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A Study on Synthesis and Applications of Vegetable De-oiled Cake Protein Based Crude Biosurfactant

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Abstract - Biosurfactant synthesis by using underutilized by-product rapeseed cake and microorganisms such as *Bacillus subtilis* and *Pseudomonas aeruginosa* had excellent ability to lower surface tension, is biodegradable, efficient replacement of petrochemical base surfactant and cost effective. Synthesis of biosurfactant was carried out by optimizing the parameters such as Carbon:Nitrogen ratio at 8%:0.9%, temperature at 37°C and crude biosurfactant was obtained using rotary evaporator at 60- 70°C and highest yield was found to be 0.095 g/ml. Crude biosurfactant was extracted using chloroform and methanol (1:2) mixture and FTIR confirms the presence of amino group at 3400-3250 cm^{-1}. Subsequently study on the application of crude lipoprotein biosurfactant in detergent and enhanced oil recovery suggests wide scope of its application which makes it economical. Surface tension was lowered to 29 mN m^{-1} when compared with SLS at0.5% concentration of biosurfactant. Applications in detergent formulation result were obtained by using tergotometer reflectance at 77.5% comparable to SLS and enhanced oil recovery at 98%.

KEYWORDS: vegetable de-oiledcake, crude biosurfactant, lipoprotein, Detergent, oil recovery

1. INTRODUCTION

Surfactants are surface active chemical compounds which are part of everyday life as they are being used in detergent and related industries, Lima et al. [1]. Surfactant has various applications as they work as emulsifiers, wetting agent, and foaming agent. Although it has various applications it also has some disadvantages such as being chemical compounds they are toxic in nature, non-degradable, they contribute in aquatic and terrestrial pollution. To overcome this problem biosurfactant term was introduced. Biosurfactant are amphiphilic compound produced on living surface, mostly on microbial cell surface. Hydrophilic and hydrophobic moiety of biosurfactant preferentially partition at interface between fluid phases. Biosurfactant helps to decrease interfacial and surface tension in liquids or different states of matter, such Properties play an important role in various sectors of biotech industries and pharmaceutics, and various applications in different industrial sectors.

Biosurfactant have various similar applications to chemical based surfactants with benefits addition of better pH and thermal stability, low or no toxicity. Faster biodegradation and structural properties of biosurfactant label them as green and sustainable material. Biosurfactant can be divided into two categories firstly low molecular weight biosurfactant which include glycolipid, lipopeptide, peptide, fatty acid and neutral lipids, and secondly high molecular weight biosurfactant composed of polysaccharide, protein, lipopolysaccharide, lipoprotein or complex mixture of this biopolymer, Leonie et al. (2016) [2]. Proteins can be used in biosurfactant as they are amphiphilic or amphiphatic in nature that is they contain both polar and non-polar moiety but they can't be directly used as commercial surfactant as they need enzymatic or chemical modification to product with surface active properties. Molecular properties like structural features, amphipathicity, size, charge and lipophilicity play important role in their surface activity, Cowell and Bluestein [3]. This amphipathic nature of protein molecule allows it to bind with surface of different chemical nature. There are numerous biosurfactants are metabolic products produced by microorganisms. For example, surfactin is a lipopeptide-type biosurfactant produced by various strains of *Bacillus subtilis* and is one of the most powerful biosurfactant so far known, Magdassi and Kamyshny [4]. Many proteins with good foaming and emulsifying capacity often do not possess the ability of stabilizing foams and emulsions. On the other hand, proteins with poor foaming and emulsifying capacity often display the ability to stabilize the dispersed systems Hancock and Lehrer [5]. Lipoprotein biosurfactant are used in various field like personal care (skin and hair care products) Steve et al. [6], in detergent and related industries (household detergent, detergent containing enzymes and wool finishing) Takehara et al. [7], Drug delivery, Bert and Barbier et al. [8], Antimicrobial agent, Lee et al. [9], in food industries,Haynie [10], in agricultural sector (pesticide formation and protection), Krog and J.am [11]. As such very less data is available on lipoprotein biosurfactant as compare to rhamnolipids and other biosurfactant. Biosurfactants are used in different industries including petroleum industry for extraction, transport, oil spillage recovery and storage. Petroleum industries lead to the

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formation of harmful oily sludge which is composed of various constituents such as oil, coarse solids and water. Accumulation of oily sludge can result in reduced tank storage capacity and can lead to corrosion which increases the necessity of periodic removal of oily sludge. This sludge settles down at the bottom of tank and removal of this will be very costly while use of crude lipoprotein biocurfactant can make it cost effective and helps to form stable oil/water emulsion while oil recovery can be achieved by breaking this emulsion. In this study we are focusing on application of crude lipoprotein biosurfactant in enhanced oil recovery and in detergency but crude biosurfactant can also be used in bioremediation and related industries, Tania et al. [12].

