloid fibres are ordered protein aggregates generally associated with many neurodegenerative diseases like Alzheimer’s, Parkinson’s and various Prion diseases. The first of these bacterial functional amyloids reported, Curli fibres of *Escherichia coli* strains had a basic subunit of CsgA components while next to be discovered, thin aggressive fimbriae (Tafi) of *Salmonella* strains also showed similar subunits (Olsén, *et al.*, 1989, Collinson, *et al.*, 1991). Similar functional bacterial amyloids were reported from *Bacillus subtilis* made up of Tas A fibrils present as a mixture with exopolysaccharides released which stabilize the bacterium’s biofilm (Soreghan, *et al.*, 1994, Gebbink, *et al.*, 2005, Nielsen, *et al.*, 2011, Blanco, *et al.*, 2012). Other species of *Bacillus* genera also were reported subsequently for amyloid like proteins for varied cellular functions (Bowen, *et al.*, 2002, Wijman, *et al.*, 2007, Jordal, *et al.*, 2009).

As their production is reported from different microorganisms like, *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroides* and also in fungi with relatively no relation to each other at the amino acid level, there seems to be a preferential selection of amyloid curve during the evolution for various functions. Although list of bacteria capable of producing functional amyloids is increasing rapidly, there exists only few reports with respect to their function in environment (Romero, *et al.*, 2010, Nielsen, *et al.*, 2011, Blanco, *et al.*, 2012). Among the few reported bacterial amyloids, many still need to be further studied in depth. Biophysical properties and ecological details of amyloids other than from *E. coli* and *Pseudomonads* and few other bacteria are still lacking (Nielsen, *et al.*, 2011). Here, an attempt to study the amyloid property of *S. silvestris* bioemulsifier AMI was also studied.

4A.2. Materials and Methods

4A.2.1. Microorganisms, media and growth conditions:

(i) *Solibacillus silvestris* AMI produced bioemulsifier in ZM medium and with peptone as the major carbon source as discussed in chapter 3. The strain was submitted to Microbial culture Collection Centre, NCCS, Pune, India (Accession No MCC 2096).
(ii) *Acinetobacter calcoaceticus* RAG-1 (MTCC 2409, ATCC 31012) was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. *A. calcoaceticus* RAG-1 was maintained on Luria agar. The production of Emulsan was conducted according to medium reported by Goldman, *et al.* (1982), in Erlenmeyer flasks at 30°C under shaking conditions for 72h.

4A.2.2. Emulsification index (%EI):

The emulsification index (%EI or E24, E48 and E72) of the emulsion was calculated using methods discussed in Chapter 2, section 2A.2.5 (Cooper & Goldenberg, 1987).

4A.2.3. Purification:

Emulsion was prepared using culture medium containing crude bioemulsifier as described above. After 24 h, the emulsion was separated by centrifuging several times at 10000 rpm to remove oil. The major aqueous portion was filtered through a membrane filter with pore size of 0.2-μm and dialyzed (10 kDa cut-off) for 24 h using distilled water with a change of medium at every 6 h; then, the sample was passed through an ultrafiltration (UF) cassette (Amicon; OMEGA filters, Millipore Inc., USA) with a cutoff of 100 kDa. The retained material was washed 2-3 times with phosphate buffer (100 mM KH$_2$PO$_4$ and 100 mM K$_2$HPO$_4$; pH 7.2 ±0.2) and collected in 5 ml buffer. This solution was then applied to a 50-cm gel filtration column with an inner diameter of 1 cm and a bed volume of 23.56 cm$^3$ filled with G200 (Sephadex, SIGMA chemical company, USA) using phosphate buffer, pH 7.2 ±0.2 as the mobile phase and a flow rate of 0.4 ml/min. After each purification step, the %EI was determined. After gel filtration chromatography (GFC), the active fractions producing emulsifier were pooled and lyophilized. The purified bioemulsifier AMI was stored at 4°C for further studies.

4A.2.4. Biochemical characterization of Bioemulsifier AM1

4A.2.4.1. Carbohydrate and protein estimation:

Carbohydrate and protein content in the purified bioemulsifier were quantified using the methods of Dubois, *et al.* (1956) and Lowry, *et al.* (1951) respectively.
4A.2.4.2. Proteinase K treatment:
The bioemulsifier was treated with Proteinase K (10 mg/ml; Bangalore GeNei, Bengaluru, India) at 37°C for 30 min; afterwards, the %EI was determined as described above.

4A.2.4.3. Thin layer chromatography:
Bioemulsifier AMI was examined for the presence of carbohydrates and lipid moieties using TLC. For this, it was then hydrolyzed for an hour with 1N HCl and run on silica gel 60 F_{254} plates (MERCK, Germany) in a chloroform–methanol–acetic acid–water system with ratios of 25:15:4:2 (Cooper & Goldenberg, 1987). α-Naphthol solution (1:1 of 10 mg α-Naphthol in 100 ml methanol and 5% H_{2}SO_{4}) was used to detect carbohydrates. For detection of lipids the bioemulsifier AMI was run in the same solvent system and detected with iodine vapours (Suthar, et al., 2008).

4A.2.4.4. High Performance Thin Layer Chromatography (HPTLC):
For HPTLC, purified bioemulsifier AMI was hydrolyzed as described above and lyophilized. Methanolic solutions of hydrolyzed bioemulsifier (50 mg/ml) and standard sugars (glucose, galactose and ribose at concentrations of 1 mg/ml and fructose, rhamnose and xylose at concentrations of 2 mg/ml) were individually applied as 8-mm-wide bands (10 μl each) on the silica plates using an automatic TLC sample application device (Linomat V, CAMAG, Switzerland) with a constant flow of nitrogen gas. The same solvent system as the TLC was used, and HPTLC was conducted in a glass twin trough chamber (CAMAG, Switzerland). Plates were developed with an α-naphthol-H_{2}SO_{4} reagent followed by heating at 110°C for 10 min and then scanning at 540 nm using scanner 3 (CAMAG, Switzerland), with integrated winCATS4 software.

