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Formulation and Applications of Biodegradable Rhamnolipid and Sophorolipid Biosurfactant

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Abstract: The hydrophilicity /hydrophobicity of sophorolipid bio surfactants has been evaluated in this research in comparison to conventional synthetic surfactants. Microemulsions of lecithin/rhamnolipid (PMNB)/sophorolipid biosurfactant were also formulated with a range of oils (varying EACN values and oil types). Sodium dihexylsulfosuccinate (SDHS) and rhamnolipid bio surfactant are less hydrophobic than sodium bis (2-ethyl) dihexyl sulfosuccinate (SBDHS), which in turn is lesser hydrophobic than the Sophorolipid biosurfactants.

Therefore sophorolipid being the hydrophobic component in lecithin/rhamnolipid/sophorolipid biosurfactant formulation. Due to no significant changes in phase behavior with isopropyl myristate with changing temperature (15, 25, 45°C) and electrolyte concentration (0.9% and 5% w/v), it makes it accessible for cosmetic and drug delivery applications. At the same surfactant active concentrations, our biocompatible formulation has higher hexadecane detergency performance compared to any commercial liquid detergent. This paper highlights the formulation of microemulsions by mixed biosurfactant systems for a range of oils and their potential applications.

Keywords: Rhamnolipid, Sophorolipid, Microemulsion, Detergency, Biosurfactant

I. INTRODUCTION

Biosurfactants include variety of structures including glycolipids, phospholipids, polysaccharide-lipid complexes, and lipopeptides and hydroxylated cross-linked fatty acids, produced by microorganisms. The most common glycolipid biosurfactants, which are of interest in this research are Rhamnolipid and sophorolipid. The glycolipid species have a composition of carbohydrate heads and lipid tails as shown in fig.1 [1, 4, and 5]

Rhamnolipid biosurfactants have two hydrophilic head groups:

- 1) Carboxylate group: give them an anionic character.
- 2) rhamnosyl group: contributes to the bulkiness of the head group. Two identical tails of C8 alkyl chains fig.1. [1, 6] are present. The hydrophilicity-hydrophobicity balance (HLB) of rhamnolipid has been reported as 22-24 [6].

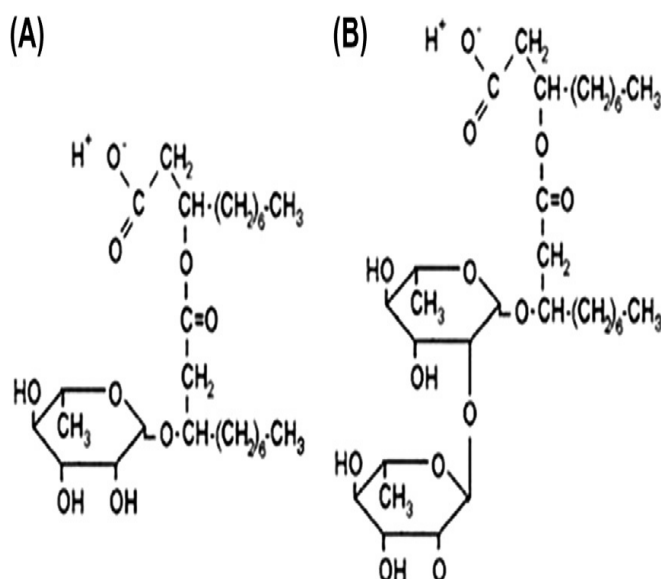


Fig.1. Structures of the Rhamnolipid : (A) monorhamnolipid and (B) dirhamnolipid [1,4]

On the other hand, Sophorolipid has only one long tail of an unsaturated fatty acid. Its two conformations are found during production:

- 1) *lactone form*: resulting from the esterification of carboxylic acid group to the disaccharide ring.
- 2) *acidic form*: In this form, two acetyl groups attached to the dimeric sugar sophorose headgroup (which also make it insensitive to temperature) and carboxylic acid head groups.

The sophorolipid are more hydrophobic due to the presence of Acetylated groups which lower the hydrophilic character.

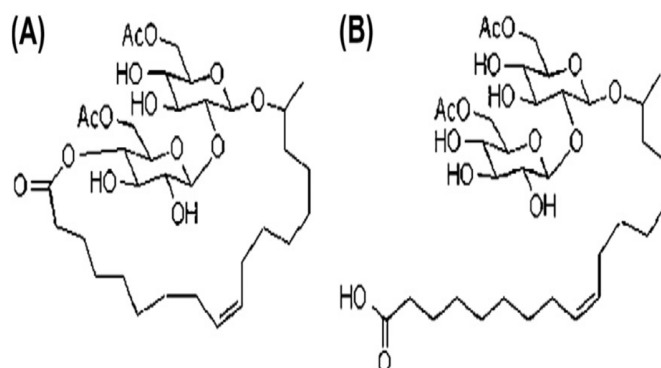


Fig.2. Structures of the sophorolipid (Ac=acetyl) (A) lactone form and (B) acidic form

Due to the biodegradability, low toxicity, antimicrobial, anti-HIV, spermicidal and moisturizing activities of rhamnolipid and sophorolipid biosurfactant, they have applications in various sectors like Bioremediation, microbial enhanced oil recovery, food and cosmetic industries, pharmaceutical industries, etc [1,9-16]

Microemulsions are isotropic dispersions of oil, water and surfactant and they are also thermodynamically stable [17]. They produce high solubilisation capacity and ultraflow interfacial tensions of oil and water. Out of the four forms of Winsor microemulsions, Type I and II are two phase systems. The tendency of Lecithin-based microemulsions to mimic the phospholipid nature of cell-membranes makes them desirable in biocompatible formulations[1].