Detergency is the process which removes contaminants from a surface by emulsifing or solublizing them. Detergents contain 1-5% surfactant and other active ingredients. Chemical surfactants are affecting both aquatic and terrestrial life. High concentration of chemical surfactant can terribly affect s growth of aquatic plant and micro-organisms thereby undermining food chain of aquatic life. Chemical surfactants through industrial effluent get into water body and forms insulating foam layer leading to reduction of dissolved oxygen. Chemical surfactant inhibits degradation of toxic substances by affecting growth of micro-organisms and contributes in water pollution, Yuan et al. [13]. Crude lipoprotein biosurfactant lowers surface tension and have emulsifying and biodegradable activity and hence it can be important part of detergent industry if cost effective synthesis is achieved. Cleaning agents like acid/base and chemical surfactant shows detrimental effect on environmental health.

Microbial biosurfactant have ability to lower the surface tension and interfacial tension between different phases of matter plus they are environment friendly and less toxic as compare to chemical surfactants, Long et al. [14]. In this study we are also focusing on brief evaluation of cleaning activity of crude lipoprotein biosurfactant in comparison of SLS using tergotometer-reflectance technique. Many studied related to biosurfactant applications but use of tergotometer to study cleaning activity of product is uncommon. Biosurfactant may have many industrial applications but there are some disadvantages which limits the use of biosurfactant such as high fermentation cost, purification cost and low productivity. In this study we have tried to overcome these disadvantages. It is known that the cost of raw material generally contributes up to 70% of selling price of product. Hence it is important to use suitable low-cost media, in order to compete with cheaper surfactant, jing li et al. [15]. In other word cost of biosurfactant can be altered by using renewable, cheaply available raw materials such as de-oiled cakes (Rapeseed cake, soya bean cake, sunflower cake etc.). World-wide production of fats and oil is about 2.4-3 million tons, 76% of which is derived from plants, which generate great quantities of oil cake, Leonie et al. (2006) [16]. De-oiled cake is an unexploited potential source of high value-added protein substrate produced after oil extraction from oil cake. The purification cost of biosurfactant can be avoided if it is efficient for applications such as detergency, oil spillage, bioremediation and oil recovery is considered. This study has focused on efficacy of crude lipoprotein biosurfactant utilizing renewable resource. Synthesis of biosurfactant and optimizing parameters using different organism and de oiled cake as carbon source give us chance to achieve good yield, activity and cost effectiveness at the same time. Every year around 4.3 million tons of de- oiled cake is produced, in other words de-oiled cake is a good cost-effective substrate for synthesis of lipoprotein biosurfactant. The synthesized lipoprotein biosurfactant also has excellent ability when used in crude form is excellent approach for replacement of harmful chemical-based surfactants, Sheshtawya et al. [17].

2. MATERIAL AND METHODS

Culture collection: Biosurfactant producing, *Bacillus subtilis MTCC 1427* and *Pseudomonas aeruginosa* MTCC 2297 was obtained from Microbial Type Culture Collection and Gene Bank (MTCC). MSM media and all solvents analytical grade procured from Hi-media.

2.1 Culture condition for synthesis of lipoprotein biosurfactant:

Bacillus subtilis MTCC 1427 and Pseudomonas aeruginosa MTCC 2297 microorganisms were utilized for the synthesis of biosurfactant. Particular organisms were inoculated in MSM media containing rapeseed de- oiled cake as carbon for four days. Different Carbon:Nitorgen ratios were studied and presence of biosurfactant was confirmed using several screening tests. The composition of mineral salt medium used was as follows (g/L): 0.2g $MgSO_4.7H_2O$, 5g $NaNO_3$, 1.5g K_2HPO_4, 2g KH_2PO_4, 0.02 g $CaCl_2$, and 0.024 g

$FeSO_4 \cdot 7H_2O$ and 1 ml of trace element solution containing:0.08g $COCl_2.6H_2O$, 0.75g $ZnSO_4.7H_2O$, 0.075g $CuSO_4.5H_2O$,0.06g $NaMoO_4.2H_2O$,0.5g $MnSO_4.H_2O$ and 0.15gH_3BO_3.