4A.2.4.5. Glycoprotein staining:
The bioemulsifier was analyzed by native PAGE to study its glycoprotein nature. Samples were prepared by dissolving lyophilized bioemulsifier AM1 in 1% SDS (sodium dodecyl sulfate) with 0.01 M dithiothreitol (DTT). Equal volumes of 2 mM sodium phosphate buffer (pH 7.1) in 8 M urea were added to the sample to obtain the final SDS concentration of 0.5%. The sample (10 μl) was loaded onto a 12% native PAGE gel. Glycoprotein staining was performed as described by Segrest & Jackson (1972). Following overnight fixing with periodic acid-Schiff (PAS) fixative...
(40:5:50 methanol: glacial acetic acid: water), the gel was treated with 0.7% periodic acid solution (in 5% acetic acid) for 2-3 h and then with 0.2% sodium metabisulfite solution (in 5% acetic acid) for 2-3 h. Subsequently, the gel was stained with Schiff's reagent for 12-18 h at room temperature and destained with PAS destaining solution (5:7.5:87.5 methanol : glacial acetic acid : water).

4A.2.4.6. LC/MS-MS:
The single largest band of untreated purified multimeric bioemulsifier with a molecular weight (MW) greater than 200 kDa was removed from the native PAGE gel and sent to the Centre for Genomic Applications (TCGA), New Delhi, India. The 30 kDa band of bioemulsifier subunit was removed from the SDS PAGE gel and sent to Centre for Cellular and Molecular Platforms (C-CAMP), Bengaluru, India for LC/MS-MS and Mascot analysis. This analysis was performed twice. The LC/MS-MS data received from all three samples were analyzed using Mascot.

4A.2.5. Physical characterization of Bioemulsifier AM1

4A.2.5.1. Fourier transform infrared (FTIR) analysis:
Bioemulsifier AM1 (1 mg) dissolved in deionized water was used to prepare a KBr pellet. FTIR spectra were recorded on a Shimadzu 8400S spectrophotometer. The spectra were obtained over the frequency range of 4000-500 cm⁻¹ after 4 scans. Deionized water was used as a background control.

4A.2.5.2. Polarizing microscope:
The purified bioemulsifier AM1 (mg/ml) was resuspended in phosphate buffer (pH 7.3) spread into a smear on the clean grease-free glass slide and stained with 50μM congo red at room temperature for 1 min, washed with the buffer and distilled water in succession. The samples were then studied with Zeis Photomicroscope equipped with LEICA DM 2500P with 500X magnification (King, et al., 1997).

4A.2.5.3. Circular Dichroism (CD) spectrum analysis:
A suspension of purified bioemulsifier AM1 (1mg/ml) was made in deionized water. CD spectra were recorded in the range of 195-250 nm wavelength on Jason J-108 (Japan) CD spectrophotometer at room temperature (26°C).
4A.2.5.4. SDS-PAGE analysis of denaturant treated purified bioemulsifier AM1:
Various denaturing substances (1-2% w/v), such as urea and dithiothreitol (DTT), and surfactants, such as SDS, Tween20, and Triton X100, were mixed with the purified bioemulsifier individually and incubated for 2 min with and without boiling treatment. The treated bioemulsifier was resolved using 12% SDS-PAGE, detected with silver staining and analyzed with AlphaEaseFC-v4.0 (Alphalnnotech, USA) software.

4A.2.5.5. Transmission electron microscope observations (TEM):
Purified bioemulsifier (500 μg) was applied to a carbon-coated grid for 1 min, stained with 1% uranyl acetate for 30 sec and subjected to transmission electron microscopy using JEOL JEM 2100 at Central Salt & Marine Chemicals Research Institute, Bhavnagar, Gujarat, India.

4A.2.5.6. Temperature, pH and salt stability of bioemulsifier:
Purified bioemulsifier AM1 (0.5 mg/ml) was examined for thermal stability by incubating in a boiling water bath (100°C) for 0.5, 1, 2, 3, 4 and 5 h. The same amount of bioemulsifier was dissolved in salt solutions of 1-5 M NaCl to examine its salt tolerance. Its pH stability was studied by preparing 0.5 mg/ml purified bioemulsifier AM1 solutions in standard buffers within the 3-9 pH range. The following buffers were used: pH 3-4 citrate buffer, pH 5-6 acetate buffer, pH 7 phosphate buffer and pH 8 and 9 Tris-Cl buffers. Bioemulsifier activity was examined by measuring emulsification, as indicated by %EI, before and after treatment in the conditions described. All tests were performed in triplicate and analyzed with one sample t-test using GraphPad Prism 5 software.

4A.2.5.7. Critical micellar dilution (CMD) measurement:
The minimum concentration of biosurfactant/bioemulsifier corresponding to the dilution at which micelles begin to form is referred to as its critical micellar concentration (CMC), which can also be expressed in terms of CMD (Makkar & Cameotra, 1997). The dilution of biosurfactant and bioemulsifier at which the %EI starts falling abruptly is the critical micellar dilution (CMD). In the absence of a direct assay test, the CMD can be used as a measure of the amount of bioemulsifier present in the original sample (Makkar & Cameotra, 1997). The CMD can be estimated by diluting the cell-free supernatant with distilled water and measuring the %EI at

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various dilutions (performed in triplicate). The previously reported bioemulsifier, Emulsan from *A. calcoaceticus* RAG-1, was used for comparison.

**4A.2.6. Congo red-plate assay:**

The method to detect the presence of amyloid proteins as shown by Romero, *et al.* (2010) was modified to observe the amyloid nature of the extracellular bioemulsifier produced by the colonies. Media used for the test were Bushnell Haas Medium (amended with 1% acetate as carbon source) and Zobell Marine medium both supplemented with congo red (25mg/l).

**4A.2.7. Statistical analysis:**

All experiments were conducted in triplicate and analyzed with ANOVA and t-test using GraphPad Prism 5 software.

**4A.3. Results and Discussion**

The results of biochemical and physical characterization of the bioemulsifier AM1 carried out to find out its chemical nature and other associated functional properties are discussed here.

**4A.3.1. Characterization of Bioemulsifier:**

Production of a bioemulsifier by *S. silvestris* AM1 was most effective in ZM medium, and less or no bioemulsifier was produced in other reported production media (Pfiffner, *et al.*, 1986, Cooper & Goldenberg, 1987, Gurjar, *et al.*, 1995, Yun & Park, 2003, Suthar, *et al.*, 2008). The presence of protein in the medium was essential (Figure 3.1) for bioemulsifier AM1 production, as was also observed in the case of *B. stearothermophilus* VR-8 (Gurjar, *et al.*, 1995). A notable feature is that the *S. silvestris* AM1 produces a potent bioemulsifier in hydrocarbon-less medium, and no inducer is required. *A. calcoaceticus* RAG-1 produces Emulsan, which has been extensively studied and has been noted for requirement of ethanol for production of bioemulsifier (Patil & Chopade, 2001, Amiriyan, *et al.*, 2004). Crude bioemulsifier AM1, crude Emulsan, and 0.1% Xanthan were found to give 62.5%, 60% and 94% emulsification indices, respectively.