The main objectives are:

- a) To check the hydrophobicity of sophorolipid biosurfactant.
- b) To use sophorolipid to test its ability to produce alcohol-free lecithin based biocompatible microemulsions for a range of oils including limonene, decane, isopropyl myristate, and hexadecane.
- c) To evaluate the lecithin-based sophorolipid microemulsions for changes in temperature and electrolyte.
- d) Comparison of commercial detergent and lecithin-based sophorolipid formulation for detergency power for removal of hexadecane. Among all studies, only detergency results are included in this paper.

II. MATERIALS AND METHODS

A. Materials

- 1) *Microorganisms and Culture Conditions*: Strain *Pseudomonas aeruginosa* (424) was used which was produced from Microbial type culture collection and Gene bank (MTCC), Chandigarh.
- 2) *Apparatus Required*
 - a) *Autoclave*: used for steam sterilization of media
 - b) *Oven*: For dry heat sterilization
 - c) *Laminar air flow*: To provide sterilize condition for culture maintenance.
 - d) *Incubator Shaker*: To maintain growth condition

To maintain temperature and to separate the phases of different densities, separating funnel and water bath are used.

- 3) *Fermentation Media*: For preparation of the inoculums, the nutrient broth was used.

The composition of the used nutrient broth was as follows: beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g in one liter of distilled water. 15.0 g of agar was added to the nutrient broth to make the nutrient agar. For about 16-18 hrs. at room temperature, the cultures were grown in this broth. Composition used for the synthesis of biosurfactant is:

Table: 2 Mineral salt medium compositions

Components	Quantity (g/l)
------------	----------------

NaNO ₃	1.0
KH ₂ PO ₄	1.0
K ₂ HPO ₄	2.0
MgSO ₄	1.0
CaCl ₂	0.02
FeSO ₄	0.002
KCl	1.0
Trace elements	1 ml/l

1.5% glycerol as sole carbon source was used to grow Bacterial culture in basal salt medium. It as incubated for 48 hrs at 100 rpm, 25°C in incubator shaker. Basal salt medium (BSM) was used to incubate culture with each substrate and yeast extract to enhance the growth. They were incubated for 48-72 hrs in incubator shaker at 100 rpm, 25°C. After the detection of the froth it was centrifuged at 6000 rpm for 15 min to obtain cell free supernatant.

4) *Surfactants used in this work:* [1,4,5,25]

- a) Sodium di hexyl sulfosuccinate (SDHS : Mol wt. 376)
 - b) Sodium bis (2 ethyl) di hexyl sulfosuccinate (SDBHS: Mol Wt. 432)
 - c) Lecithin (Mol Wt. 770)
 - d) Rahmnolipid synthesized from pseudomonas aeruginosa (PMNB)
 - e) Sophorolipid – Oleic Acid (SPL-O)
 - f) Sophorolipid – Palmitic Acid(SPL-P)
- 5) *Oil Used:* Benzene, Limonene, Decane, Isopropyl Myristate, Hexadecane [1,18,25,28]

B. Methods

- 1) *NMR and FTIR Analysis:* Magnetic nuclear response (NMR) and FTIR are used to analyze purified rahmnolipid (PMNB-synthesized from Pseudomonas aeruginosa) samples. JEOL JNM-ECS400 spectrometer manufactured by Tokyo, Japan was used for ¹H and ¹³C spectrum. PerkinElmer Spectrum Version 10.03.06 manufactured by USA was used to study FTIR.
- 2) *Phase Study:* Equal volumes of the aqueous and oil phase (6ml of each phase) were placed in 15ml glass tubes (diameter of 12mm) to do the Phase studies. Teflon screw caps were used to minimize evaporation when benzene was used as the oil phase. Sample tubes were hand-shaken for one minute once a day for the first three days, and then left in a water bath to get equilibrated at desired temperatures for two weeks [1,25]. By passing light through the phases, the microemulsions were identified as visual. All experiments have been done at 25C unless stated otherwise.
 - a) *Sophorolipid Hydrophilicity / Hydrophobicity Study:* Using Rhamolipid as the reference, it was mixed with SBDHS, SDHS, SPL-P, and SPL-O individually. The total surfactant concentration was fixed at 0.1M. Salinity was scanned to determine the optimum formulation for each surfactant ratio where, in middle phase microemulsions, equal amounts of oil and water were solubilized. In this study, we considered benzene to be the oil. The height attained after equilibrium to each phase in the middle phase micro emulsion was the phase volume.[1,25]
 - b) *Microemulsion Formulations Of Surfactant Mixtures With Various Soils:* Combination of lecithin, SPL-O, and PMNB were used to study the surfactant mixtures. Keeping the Lecithin to SPL-O ratio constant at 1/1 by weight and the electrolyte concentration at 0.9% w/v for most experiments. As this is the maximum salinity concentration that doesn't cause irritation in human cosmetic and pharmaceutical products. The PMNB concentration was varied for each lecithin concentration to delineate the transition between the different microemulsion types. Based on the varying lecithin concentration with varying PMNB concentration Fish diagrams were constructed for Limonene and IPM. The lecithin/SPL-O concentration was fixed at 5/5%wt for decane and hexa-decane keeping the PMNB concentration variable. For each oil, the resulting interfacial tension values were measured. The phase studies with IPM were done at 15 and 45° C and the rest were done at 25C.
- 3) *Interfacial Tension:* Glass capillary tubes and a spinning drop tensiometer (USHA Instruments, Kolkata) are used to measure the interfacial tension value between the excess oil and the excess water phase. The capillary tube is 2 mm in diameter and has a volume of 300 microL. After filling the excess water (the denser phase) in the tube, 1-5 microL of the excess oil (the less dense phase) was injected into the aqueous solution to form a droplet [1,29]. Thus to measure the droplet size, the filled tube was placed in the spinning drop tensiometer.
- 4) *Detergency Test:* The model 7243 US Testing machine and ASTM standard D3050-98, "Standard Guide for Measuring Soil Removal from Artificially Contaminated Soils", were used for the detergency test[1,30]. Fabrics were artificially stained of