2.2 Recovery of Crude lipoprotein biosurfactant

Cell free culture broth was used for extraction of biosurfactant. The bacterial cell was removed by centrifugation at 5000 rpm, 4°C for 30 minutes. 1 M sulphuric acid was used to adjust pH of supernatant to 2. Equal volume of chloroform and methanol (2:1) were added to the solution and mixture was incubated in rotary shaker for 3 hours at 200 rpm and 30°C. The crude lipoprotein biosurfactant was separated from organic

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phase and was concentrated using rotary evaporator at60-70°C,Nalini et al. [18].

2.3 Screening test:

Screening test such as oil displacement test, foam stability, emulsification index (E24) gives quickly the efficiency of surfactant. Firstly, Oil displacement test was carried out, for this Petri dish was filled with water and on this few drops of coconut oil was layer uniformly. Further, test sample was added at different spots on oil layer to observe occurrence of clear zone. The presence of clear zone in cm was indication of biosurfactant presence, Nalini et al. [18]. Furthermore, Foam stability of biosurfactant was also checked by taking 5 ml of biosurfactant solution in 100 ml glass stopper cylinder and 30 vertical strokes was given. Glass stopper was removed and volume of foam at particular time interval was noted. Finally, Emulsification index of biosurfactant was determined to check the ability of crude lipoprotein biosurfactant to form stable emulsion and for that 2 ml of sample was added in equal amount of culture and emulsification index (E24) was determined after 24 hours by using following formula Eq. (1).

E24 = Height of emulsion / Total height ×100 Eq. (1)

2.4 Surface tension

MSM media containing 24-hour old inoculum was centrifuged at 1000 rpm for 20 min. for surface tension analysis cell free supernatant was used. The surface tension was measured by wilhelmy plate method using kruss tensiometer K11, Nalini et al. [18]. The critical micelle concentration (CMC) was measured as the surface tension at different concentrations of biosurfactant. All measurements were performed at 25°C. The results are expressed as the means of three repetitions.

2.5 FTIR

The FTIR of the samples were determined for functional group and presence of bond using FTIR model Miracle 10, Shimadzu**.** The spectral measurements were conducted in transmittance mode in the range of 4000-500 cm^{-1}Nalini et al. [18]

3. APPLICATION STUDY OF CRUDE BIOSURFACTANT

Although biosurfactant have various applications over chemical surfactant their purification cost limits their industrial use. In this study we are trying to use crude biosurfactant for its application in detergency and oil recovery as this are two largely growing industries and use of chemical surfactant in this industries causing aquatic and terrestrial pollution use of cost effective crude biosurfactant can overcome this problem.

3.1 Detergent formulation

Table 1: Detergent formulation

INGREDIENTS	PERCENTAGE
Citric acid	0.70
Protease	0.25
Borax	0.70
NaOH	1.6
Na formate	1.24
Perfume w/ benzyl salicylate	0.43
Monoethanol amine	0.34
AS	5.0
FWA	0.02
Propylene glycol	0.30
Crude biosurfactant	5.00
Water	Balance

Detergent was formulated using Citric acid, Protease, Borax, NaOH, Na format, Perfume, Monoethanol amine, AS, fluorescent whitening agent, Propylene glycol, water and crude lipoprotein biosurfactant according to Table1,[19]

3.2 Analysis of detergent

Prepared detergent formulation was analysed by following methods.

3.2.1 Active alkalinity as Na2o

To determined alkalinity of detergent 10 ml of sample was added in a conical flask containing 20ml of distilled water. Solution was gently warm on water bath and titrated against N/2 HCl. Methyl orange was used as indicator. Alkalinity was calculated using following formula Eq. (2)

Total alkalinity content as Na2o = volume of HCl × Normality of HCl× 3.1/ volume of sample Eq. (2).

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3.2.2 pH

pH of the detergent should be optimum hence it is important to determined pH of the detergent. pH paper analysis was used to determined pH for accurate results pH meter can be used.