**4A.3.2. Purification:**

The culture supernatant containing bioemulsifier AM1 was dialyzed to remove residual salts from the medium. It was then concentrated using ultrafiltration cassettes.
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with 100 kDa cutoff. Following gel filtration chromatography (GFC) of the concentrate, the emulsification activity was found in 7-9th fractions of the flow through of a 30 ml gel column and was enhanced by 49.6-fold (Table 4.1). Gel filtration beads appeared to exclude the bioemulsifier because the emulsification activity was repeatedly observed in the flow through indicating that bioemulsifier AM1 was larger than 200 kDa.

Table 4.1. Purification of bioemulsifier AM1

<table>
<thead>
<tr>
<th>Purification stages</th>
<th>Total protein (mg/ml)</th>
<th>EI (mL)</th>
<th>Sp. Activity (EI/mg)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>8.73 (±1.14)</td>
<td>61.8 (±0.69)</td>
<td>7.15 (± 0.86)</td>
<td>1</td>
</tr>
<tr>
<td>Dialysate</td>
<td>8.04 (± 0.53)</td>
<td>60.99 (±0.12)</td>
<td>7.60 (±0.51)</td>
<td>1.07 (±0.05)</td>
</tr>
<tr>
<td>Ultra filtration (UF)</td>
<td>3.50 (±0.34)</td>
<td>57.2 (±0.68)</td>
<td>16.4 (±1.8)</td>
<td>2.3 (± 0.02)</td>
</tr>
<tr>
<td>Gel filtration (GFC)</td>
<td>0.15 (±0.03)</td>
<td>52.38 (± 0.25)</td>
<td>344.8 (±83.74)</td>
<td>49.6 (±17.79)</td>
</tr>
</tbody>
</table>

Values in parenthesis = Standard deviation (SD)

The elution profile of GFC is given in Figure 4.1 (a) and (b). Bioemulsifier from each of the active fractions was resolved into multiple bands using SDS PAGE (Figure 4.1a and b), and these bands could not be eliminated even after additional ultrafiltration and gel filtration steps. Surprisingly, an additional band of proteins of...
less than 100 kDa were always visible in SDS PAGE, even after repeatedly recycling the filtrate through 200 kDa gel columns (Figure 4.2). Therefore, it can be inferred that the bioemulsifier eluted from the gel-filtration column must be a multimer that dissociated into smaller subunits during electrophoresis, appearing as less than 100 kDa bands (Figure 4.2).

![Figure 4.2. SDS-PAGE analysis of purification steps of bioemulsifier AMI: Lane 1- crude supernatant, Lane 2- dialysate, Lane 3 ultrafiltrate (UF), Lane 4- active fraction from gel-filtration chromatography (GFC) and Lane 5- molecular markers in SDS-PAGE (10%).](image)

4A.3.3. Biochemical characterization of bioemulsifier AM1:

4A.3.3.1. Chemical analysis of bioemulsifier AM1:

The total carbohydrate content of purified bioemulsifier AM1 showed 36.5 µg of sugars per mg of protein. The carbohydrate component in this proteinaceous bioemulsifier is 3.6%, which is much less compared to the glycoprotein bioemulsifier reported by Gutiérrez et al. that contains 15.4% carbohydrate (Gutiérrez, et al., 2007b). Thus, the bioemulsifier from *Solibacillus silvestris* AM1 is proteinaceous with a minor carbohydrate component.

4A.3.3.2. Proteinase K treatment:

Purified bioemulsifier AM1 lost its activity after Proteinase K treatment (Figure 4.3). Loss of bioemulsifier activity after enzymatic treatment indicates that it contains an active protein component.
Figure 4.3. Emulsification activity of bioemulsifier AM1 after Proteinase K treatment

4A.3.3.3. Thin Layer Chromatography and HPTLC:

The purified bioemulsifier AM1 does not contain lipid, as no spot was observed in thin layer chromatography (Figure 4.4a). The solvent system described by Cooper and Goldenberg for TLC and HPTLC separated all the standard reported sugars effectively (Cooper & Goldenberg, 1987). The spots for crude bioemulsifier were observed near fructose on TLC but differed with respect to RF (Figure 4.4b). In figure 4.5 HPTLC densitogram of bioemulsifier AM1 along with standard sugars is shown in Figure 4.5. The software used for this analysis, winCATS, predicted the presence of galactose and xylose or ribose in the bioemulsifier AM1 with high probability.

Figure 4.4. TLC of bioemulsifier from S. silvestris AM1 for (a) lipid, run with standard bioemulsifier from B. licheniformis K125 and (b) carbohydrate standards and bioemulsifier AM1. The red box indicated the spots for crude bioemulsifier
As seen in figure 4.5 the peak I of bioemulsifier AM1 coincides with galactose and peak II with ribose or xylose. Because ribose is a C3 epimer of xylose, these two sugars could not be distinguished using HPTLC. Similar protein-sugar conjugates are reported to be responsible for the stability of many extracellular bacterial proteins, such as flagellin (Schirm, et al., 2004, Taguchi, et al., 2010).

Figure 4.5. High performance thin layer chromatography (HPTLC) densitogram of bioemulsifier AM1 and standard sugars at 540nm. Peak I and Peak II represent the two peaks for sugars represented by bioemulsifier AM1.

4A.3.3.4. Carbohydrate analysis of the bioemulsifier AM1:

To determine the nature of the carbohydrate component of the glycoprotein bioemulsifier, native PAGE and further HPTLC was performed. A pink band detected by PAS staining was observed, corresponding to bioemulsifier subunits above the 100 kDa position in native PAGE, as shown in Figure 4.6.

*Solibacillus* sp. has not been reported to produce any extracellular proteinaceous bioactive molecule to date. Our work presents the first report of the production of bioemulsifier from *Solibacillus* sp.
Figure 4.6. Native PAGE(12%) of active fraction GFC. Lane 1- silver staining, Lane 2- Periodic acid-Schiff’s stain (PAS) staining for glycoprotein and Lane 3- control lane.

4A.3.3.5. LC/MS-MS:
Mascot analysis (Figure 4.7) of the LC/MS-MS spectrum of the single largest (200 kDa) band of purified bioemulsifier AM1 from Native PAGE provided the highest peptide score, matching two peptides: VIGPVVDVEFPR and FTQAGSEVSTLLGR of the β-subunit of F0F1 ATP synthase from Corynebacterium diphtheriae NCTC 13129 (NP_939413), as shown in Table 4.2a. Similarly, LC/MS-MS followed by Mascot analysis of a single band of 30 kDa provided a high peptide score, matching with three flagellin hag peptides (AGDDAAGLAISEK, INRAGDDAAGLAISEK and LGAYQNR) from Bacillus halodurans C-125 (FLA_BACHD). This analysis was performed twice to confirm the results (Table 4.2b).