chloroform by immersing the already cut piece of 3*4 in. into a solution containing 20% by volume of hexadecane dyed with 200 ppm of oil red O. The ventilated hood was used to dry these fabrics [1, 31-32]. Agitation speed of 120 rpm, room temperature, washing time of 20 minutes and surfactant formulation solution of 1L are the desired test specifications. Two rinse steps with deionized water were followed; 1st rinse for 3 min and the 2nd rinse for 2 min [33]. These detergency tests are done in triplicate. The post-wash fabrics were dried overnight before determining detergency efficiency; calculated based on the reflectance of the pre-wash and post-wash stained fabrics. The ultrascan sphere spectrophotometer was used to measure the reflectance at 520 nm(Hunter Lab). The detergency(%) calculated was based on the following equation:

$$\text{Detergency}(\%) = [(A - B)/(C_0 - B)] \times 100$$

Here, A is the average reflectance of the soiled fabrics after washing, B is the Average reflectance of the soiled fabrics before washing, And, C is the average reflectance of the unsoiled fabrics before washing[1,33]. For comparison purposes, study was also on commercial detergents.

III. RESULTS AND DISCUSSION

A. Chemical Characterization Of Purified Biosurfactant

Following inferences were obtained from the figure below, the characteristic stretching of -OH group is observed at 3417 cm⁻¹. The symmetric stretch of (-C-H) of -CH₂-and -CH₃ groups of aliphatic chains are represented by absorption bands around 2927 cm⁻¹, 2856 cm⁻¹ and 1402 cm⁻¹, and absorption band at 722 cm⁻¹ was assigned to -(CH₂)_n- (n = 6) group. The -C=O group and -C-O-C- group were indicated by intense absorption bands at 1722 cm⁻¹ and 1068 cm⁻¹ respectively. The weak absorption band at 1722 cm⁻¹, 1651 cm⁻¹, 1377 cm⁻¹, 1124 cm⁻¹ and 982 cm⁻¹ were the unsaturated bond (-C=C-) of aliphatic chains. On comparing, these functional groups are in accordance to the characteristic structure of rhamnolipid.

NMR was used to identify the detailed chemical structure, with the basic three parameters; chemical shifts of the absorption frequency, coupling (mutual influence of adjacent nuclei), and integral height [24].