3.2.3 Wetting power

Canvas disk sinking test: A mount veron cotton duck 6 canvas disk 1 inch in diameter; is floated on the surface of a solution, and the time required for it to sink is measured accurately.

3.2.4 Soil removing properties

Soil removing property was checked by calculating percentage soil removal using tergetometer reflectance analysis using method mention in following section.

3.2.5 Tergetometer-Reflectance study

To check the cleaning efficiency of crude lipoprotein biosurfactant, cloth of 10cm×10cm in size was stained with desired stain and washed with detergent containing crude biosurfactant in tergetometer reflectance (Tergetometer – Wadegati Lab equip (P) LTD.) for 15 minutes at 100 rpm. After drying of cloth reflectance of washed and dried cloth

$$\%SR = [(Rw - Ru)/(Rn - Ru)] \times 100 \quad \text{Eq. (3)}$$

Where;

R – Reflectance

n= normal unstained unwashed fabric

u= stained unwashed fabric

w=stained washed

3.3. Enhanced oil recovery

Crude lipoprotein biosurfactant was used to check its application in oil recovery by sterilizing oily sludge and crude biosurfactant. 50 ml of tap water was added to flask containing sterile oily sludge and biosurfactant and was incubated in rotary shaker at 200 rpm for five days. Oil emulsion was break by adding 1ml of 20% magnesium nitrate on 5^{th} day. Oil was recovered by discarding aqueous and percentage oil recovered was calculated by using following formula Eq. (4) Tania et al. [12].

$$\%\ \text{Oil recovered} = \{(V1 - V2)/V1\}*100 \quad \text{Eq. (4)}$$

V1= Volume of oil added to form sludge

V2= Volume of oil recovered

4. Results and Discussion

Lipoprotein biosurfactant synthesis

The synthesis of Lipoprotein biosurfactants by using various bacterium microorganisms have been investigated for many years. In addition of having capability of interaction with cell membranes that imparts antimicrobial properties; they also act as surfactant in the presence of hydrophobic compounds. Microorganisms from bacillus species are commonly applied for synthesis of lipoprotein there is comparatively less data on pseudomonas species for synthesis. Lipopeptide structure consist cyclic structure of 7-10 amino acids linked to fatty acids. The sequencing of amino acids and peptide linkage vary in variety of isoforms, Ongena et al. [20] in production of biosurfactant yield and cost is limiting factor for commercial applications

Yeh et al.(2005) [21] studied enhancing yield of biosurfactant called surfactin using *B.subtilis* ATCC 21332 with utilizing activated carbon in growth medium to achieve yield of 3.6 g/L of surfactin that could reduce surface tension to 27mN/m with a CMC of 10mg/L. Commercially only surfactin is reported most effective protein based biosurfactant for bioremediation and microbial enhanced oil recovery It is also reported that surfactin shows greater efficiency than rhamnolipids and thus have potential of research for commercial applications.

In order to economize the biosurfactant production, the cheaper carbon source rapeseed de-oiled cake was used for the synthesis of biosurfactant with *Bacillus subtilis* and *Pseudomonas aeruginosa*. Fig 1 showed the complete observations on variations in percentage yield of biosurfactant production influence by C:N ratio with respect to different microorganism. The maximum Crude biosurfactant was obtained by using 8% carbon source and 0.9% nitrogen source and was extracted using chloroform and methanol (1:2) observed after 3hours of incubation. Concentration of biosurfactant was affected by optimization of carbon: nitrogen ratio, directly leads to an increase in extracellular biosurfactant concentration in the culture medium. During fermentative processes appropriate the C; N ratio favour cell metabolism towards the production of metabolites. Parameters such as temperature, pH, agitation speed and oxygen also influence on growth conditions of biosurfactant production wit highest yield at 0.095 g/ml. The examination stated that vegetable de-oil cake is productive carbon source at the same time microorganism are proficient.