As described by Hirose et al., the fragment of flagellin AGDDAAGLAISEK is contained within the sequence INRAGDDAAGLAISEK (Hirose, et al., 2000). These sequences show 94.44% homology with the flagellin sequence from Solibacillus silvestris StLB046, present in NCBI database with a few variations in the amino acids (Table 4.2b). According to Sakamoto, et al. (1992), the amino acid sequences of B. subtilis flagellin shows a pattern of similarities in the N- and C-terminal regions and dissimilarity in the central regions. B. halodurans C-125 flagellin, with which the peptide match for bioemulsifier AM1 was obtained, has a similar variable central region as observed in B. subtilis flagellin (Sakamoto, et al., 1992).
Table 4.2. Comparison of amino acid sequence of peptides provided by LC-MS/MS and MASCOT analysis of the 200kDa (a) and 30kDa (b) proteins comprising bioemulsifier AMI.

<table>
<thead>
<tr>
<th>Analysed Peptide</th>
<th>Source</th>
<th>Organism</th>
<th>% Peptide similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) VIGPVVDVEFPR</td>
<td>This study</td>
<td><em>Solibacillus silvestris</em> AM1</td>
<td>100%</td>
</tr>
<tr>
<td>FTQAGSEVSTLLGR</td>
<td>NCBI</td>
<td><em>Corynebacterium diphtheriae</em> NCTC 13129</td>
<td>100%</td>
</tr>
<tr>
<td>VIGPVVDVEFPR</td>
<td>NCBI</td>
<td>F0F1 ATP synthase subunit beta</td>
<td>84.61%</td>
</tr>
<tr>
<td>FTQAGSEVSTLLGR</td>
<td>(NP_244621)</td>
<td><em>Bacillus halodurans</em> C-125</td>
<td>80.77%</td>
</tr>
<tr>
<td>VMGPVVDVPQOS</td>
<td>NCBI</td>
<td>F0F1 ATP synthase subunit beta</td>
<td>97.22%</td>
</tr>
<tr>
<td>FTQAGSEVSAOALLGR</td>
<td>(NP_244621)</td>
<td><em>Bacillus halodurans</em> C-125</td>
<td>94.44%</td>
</tr>
<tr>
<td>VMGPVVDVPKAN</td>
<td>NCBI</td>
<td>F0F1 ATP synthase subunit beta</td>
<td>100%</td>
</tr>
<tr>
<td>FTQAGSEVSAOALLGR</td>
<td>(YP_006463939)</td>
<td><em>Solibacillus silvestris</em> StLB046</td>
<td>100%</td>
</tr>
<tr>
<td>(b) INRAGDDAAAGLAISEK</td>
<td>This study</td>
<td><em>Solibacillus silvestris</em> AM1</td>
<td>100%</td>
</tr>
<tr>
<td>LGAYQNR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGDDAAGLAISEK</td>
<td>NCBI</td>
<td>Flagellin <em>Bacillus halodurans</em> C-125</td>
<td>97.22%</td>
</tr>
<tr>
<td>INRAGDDAAAGLAISEK</td>
<td>(NP_244483)</td>
<td>Flagellin <em>Bacillus halodurans</em> C-125</td>
<td>94.44%</td>
</tr>
<tr>
<td>LGAYQNR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGDDAAGLAISEK</td>
<td>NCBI</td>
<td>Flagellin <em>Solibacillus silvestris</em> StLB046</td>
<td>100%</td>
</tr>
<tr>
<td>INRAGDDAAAGLAISEK</td>
<td>(YP_006463850)</td>
<td>Flagellin <em>Bacillus silvestris</em> StLB046</td>
<td>100%</td>
</tr>
<tr>
<td>LGAYQNR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGDDAAGLAISEK</td>
<td>NCBI</td>
<td>Flagellin <em>Bacillus</em> sp. Kps3</td>
<td>100%</td>
</tr>
<tr>
<td>INRAGDDAAAGLAISEK</td>
<td>(BAH80325)</td>
<td>Flagellin <em>Bacillus</em> sp. Kps3</td>
<td>100%</td>
</tr>
<tr>
<td>LGAYQNR</td>
<td></td>
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</tbody>
</table>
Figure 4.7. LC/MS-MS spectra of three peptides sequences (a, b & c) obtained from purified bioemulsifier AM1
Similar observations were reported for other sequenced flagellins. While conserved regions of the flagellins play an important structural role by forming hairpin loop structures, the central variable region forms the outer surface of the flagellin filament (Homma, et al., 1987). Reports suggest that flagellins produced by bacteria inhabiting extreme environments are stabilized by their central regions. Albertini, et al. (1991), studied the entire complex of genes involved in flagellation and motility and reported an open reading frame (ORF) that translated into a protein with almost 30% identity to the β-subunit of E. coli ATP synthase. Ours is the first study reporting the similarity of a bioemulsifier to flagellin.

4A.3.4. Physical characterization of bioemulsifier AM1:

Functions of amyloids in bacteria are still not well described but seem to include fimbriae and other cell appendages for adhesion and biofilm formation, cell envelope components, spore coating, formation of large extracellular structures, amyloids acting as cytotoxins and probably several others, as yet unknown. Amyloids assembly is known to be depicted by proteinaceous surface structures of bacteria (Neilson et al. 2011), therefore bioemulsifier AM1 was subjected to various analysis performed for amyloids.

4A.3.4.1. Fourier transform infrared (FTIR) analysis:

FTIR spectroscopy was used to study the chemical structure of purified bioemulsifier AM1. Figure 4.8a represents the spectrum of purified bioemulsifier AM1 at 500-4,000 wavenumbers (cm⁻¹). Peak assignment was performed as per Jagmohan (2001), Suthar, et al. (2008). The FTIR spectrum in Figure 4.8a depicts – OH hydrogen bond stretching of 3400 cm⁻¹ (a), –CH stretching of 2930 cm⁻¹ (b), NH-CO stretching of 1690 cm⁻¹(c), -OH deformation bending of 1274 cm⁻¹ (d) and C-O-C ester stretching of 1066 cm⁻¹(e).