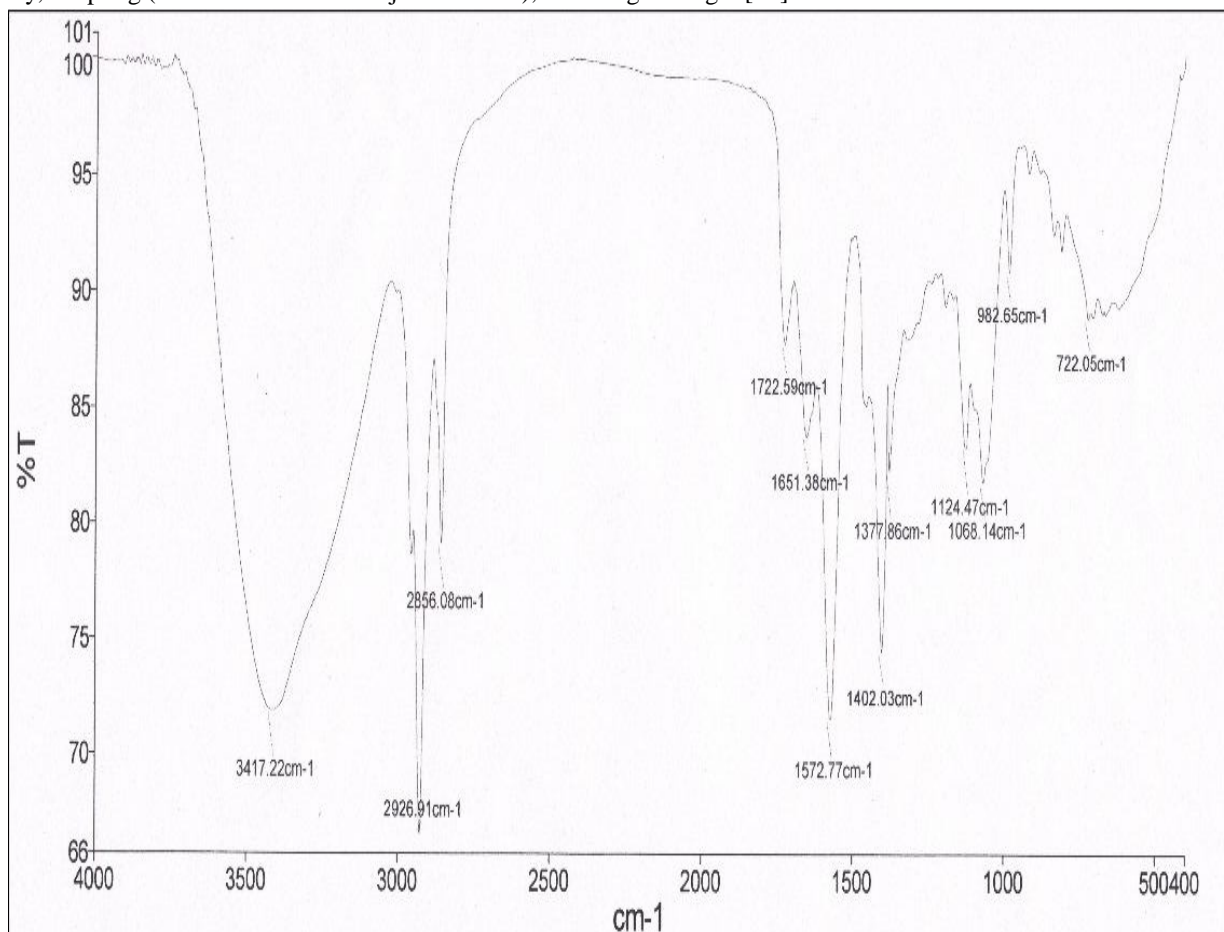
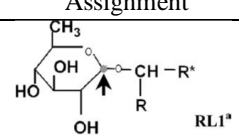
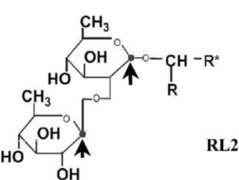


Figure 2: FT-IR spectra of the purified biosurfactant produced by Pseudomonas ae. 424.

Table: 7 Chemical shifts of purified rhamnolipid in ¹H NMR and ¹³C NMR spectra

¹ H chemical shift (ppm)	Multiplicity	Assignment
0.856	Triplet	--CH ₃
1.232	Multiple	---(CH ₂) ₆ ---
2.742	Doublet	--CH ₂ —COO--
4.100	Multiple	--O—CH--
5.308	Multiple	--COO—CH—
¹³ C Chemical shift (ppm)	Assignment	
76.773		
77.412		

^aRL1 (rhamnolipid 1): L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate

^bRL2 (rhamnolipid 2): L-rhamnosyl L-rhamnosyl-bhydroxydecanoyl-b-hydroxydecanoate

Figure 3 shows the NMR analyzed pure rhamnolipid. Following characteristic shifts were observed from ¹H NMR analysis were 0.856 ppm (for -CH₃), 1.232 ppm (for -(CH₂)₆), 2.742 ppm (for -CH₂-COO-), 4.100 ppm (for -O-CH-), and 5.308 ppm (for -COO-CH-). The ¹³C NMR also displayed characteristic shifts of 76.773 ppm (characteristic of RL1) and 77.412 ppm (characteristic of RL2). All those results indicate the molecular structures of common rhamnolipid types, produced by *P. aeruginosa* strains, L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (RL1) and L-rhamnosyl-L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (RL2)[25,26].

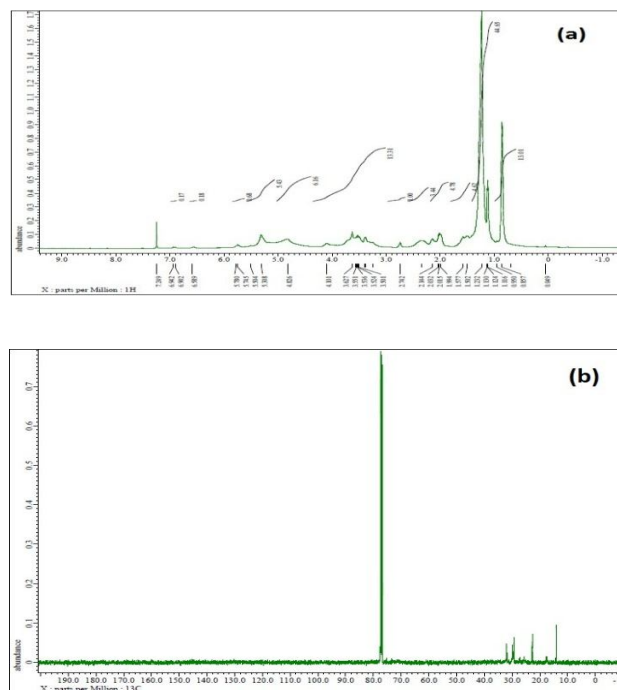


Figure 3: ¹H NMR spectra (a) and ¹³C NMR spectra (b) of the purified biosurfactant produced by *Pseudomonas ae.* 424.