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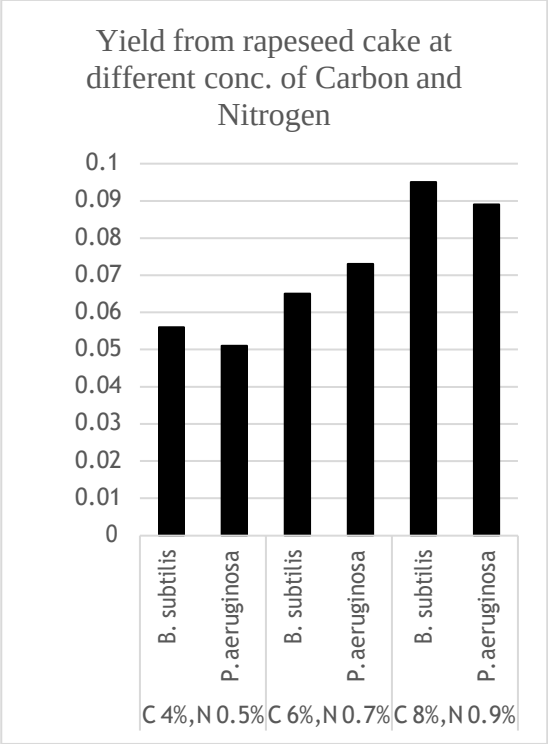

Fig. 1: Yield of biosurfactant

Lipoprotein crude biosurfactant analysis.

The biosurfactant production was confirmed after sub-culturing by screening methods. The screening methods gave positive results for all biosurfactant in cell free culture supernatant for all synthesis modes. From fig 2 Foam heights was moderate and stable. The emulsifying ability is one of the most important properties for application of surfactant in detergent for dirt removal.

The emulsification degree of produced lipoprotein biosurfactant was analyzed in comparison with SLS. The emulsification index of lipoprotein is comparable with SLS shown in Fig. 3. It was recorded as 66% for initial screening. Biosurfactants produced by different microorganisms are substrate specific, emulsifying diverse hydrocarbons at various rates, Ilori et al. [22]. Biosurfactants are known for their potential emulsification activity even at very low concentration oil displacement method was considerably good, since the oil displacement area (clearing zone) in this assay is directly proportional to the concentration of the biosurfactant in the solution, Morikawa et al. [23]. Many researchers have reported use of these screening methods to study biosurfactant production efficiency.

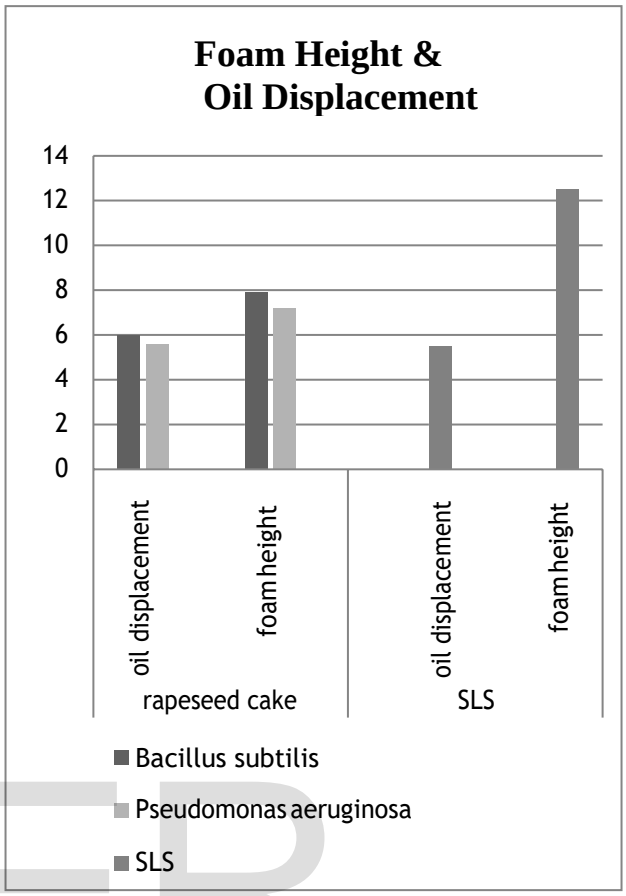

Fig 2: Comparison of foam height and oil displacement of various surfactants

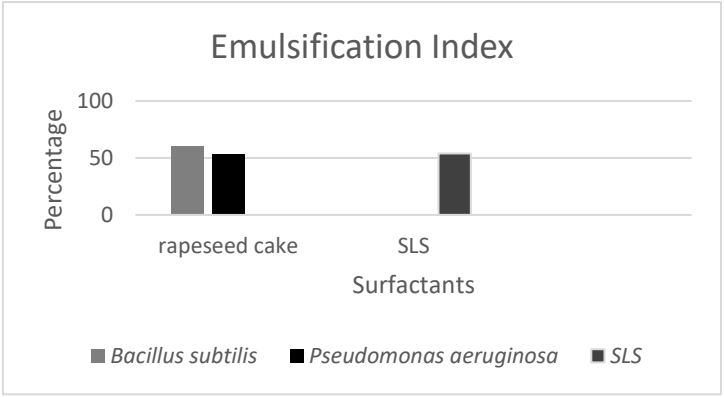

Fig 3: Comparison of emulsification index of various surfactants.