In Figure 4.8b, FTIR spectrum of bioemulsifier AM1 from the amide I region of the spectrum (1600-1700 cm⁻¹) shows the peptide carbonyl stretching frequency, which is sensitive to the local conformation,(Jagmohan, 2001) and additionally, carbohydrate ester bond stretching was observed. This implies towards the glycoprotein nature of bioemulsifier AM1.
Figure 4.8. Fourier Transform Infrared (FTIR) Spectroscopy of the bioemulsifier AM1 depicted in (a) transmittance (%) for a range of 500-4000 cm\(^{-1}\) and (b) absorbance of amide I region for the region of 1600-1800 cm\(^{-1}\).

Amyloid fibres are known to possess a quaternary structure of characteristic cross-\(\beta\) sheets with \(\beta\) strands oriented perpendicular to fibril axis. Amphipathicity is also a common property seen in amyloids with some reported to be able to reduce surface tension (Soreghan, et al., 1994, Gebbink, et al., 2005, Wang & Chapman, 2008, Nielsen, et al., 2011, Blanco, et al., 2012). Although there have been reports of benign amyloid proteins, recent studies have revealed a new class of amyloid proteins extensively employed by microorganisms termed as "functional amyloids". Presence of characteristic amyloid-like \(\beta\) sheet structure of the bioemulsifier AM1 was supported by FTIR spectroscopy results. The major wavenumber was found to be around 1631 cm\(^{-1}\) and a minor peak of 1693 cm\(^{-1}\) was also seen which were suggested to be indicative for antiparallel \(\beta\) strands of an amyloid \(\beta\) sheet (Nilsson, 2004, Cerf, et al., 2009, Zanetti Polzi, et al., 2011).

4A.3.4.2. UV Circular Dichroism (CD) spectrum analysis:

FTIR and CD spectra are usually used for secondary structure analysis of proteins. The far UV CD spectrum of purified bioemulsifier AM1 as depicted in figure 4.9, shows absorption minimum near 220-225nm. As per Jordal et al., (2009), this is a typical amyloid spectral property indicating a \(\beta\) sheet secondary structure in agreement with the expectations for the cross \(\beta\) amyloid fibrils. Therefore bioemulsifier AM1 can be positively said to have amyloid like structural property. These \(\beta\)-type spectral features are known to be predominantly associated with

Figure 4.9. Circular Dichroism (CD) spectrum of the bioemulsifier AM1

4A.3.4.3. SDS-PAGE analysis of denaturant treated bioemulsifier AM1:

A multimeric protein, upon surfactant treatment, should dissociate into monomeric subunits, as was observed with bioemulsifier AM1. To further study this dissociation, bioemulsifier AM1 was treated with various denaturants. Only SDS treatment at high concentration was found to dissociate bioemulsifier AM1 into its individual subunits. At 2% SDS concentration, AM1 dissociated completely, as was shown by the appearance of a single band of approximately 30 kDa molecular weight (Figure 4.10).

High SDS concentration dissociated the multimers into individual subunits of 30 kDa, which was not observed with other denaturants, such as urea, dithiothreitol (DTT), Triton X100, Tween 20 and even low concentration of SDS (1%) with or without boiling (Figure 4.11). SDS disrupted the tertiary structure of the bioemulsifier and resolved the multimer into its monomeric subunits that were localized in a single band of 30 kDa. Upon boiling and 1% SDS treatment, the bioemulsifier separated into five distinct bands after SDS-PAGE: 28, 57, 83, 114 and 136 kDa in approximately 30 kDa increments. From these results, we can predict that protein bioemulsifier AM1 is a homopolymer composed of 30 kDa monomeric subunits. On the contrary, the disappearance of the bands observed in presence of complexing agents like
glutaraldehyde which can bind closely related proteins indicated strongly towards the multimeric nature that too a homopolymer of the protein (homo-multimer).

Figure 4.10. SDS-PAGE (10%) analysis of purified bioemulsifier AM1. Lane 1- Higher range protein marker (M), Lane 2- 0.2% SDS treated active fraction of Gel filtration chromatography (GFC), Lane -3 active fraction of GFC, Lane 4- 2% SDS treated active fraction of GFC.

Figure 4.11. SDS-PAGE (10%) analysis of denaturant treated purified bioemulsifier AM1 (after treatment with urea, glutaraldehyde and also dithiothreitol (DTT), Triton X100 and Tween 20 with boiling (b) and without boiling (nb) in 10% SDS-PAGE)
4A.3.4.4. Transmission electron Microscopy (TEM) and Polarizing microscopy:

The glycoprotein bioemulsifier AM1 was checked for its aggregation properties. The electron microscopic structure of the bioemulsifier aggregate was found to be filamentous with ≈5nm width as seen in bacterial functional amyloids (Figures 4.12a and b). Two or more filaments could laterally associate to give a wider width than that seen in typical amyloid filaments.

After TEM, variety of techniques were used to confirm the amyloid nature of bioemulsifier AM1. Congo-red birefringence is considered to be one of the standard assays for amyloids (Wolman & Bubis, 1965). Bioemulsifier AM1 stained red with Congo-red dye and exhibited typical amyloid-like greenish-yellow to greenish-blue colour in polarized light microscope (Figure 4.13).

Figure 4.12. Transmission electron micrograph of purified bioemulsifier AM1 (a, bar = 50 nm; b, bar = 5 nm).

Figure 4.13. Polarizing microscope images of bioemulsifier AM1 stained with congo red. (a) Light microscopy and (b) polarization microscopy (bar = 50μm)
A total score of 4 must be achieved by satisfying a combination of the following criteria: β sheet secondary structure (score=2), congo red binding (score=2), denaturant or protease resistance (score=1), ThT or ThS binding (score=2), gel formation (score=1) and protofibril intermediate (score=1). Using these standard criteria and scoring technique set by Nilsson, (2004) for identification of amyloid fibrils, the bioemulsifier AMI gets a score of 5 that is the first three criteria. With minimum score for 4 required to be achieved by the protein in question for categorization into amyloid proteins, bioemulsifier AMI can be considered as a glycoprotein amyloid with emulsification properties.

4A.3.4.5. Congored-plate assay

Bacteria producing amyloids on their surface are known to be taking up the congo-red stain when grown in its presence (Romero, et al., 2010). As discussed, the bioemulsifier produced by S. silvestris AMI has amyloid properties. S. silvestris AMI is known to be producing cell bound and cell free bioemulsifier AMI in protein rich medium like ZM medium and negligible or no bioemulsifier in medium containing no protein-carbon source. Figure 4.14a does not show the typical red coloration of the S. silvestris AMI colonies when it produces bioemulsifier. When such media were amended with congo-red, the colonies producing bioemulsifier AMI in ZM agar medium exhibited typical red colour (figure 4.14b), demonstrating the presence of amyloid bioemulsifier AMI on their surface. Thus indicating amyloid like properties associated with the cell bound bioemulsifier.