B. Sophorolipid Hydrophilicity/Hydrophobicity

The given fig.3 plots the optimum salinity (S^* is the salinity at which the optimum formulation is obtained) for each surfactant mixture as a function of molar fraction of PMNB, a common surfactant in all surfactant mixtures. The observations were made for all the four studied surfactants, which concluded that increasing the PMNB mole fraction increases the optimum salinity for the mixture. The simplified Winsor R-ratio is defined as follows:

$$R = A_{co} / A_{cw}$$

Here, A_{co}/A_{cw} implies the ratio of interaction between the surfactant adsorbed at the interface with the oil phase and the water phase respectively [1,34].

For $R > 1$, the oil-surfactant interaction is stronger than the water-surfactant interaction while the opposite is true for $R < 1$. Hence, the system for which $R < 1$, type I microemulsion system is formed and the system for which $R > 1$, type II is formed. At $R = 1$, the water-surfactant and the oil-surfactant interactions are balanced, thus forming optimum formulation (type III microemulsion). Tuning parameter such as salinity or temperature may change at least one of the interactions for any change in them [1,35]. For example, as the salinity decreases, A_{cw} will increase and thus the R ratio will decrease which results in a phase transition from type III to type I. Therefore to increase the R ratio to 1 for a given oil phase, higher salinity is required for a more hydrophilic surfactant system.

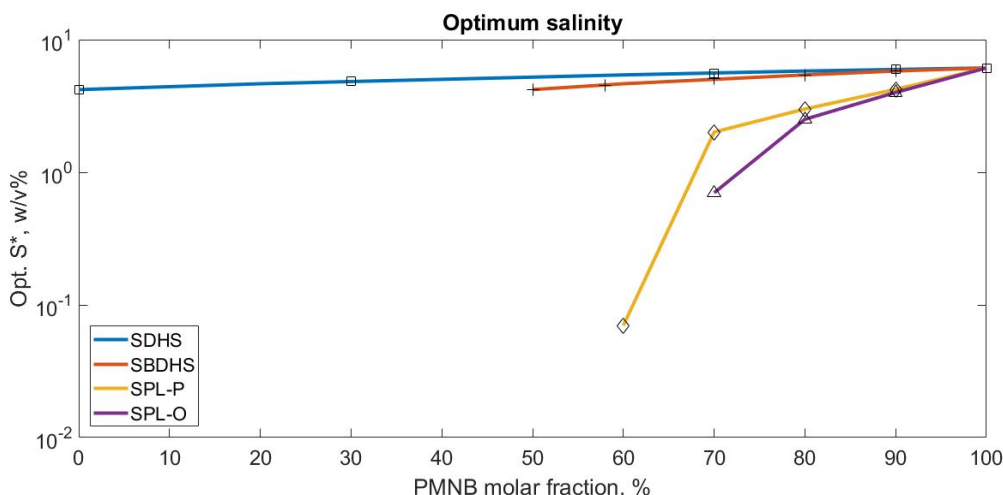


Fig.3. optimum salinity(S^*) for varying fraction of rhamnolipids

According to Winsor concept, the optimum salinity decreases in mixture with other surfactants in order: SDHS, SBDHS, SPL-P and SPL-O for any given molar fraction of PMNB. The optimum salinity value is dependent on the hydrophilicity/hydrophobicity of the other surfactant in the mixture for a given concentration of PMNB. S^* is at its highest (6.5%) for 100% PMNB which implies that amongst the studied surfactants, PMNB is the most hydrophilic surfactant.

Hence, the order of hydrophilicity is: PMNB > SDHS > SBDHS > SPL-P > SPL-O

An optimum middle phase microemulsion with benzene can be produced by SDHS without the presence of PMNB. Type II microemulsions or no microemulsions are formed by higher molar fractions of SBDHS, SPL-P, SPL-O because these are too hydrophobic for benzene and / or rather than forming microemulsions they form mesophases (eg.- liq. crystals). thus it can be concluded that sophorolipid biosurfactants are hydrophobic surfactants.

C. Effects Of Temperature And Salinity On Ipm-Based Biocompatible Micro Emulsions

To evaluate the change in phase behavior of IPM-based micro emulsions, we have studied its phase behavior at 3 temperatures (15, 25 and 45 C- Fig.4A) and at 2 electrolyte levels (0.9% and 5% w/v). The phase behaviour changed from type II to III to I with increase in PMNB/lecithin weight ratio.

Type II was observed at low ratio which suggests that the interaction of the surfactant with oil phase (A_{co}) is stronger than the water phase (A_{cw}). Due to hydrophilic nature of PMNB, A_{cw} increases and A_{co} decreases with increase in PMNB or ratio. When $R = 1$, this leads to a transition from type II to type III. On further increase in the PMNB in mixture, it causes the A_{cw} parameter to dominate and thus $R < 1$ and forming type I.