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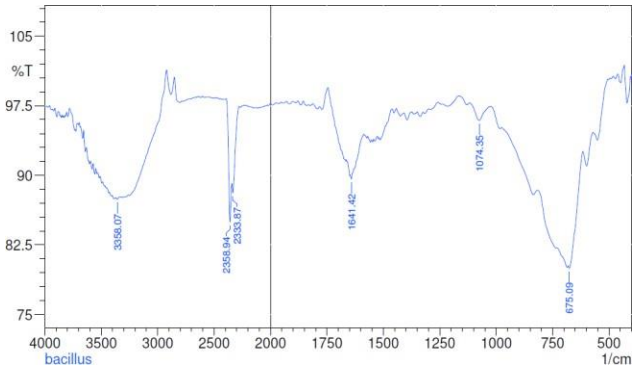

Fig. 4: FTIR graph of crude lipoprotein biosurfactant made from Rapeseed cake and *Bacillus subtilis*

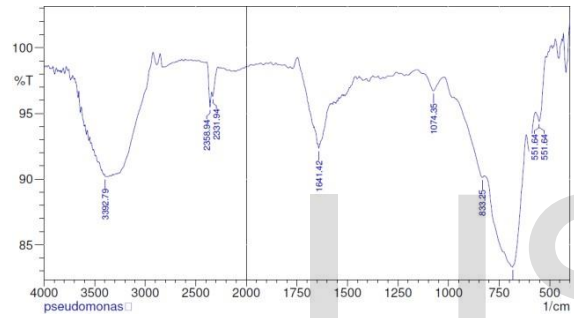

Fig. 5: FTIR graph of crude lipoprotein biosurfactant from Rapeseed cake and *P. aeruginosa.*

FTIR confirms the presence of expected components in biomaterials where IR spectrum is obtained as function of time which is converted by Fourier transform as frequency domain. In this molecular composition of biosurfactant evaluated by FTIR all the samples revealed that the most important N-H stretch bands were located at 3400-3250 frequency cm^{-1} confirming the presence of protein moieties.

Crude biosurfactant fig 4 and 5 with optimum yield obtained from vegetable de-oiled cake with specific microorganism preferred in this study was classified as a lipoprotein based biosurfactant.

Study revealed that 0.5% of crude biosurfactant concentration resulted lowering surface tension beyond 0.5% there was no significant change observed due to CMC. Efficiency is measured by the CMC, whereas effectiveness is related to surface and interfacial tensions. The CMC of biosurfactants ranges showed in fig 7 and 8 whereas interfacial and surface tensions are respectively showed in fig 6.

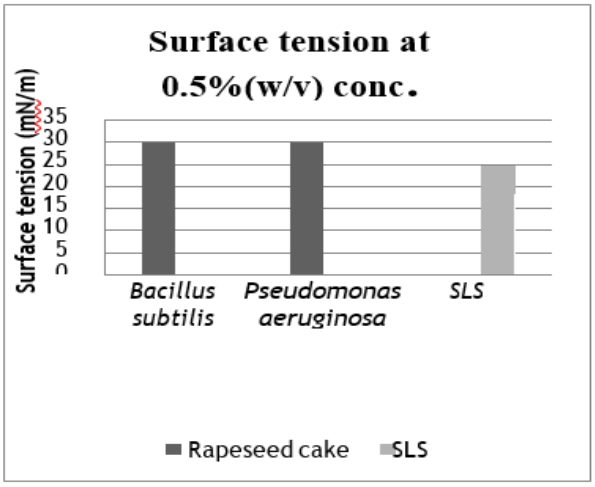

Fig.6: Surface tension of various surfactants.