Figure 4.14. S.silvestris AMI grown in (a) Bushnell Haas (amended with 1% acetate) medium and (b)Zobell Marine agar medium
4A.3.4.6. Temperature, pH and salt stability of bioemulsifier AMI:

As evident in Figure 4.15a, bioemulsifier AMI is highly thermostable, retaining 33.81% $E_{24}$ even after 5 h of treatment in boiling water (100°C). However, the emulsion formed was not stable after 24 h. The $E_{24}$ remained between 55.1 and 40.5% after treatment with pH values ranging from 4 to 9 (Figure 4.15b). The emulsification activity was negligible below pH 4. The $E_{24}$ of the bioemulsifier dropped from 47 to 30% after 2 M NaCl treatment but remained constant up to 5M NaCl treatment (Figure 4.15c). Nevertheless, the $E_{24}$ of the bioemulsifier never dropped below its half-life in any of the conditions tested (other than pH 3).

The results of the experiments described here and in the previous section (SDS PAGE analysis) show that the bioemulsifier resists extreme temperatures, NaCl concentration, pH, and dissociation by detergents. Multimeric association of the emulsifier could be due to either hydrophobic interactions or to ionic, hydrogen or covalent bonds. Because the bioemulsifier AMI multimer could not be disrupted completely by detergents, it may not be dependent on hydrophobic interactions. Additionally, it was previously observed that boiling in the presence of detergents results in the dissociation of the multimeric complex; therefore, we can conclude that hydrophobic interactions are only partially responsible for multimer stability, which was also reported by Law & Levine (1977). Stability in high salt concentrations and at high pH values indicates that inter-subunit interactions may not to be due to ionic or hydrogen bonds. Amyloid fibre formation and stability are reported to be also dependent on hydrophobic interactions (Marshall, et al., 2011).

As described by Dimmitt & Simon (1971), purified flagellins are relatively stable compared to those obtained by shearing flagellar filaments. The variable region of flagellin from *B. halodurans* C-125 is hypothesized to give stability to the protein in the host’s alkaline environment (Sakamoto, et al., 1992). There exists a high probability that the resilience to NaCl concentration and pH, as well as to temperature (thermostability) is properties imparted to a bioemulsifier because of its primary structure bearing similarity to flagellin. The best match of the flagellin-like peptide of bioemulsifier AM1 with *B. halodurans* C-125 and *Bacillus* sp. Kps3 that are grown in extreme environments also supports this observation (Table 2.2).
Critical micellar dilution (CMD):

If the concentration of bioemulsifier produced by a bacterium is above its CMC, an increase in its concentration cannot be detected. Consequently, two cultures with very different concentrations of bioemulsifier may display the same activity. This problem can be overcome by serially diluting until a sharp decrease in emulsification is observed, in this case by measuring critical micellar dilution (CMD) (Walter, et al., 2010).

As shown in Figure 4.16, the CMD of bioemulsifier AM1 was twenty times diluted as compared to Emulsan which was diluted 6.66 times, i.e., a three times
higher dilution is required to reduce the activity to zero comparing to Emulsan. According to Oliveira, et al. (2006) and Makkar & Cameotra (1997), CMD is an indirect means of measuring the surfactant production relative to the CMC range. Thus, it can be inferred from Figure 4.16 that S. silvestris AM1 has significantly higher (P = <0.001) bioemulsifier production than A. calcoaceticus RAG-1.

**Figure 4.16. Critical Micellar Dilution (CMD) of bioemulsifier produced by S. silvestris AM1.**
Chapter 4: Characterization studies - Characterization of bioemulsifier BM1 and its emulsions

4B. Characterization of emulsion

4B.1. Introduction

Dispersion, usually temporary mixture of two immiscible phases and if it lasts long, it is said to be a suspension. Thus the difference between dispersion and suspension is indistinct but it is known that more uniform the specific gravities of the two phases, and smaller the particle size of the dispersed phase, the dispersion lasts long. When the particle size is very small (colloidal), the system approaches the appearance of a solution. When there is a strong attraction between dispersing medium and the particles, the system is called an emulsoid. When this emulsoid (or Lyo-philic or showing affinity) requires a stabilizing (emulsifying) agent for greater/lesser stability, the colloid is called an emulsion (Harkins, 1947, Bennett, et al., 1968).

Surface active compounds, when added into water, are adsorbed at the surface resulting in decreased surface tension. When the liquids are shaken, a large area free of surfactant is created. The molecules from the bulk solution, the air-water or oil-water interfaces adsorb and cover the equilibrium monolayers and prevent bubbles and droplets from coalescence, thus forming emulsions and foams. Depending on the laboratory method used, the interfaces are stretched rapidly and ruptured in different flow conditions and forming emulsions. Hence different types of flow used lead to different rupture mechanisms. Surface rheology plays role in the formation of emulsion once the threshold for rupture is crossed in a well defined flow. The energy used during emulsification process is directly proportional to elasticity and affect emulsion stability. Thus the importance of emulsification is a direct result of high surface area to volume relationship. An emulsion can be defined as a dispersion of two mutually immiscible liquids usually containing an emulsifying agent (surface active agents) (Harkins, 1947, Bennett, et al., 1968, Langevin, 2000).

Emulsions have been historically considered to be dispersion of oil and water. Hence emulsions are also classified into oil-in-water (O/W), water-in-oil (W/O) and dual emulsions. An oil-in-water (O/W) emulsion has oil as the internal phase and water as the external phase and can be easily diluted by adding more water at the cost of stability. A water-in-oil (W/O) emulsion consists of dispersion of oil external phase with water internal phase and cannot be diluted by water. By process of inversion,
simultaneous switching of internal and external phases occurs as a result of mechanical or chemical action. Thus the relative concentration of the phases drive the change and an emulsifying agent fairly soluble in both the phases can help in formation of these dual emulsions (Harkins, 1947, Bennett, et al., 1968).

The particle size of an emulsion is one of its most important characteristics. It is the function of nature of the phases in mixture, their quantity, also type of the emulsifying agent and the processing method used. For an emulsion droplet, the most thermodynamically stable energy configuration in an emulsion is spherical as it minimizes the surface area thus reducing the surface energy of the particle. This same minimizing surface energy tries to stabilize the configuration of emulsion as a whole which is as two distinct phases with lowest thermodynamic energy levels. Hence the type and concentration of emulsifier should provide sufficient thermodynamic energy to maintain the particulate dispersion of the internal phase. Increasing the concentration of emulsifier in the emulsion will result in smaller particle size (Bennett et al., 1968).