The range of operating conditions found in detergency were reflected by the temperature taken. So, non-ionic surfactants having ethylene oxides group are affected by temperature to a greater extent than ionic surfactants. As the sugar head group hydration has shown negligible change with temperature, its presence makes SPL less temperature sensitive. Therefore it explains the insensitivity of IPM-based microemulsion to temperature.

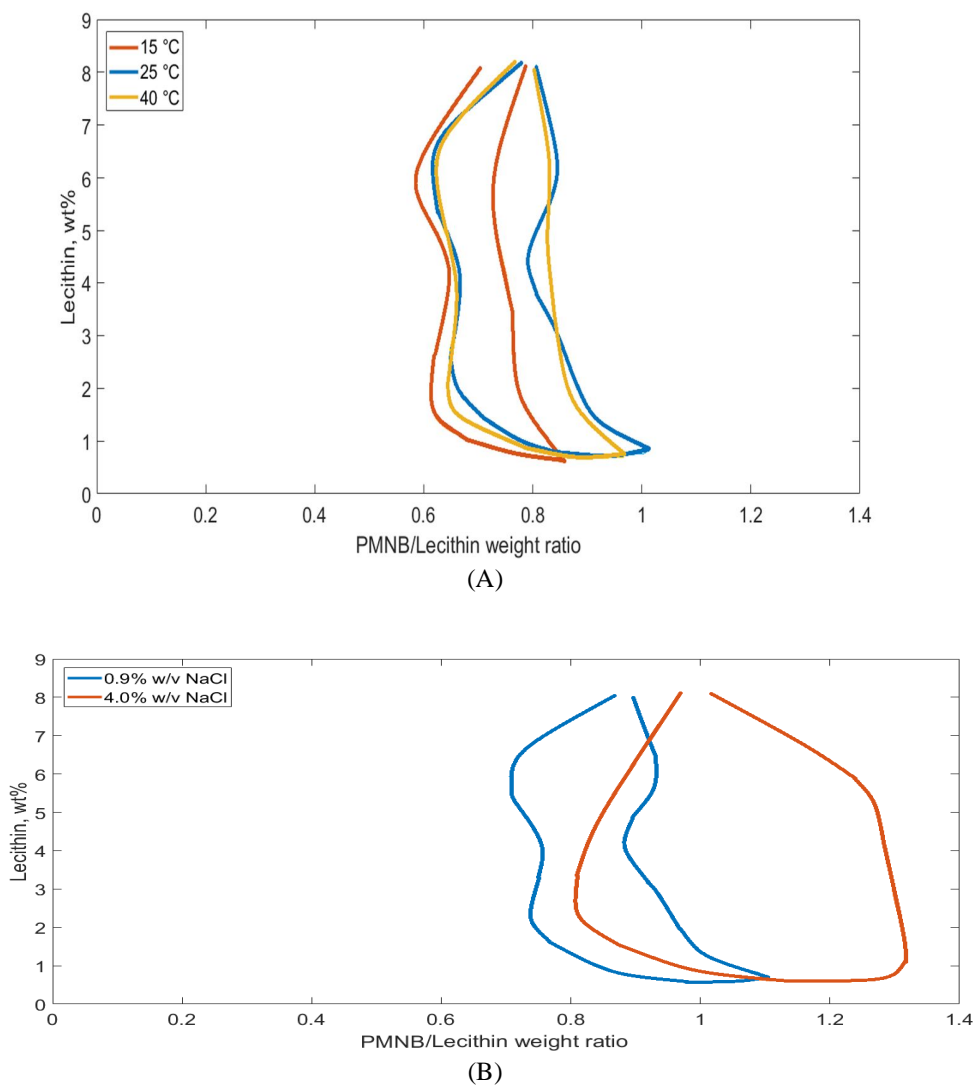


Fig.4. Phase behavior diagrams for biocompatible IPM-based microemulsions at different formulation conditions: (A) effect of temperature at 0.9% w/v salinity and (B) effect of electrolyte concentration at 25°C

The effect of electrolyte concentration on the phase behavior of IPM-based microemulsions is clearly visible in the other figure. To evaluate the impact of varying and higher concentrations, the electrolyte concentrations of 0.9% (conc. Of isotonic solution) and 5% was selected. The PMNB to lecithin weight ratio slightly increases on increase in concentration, but due to the increase of hydrophobicity of the formulation at higher salinity the window of middle phase microemulsion almost doubles.

Hence it can be concluded that SPL/PMNB/lecithin microemulsions aren't significantly affected by change in temperatures and electrolyte concentrations making these systems desirable in cosmetic and drug delivery applications. It should be noted that any of these single biosurfactants was not able to produce microemulsions for any of the studied oils.

On a note, these single biosurfactants weren't able to produce microemulsions for any of the oils we studied. Thus, these results summarize the synergistic effect of the mixed biosurfactants.

D. IPM-based Versus Limonene-Based Biocompatible Microemulsions

As IPM is more hydrophobic, the optimum PMNB weight ratio is lower for IPM than limonene as inferred from the diagram. In order to balance the interaction between oil surfactant and water surfactant, higher concentration of the hydrophilic component (oil) in the formulation is required [18]. The total surfactant concentration increases as the oil becomes more hydrophobic. From fig-5, the less hydrophobic oil Limonene requires less total surfactant concentration to form type IV single phase microemulsion thus making its applications more economically accessible.

E. Biocompatible Microemulsion Formation For A Range Of Oil Types And Oil Hydrophobicity

It is noticed higher EACN values can be found in more hydrophobic oils. Only limonene was able to form single phase type IV microemulsion, as observed, due to its hydrophilicity as compared to other oils. Therefore for limonene microemulsions, higher optimum PMNB to lecithin ratio is preferred.

For microemulsions of all oils, ultra-flow interfacial tension(<0.1Mn/m) was produced and the low IFT values make microemulsions applicable in cosmetics and cosmetics and pharmaceuticals(IPM), hard surface cleaners(limonene), and detergents(hexadecane).

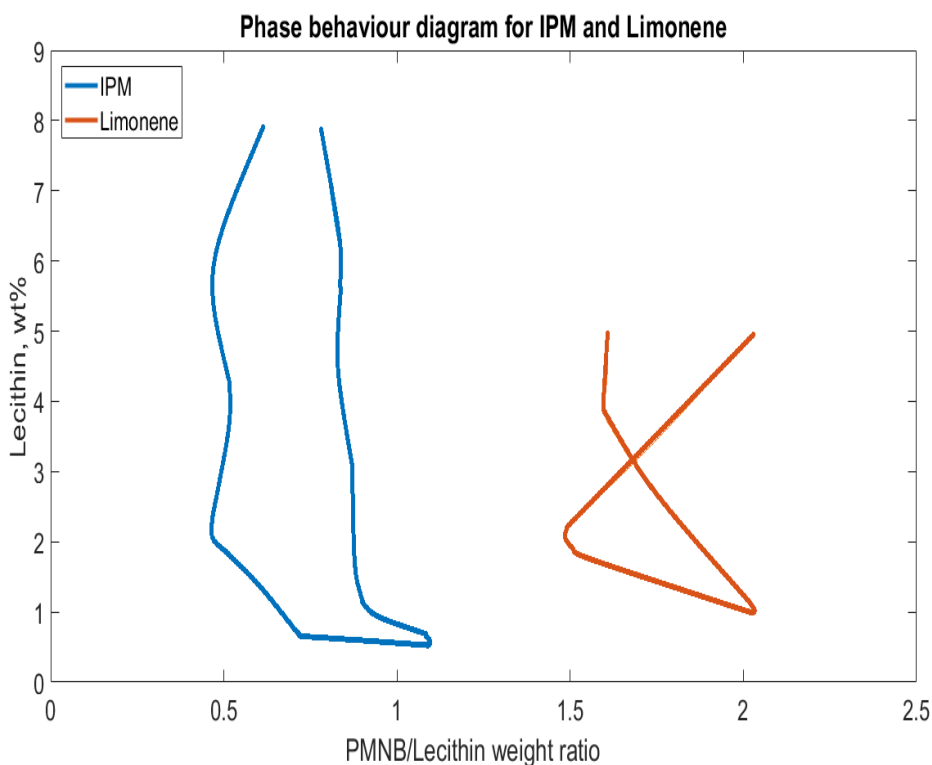


Fig.5. Phase behavior diagram for IPM and limonene microemulsions at 25°C

F. Detergency Performance On Hexadecane Removal Using Biocompatible Formulation

To check the potential in detergency applications, detergency tests were performed on hexadecane by this formulation and then were compared to the commercial detergents. For a range of concentrations (0-2000ppm or 0.2%w/v) with formulation of lecithin/SPL/PMNB = 1/1/0.3 by weight ratio and 0.9% w/v NaCl, various total surfactant concentration were prepared. Whereas the same active surfactant concentration range is used for commercial detergent (CD) but without electrolyte.

The comparison of the detergency power is shown in the figure for hexadecane removal between our formulation and the CD. On increasing the total active concentration for both, the detergency performance was observed to be directly proportional whereas the indirectly proportional for dynamic IFT (fig.7(B)). Overall, our formulation had the higher detergency than CD. CD showed 47±4.3% (IFT=2.6m N /m) whereas our formulation showed a highest of 66±1.8% at 1000ppm or 0.1% w/v (IFT=3.4×10⁻² m N /m). Lower IFT values of prewash solutions against hexadecane are obtained with our formulation than with CD. So, both formulations are indirectly proportional to detergency.