Rheology can be defined as the study of the deformation and flow of matter. In rheological terms, the tendency of a fluid to flow is called fluidity and the measure of its resistance to flow is called viscosity. Viscosity can also be defined as the force required to compel two parallel liquid surfaces of unit area separated by a distance of one differential unit to slide past each other with a constant unit of velocity. Thus viscosity is the factor of proportionality between rate of shear and shearing stress. When a fluid's flow is independent of the amount of shear, the fluid is termed as a Newtonian fluid while when a fluid has a viscosity as a function of shear and time of its application, it is called as a non-Newtonian fluid. A non-Newtonian fluid can be further classified into, plastic, pseudoplastic, thixotropic, dilatants and rheopectic. The shear stress of a plastic fluid should first exceed a minimum threshold value before it flows as seen in some synthetic resins. Pseudoplastic fluid viscosity decreases proportionally to the increase in shear rate irreversibly. The apparent viscosity of a thixotropic substance decreases reversibly with time to particular minimum value with constant increase in shear. Dilutant fluid's apparent viscosity is known to increase instantaneously with increase in shear rate as seen in starch in water while in rheopectic fluid, the viscosity increases to a maximum value at any constant rate of shear. The application of the emulsion industrially is dependent upon the emulsion's
rheological properties. Emulsion characteristics are important for its applications hence the characterization of emulsion formed by bioemulsifier AM1 was done (Bennett et al., 1968).

4B.2. Materials and Methods

4B.2.1. Comparative relative emulsion stability (%ES) in different solvents:

The emulsification activity of bioemulsifier AM1 was compared with Emulsan in 14 different solvents and was examined for up to seven days. Xanthan (0.1%) was used as a positive control. The following solvents were used: hexane (H), heptane (Hp), decane (D) and hexadecane (Hd), benzene (B), toluene (T), xylene (X) and trichlorobenzene (TCB), paraffin oil (Po), cottonseed oil (Co), groundnut oil (Go), silicone oil (So), white oil (Wo) and kerosene (K). Each test was performed in triplicate.

Relative emulsion stability (%ES) was calculated using the following formulae (Das, et al., 1998, Kebbouche-Gana, et al., 2009):

\[
\% \text{ Emulsion volume (EV)} = \frac{\text{Emulsion height (mm) } \times \text{ Cross-section area (mm}^2\text{)} } {\text{Total volume of mixture (mm}^3\text{)}} \times 100
\]

\[
\% \text{ Emulsion stability (ES)} = \frac{\text{EV at time } t \times h}{\text{EV at time } 0h} \times 100
\]

4B.2.2. Bright-field microscopy of emulsion droplets:

Microscopic examination of emulsions formed with the previously listed fourteen solvents was performed using a bright-field Olympus microscope, model CX41, at 100X magnification. The images were analyzed using ProgRes CapturePro 2.7 imaging software (JENOPTIK optical systems, USA).

4B.2.3. Particle size analysis and Shear rate of the emulsion droplets:

Emulsions were prepared by homogenizing 0.5 mg/ml purified bioemulsifier with paraffin oil and TCB (1:1). Particle size analysis of the emulsion droplets was performed using Sympatec HELOS –BF particle size analyzer. Rheological properties of the emulsion, such as shear stress and shear viscosity, were measured by a Brookfield LV DV-III rheometer using CPE52.
4B.3. Results and Discussion

In this section, results of the characterization of emulsion produced by bioemulsifier AM1 are presented.

4B.3.1. Characterization of the emulsion formed by bioemulsifier AM1:

The relative emulsion stability (%ES) of bioemulsifier AM1 was found to be 89.7, 84.8, 81.7 and 79.32% with TCB, decane, groundnut oil and xylene, respectively, and these values were greater than those given by Emulsan (Figure 4.18). As shown, the best emulsion stabilization by bioemulsifier AM1 was in TCB, while the most negligible was in hexadecane. In hexane, heptane, toluene, silicone oil and paraffin oil, %ES values between 47.48 and 61.30% were found. In benzene, cotton seed oil, kerosene and industrial white oil, the relative %ES range was between 24.51 and 35.26%. The maximum relative emulsion stability of 107.7% was found to be Emulsan in silicone oil (Figure 4.18). Bioemulsifier AM1 showed stability in all types of solvent, whether aliphatic, aromatic or in oils. However, Emulsan showed better stability with oils and very low stability in aliphatic and aromatic solvents. The differences between all values of solvents used for bioemulsifiers AM1 and RAG-1 has a 0.37% chance of random occurrence in this experiment (p-value = 0.0037).

Bright-field microscopic studies of the emulsions formed by bioemulsifier AM1 corresponding to E24 and E168 in the solvents studied, showed a marked increase in droplet size, except in the case of TCB where there was a decrease in droplet size and in the case of hexadecane where no emulsion was formed (Figure 4.19). Droplet size variation was least in the emulsion with decane. Figure 4.19 shows benzene losing most of its smaller emulsion droplets after 168 h, while in trichlorobenzene, bigger droplets became less common after 24 h. Among oils, the emulsion droplet size increased in kerosene and white oil (Figure 4.20). The density of droplets increased in the emulsion formed at 168 h for stable emulsions, such as those formed in TCB and decane.

A bioemulsifier capable of producing a good and stable emulsion with all types of hydrophobic solvents (aliphatics, aromatics and oils) would be a versatile process material in industries. The results herein achieved are in accordance with the previous report that describes the inability of A. calcoaceticus RAG-1 to effectively emulsify low-molecular weight benzene derivatives, aromatic compounds containing
more than one ring, branched chain aliphatics from pentane to octadecane and unstable emulsions formed with kerosene and gasoline (Zuckerberg, et al., 1979).

Figure 4.18. Comparative relative emulsion stability (ES%) of bioemulsifier from AM1 and Emulsan in 14 different solvents with respect to the standard emulsifier Xanthan. Hexane (H), heptane (Hp), decane (D) and hexadecane (Hd), benzene (B), toluene (T), xylene (X) and trichlorobenzene (TCB), paraffin oil (Po), cottonseed oil (Co), groundnut oil (Go), silicone oil (So), white oil (Wo) and kerosene (K).