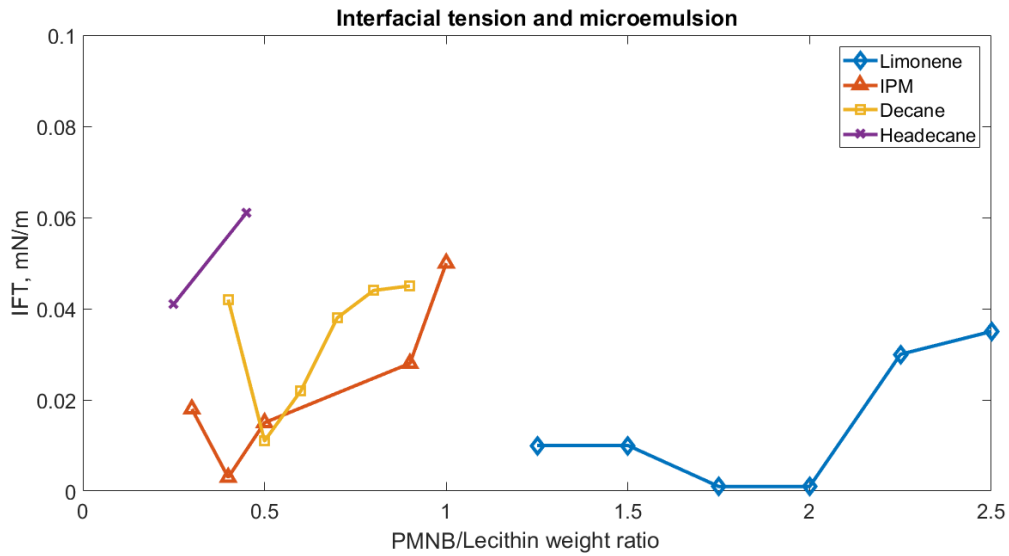
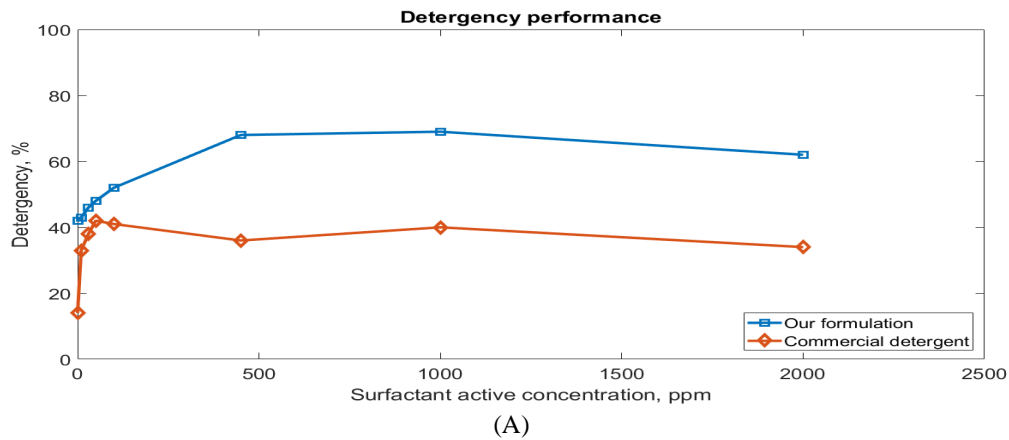
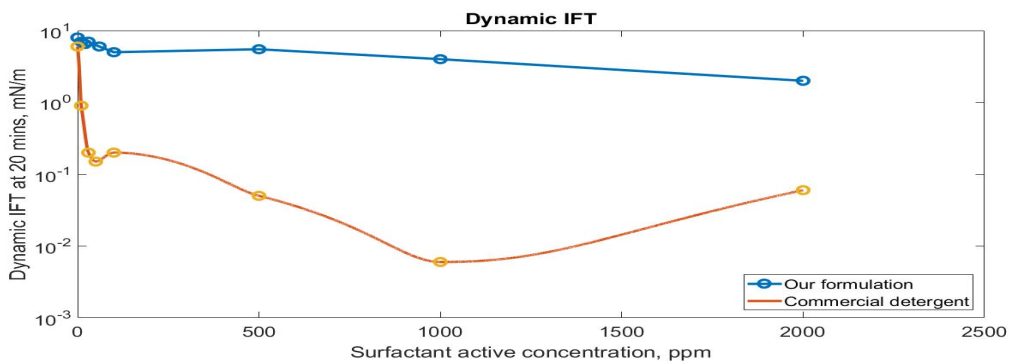


Fig.6. Interfacial tension and microemulsions for four different oils

Our formulation is better than as that by Tongcumpou et al.[33] in terms of lower active concentration and lower electrolyte concentration, example: the same detergency power (~65%) was attained at these specifications in our formulation: 0.1.8% w/v total active concentration and 0.9% w/v electrolyte concentration, whereas the CD has these specifications: 0.25% w/v total active concentration and 5% w/v electrolyte concentration[33].



(A)



(B)

Fig.7. Detergency performance (A) and dynamic IFT (B) for our formulation versus commercial detergent at different total surfactant active concentration

IV. CONCLUSIONS

This paper evaluates hydrophobicity of sophorolipid biosurfactants and chemical characterization of purified rhamnolipid biosurfactant on the basis of salinity due to their phase behavior. Sophorolipid is used as a hydrophobic component in surfactant mixtures with lecithin because of its dominant hydrophobic nature and rhamnolipid in microemulsions systems with a range of oil EACNs and oil types. Various other applications of these biocompatible microemulsions are in cosmetics, drug delivery and evaluation on detergency has also been done. Despite of insignificant changes, phase behaviors were observed, for different temperatures and electrolyte concentration. Microemulsions were formed using biosurfactant mixtures. For all studied oils, ultra flow IFT was also produced. CD and other formulations did not proved to give better results than our formulations. So this paper discusses about the characteristics of biosurfactants and their mixtures in various fields.

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