Many reported organisms, such as Planococcus maitriensis Anita I (Kumar, et al., 2007), Antarctobacter sp. TG22 (Gutiérrez, et al., 2007) and Penicillium citrinum (De Morais, et al., 2006), displayed good emulsification with oils. The yeasts Torulopsis petrophilum (Cooper & Paddock, 1983) and Saccharomyces cerevisiae (Cameron, et al., 1988) were reported to produce compounds that emulsify in aliphatic and aromatic compounds. However, reports of microorganisms producing stable emulsions in all the three are rare. Thus, the ability of bioemulsifier AM1 to emulsify aliphatics, aromatics and oils is a significant property, and its efficiency can be increased further by optimizing the medium components and by yield enhancement.

4B.3.2. Particle size analysis and shear rate of emulsion droplets:

The particle size distribution patterns of emulsion droplets in paraffin and TCB are shown in Figure 4.21. A particle size shift was observed in TCB, while no
shift was observed in paraffin oil. Particle size of the emulsion with TCB was increased at 24 h (Sauter Mean Diameter, SMD= 127.69 μm) as compared to at 168 h (SMD= 97.47 μm), coinciding with a shift from a bimodal distribution at 24 h to a unimodal distribution after 168 h. Conversely, particle size in the emulsion formed using bioemulsifier AM1 and paraffin light oil (Po) was relatively small at 24 h (SMD= 49.18 μm) and increased thereafter, showing larger particles in emulsion at 168 h (SMD= 68.25 μm).

In an emulsion, smaller droplets are considered to be more rigid than larger droplets because they are deformed into polyhedral shapes. The formation of two distinct phases by coalescence of these larger droplets leads to a greater decrease in free energy than is achieved by mere aggregation, as observed in particle size shifts in these experiments (Figure 4.21). When two droplets come together, either by Brownian collision, shear-induced collision or gravitational force, the interaction of the two droplets (inter-particle forces) and the viscous force from the fluid drainage increase the contact area between the two particles, thus increasing the area of interface. This rapid stretching of the interface increases the local interfacial tension and opposes stretching (Goodwin, 2009).

Differences in static values and dynamic surface tension resist expansion and force the movement of the stabilizing agent (bioemulsifier) into the interaction zone, resulting in the Marangoni effect (drainage of fluids is opposed by fluids drawn into the thin interfacial film by viscous drag). Stability is sustained if the emulsifier is strongly adsorbed at the interface when the droplets come together. In a polydispersed emulsion, larger particles will have lesser solubility than smaller particles and tend to grow at the expense of the latter, which tend to dissolve and lead to Oswald ripening (Shaw, 2003). TCB, having higher density than water, forms a layer at the bottom of the test-tube. Thus, the emulsion formed with it is always in a compressed form because of the constant presence of an aqueous phase over it, leading to a size shift during the incubation and stabilization of the emulsion by smaller droplet packing and separation of the larger droplets from the emulsion into the immiscible phase.

As described by Bennett et al. (1968), the viscosity of an emulsion is a factor of proportionality between the rate of shear and shearing stress. The gradual reduction in viscosity (η) of both emulsions formed in paraffin and TCB shows their non-Newtonian nature and shear thinning effect for shear rate values used in this study.
Figure 4.19. Brightfield micrographs of the emulsion of *S. silvestris* AM1 with aliphatic (first row) and aromatic (second row) solvents at 24h & 168h
Figure 4.20. Brightfield micrographs of emulsions of *S. silvestris* AM1 with different oils as solvents at 24h & 168h
The loss of emulsion viscosity and the shear thinning phenomenon exhibited by the emulsion formed using the two solvent systems can be attributed to the proteinaceous bioemulsifier AM1 and hydrocarbon system. Pseudoplastic non-Newtonian emulsions, as observed in paints, show an instant decrease in viscosity with an increase in shear rate, as is observed in the rheogram of bioemulsifier AM1 depicted in Figure 4.22. This result means that the emulsion formed by bioemulsifier AM1 is non-adhesive, viscous and stable to temperature and stress over extended time periods, which is a distinct advantage in cosmetic, nutritive and pharmaceutical formulations.

**Figure 4.21.** Particle size analysis of emulsion droplets formed using paraffin light oil and trichlorobenzene after 24 h and 168 h against bioemulsifier AM1.

**Figure 4.22.** Analysis of shear rate and viscosity change of the emulsion produced by bioemulsifier AM1 with paraffin and TCB.
A novel estuarine bacterial strain, *Solibacillus silvestris* AM1, was found to produce an extracellular, multimeric glycoprotein bioemulsifier, termed AM1, with a MW of 200 kDa and containing 30 kDa monomeric subunits. The bioemulsifier contained 3.6% of the minor carbohydrate components galactose and ribose/xylose. LC/MS-MS of the 30 kDa subunit revealed its homology with a flagellin-like protein arranged in the form of fibers, as shown by a transmission electron micrographs. This is the first report of a flagellin-like protein that exhibits bioemulsifier activity being produced from a member of the *Solibacillus* genus. Bioemulsifier AM1 has a high emulsification index of 62.5% with $10^{-2}$ critical micellar dilution. It was found to be thermostable and active in the pH 5-9 and 0-5 M NaCl ranges. Moreover, AM1 formed stable emulsions with a broad range of solvents, including aliphatics, aromatic hydrocarbons and oils, performing better than the well-known bioemulsifier Emulsan. Emulsions formed with trichlorobenzene and paraffin oil have a pseudoplastic non-Newtonian rheological property, as observed by particle size and shear stress analysis. AM1, an eco-friendly bioemulsifier, formed stable emulsions in varied physical conditions, and these attributes may prove to be advantageous in cosmetic, pharmaceutical and environmental applications.

An interesting observation in this chapter is regarding the amyloid nature of bioemulsifier AM1. Picking the cue from TEM observations of fibre-like bioemulsifier conformation, secondary structure analysis was done by FTIR, CD and Congo red birefringence in polarizing microscopy, where the antiparallel $\beta$ strands observed established the amyloid nature of bioemulsifier AM1. The amyloid nature of the cell bound bioemulsifier was also shown in Congo red plate assay, where the cell surface amyloid biomolecules are stained red. According to Neilson *et al.* (2011), the functions of bacterial amyloids are not yet clear, but the list of bacteria producing them is expanding. Since bioemulsifier AM1 production was triggered only in certain nutritional conditions, it can be said that it is produced only when its role is needed by the bacterium, envisaged as in adhesion and thereby biofilm formation as observed in chapter 5.
References:


Chapter 4: Characterization studies - Characterization of Bioemulsifier J44M1 and its emulsions