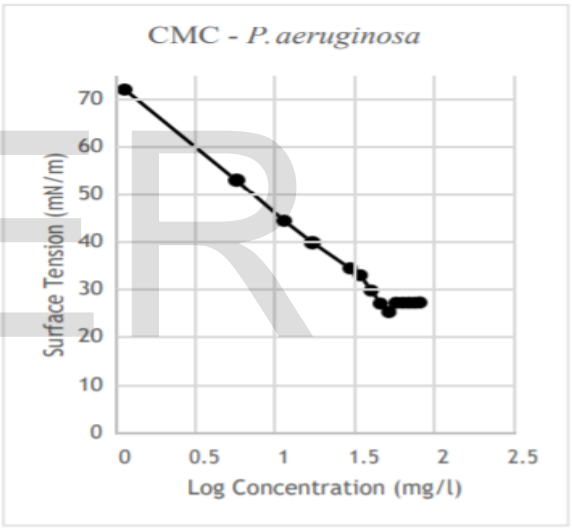

Fig. 7 CMC graph of surfactant made from Rapeseed cake and *B. subtilis.*

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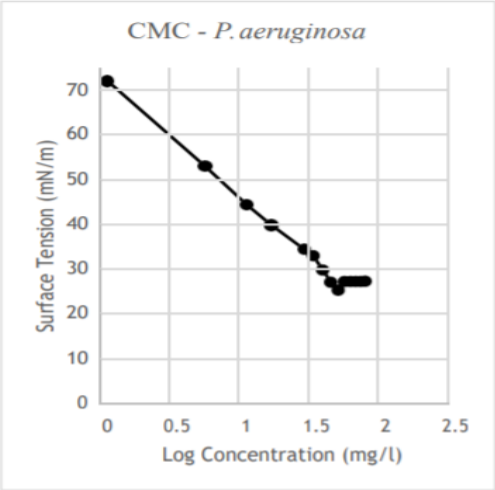

Fig. 8 CMC graph of biosurfactant made from Rapeseed cake and *P aeruginosa.*

Our crude lipoprotein biosurfactant fall under range of good surfactants that is able to reduce water
Observing foaming, emulsification and tergetometer reflectance studies shows the ability of detergent for dirt removal. The foaming height of detergent formulation was measured according to method, moderate foaming was observed. High foaming is not acceptable everywhere which cause of environmental pollution. The detergent formulation of 0.1 % showed good detergent activity which was confirm by tergometer- reflectance analysis as 77%. The wetting percent of our detergent was found to be 60%. The 30% alkalinity of our detergent is efficient in removing carbon and tea stain surface tension to 29mN/m. The CMC value of biosurfactant is a key to open its industrial perspectives. Cleaning, detergency and solubilization properties are determined by its CMC values and molecular structure. Thus, 0.5% crude lipoprotein biosurfactant concentration was used as the optimum level in cleaning activity experiments carried out in this study.

Analytical application of lipoprotein biosurfactant.
After successful Optimization of synthesis parameters and analytical parameters on crude lipoprotein biosurfactant application studies was done to showcase wide scope of commercial application that will be cost effective and efficient. Two major application observed in this studied firstly detergency and secondly enhanced oil recovery.

Detergency
The synthesized lipoprotein biosuurfactant was incorporated in detergent formulation show in table 1detergent formulation. The result of detergent formulation table 2 was analogous to lipoprotein biosurfactant result. The difference in fabric after washing in tergetometer with formulated detergent is shown in fig 10. Most commonly used liquid in our everyday cleaning is water, our crude biosurfactant has reduce surface tension to 29mN/m this surface tension causes water to wet the fabric surfaces and initiate cleaning process.

Table 2- Results of detergent analysis

Tests	Results
Active alkalinity as Na_2o	**30%**
pH	**7.02**
Wetting power	**60%**
Tergetometer-Reflectance study	**77%**
Foam stability	**For 2 hrs**
Foam height	**7.9cm**

Enhanced Oil recovery

On day 5, after addition of magnesium nitrate aqueous phase was discarded and 98% oil was recovered. Several enhanced oil recovery processes are currently employed worldwide: thermal, chemical, physical, etc. However, these processes are very expensive as well as environmentally harmful. Thus, the search for alternative, cost-effective eco-friendly alternatives to the chemical and thermal enhanced oil recovery methods is necessary. Crude lipoprotein biosurfactants have applications in this realm, as these are natural compounds, cost effective and eco-friendly.

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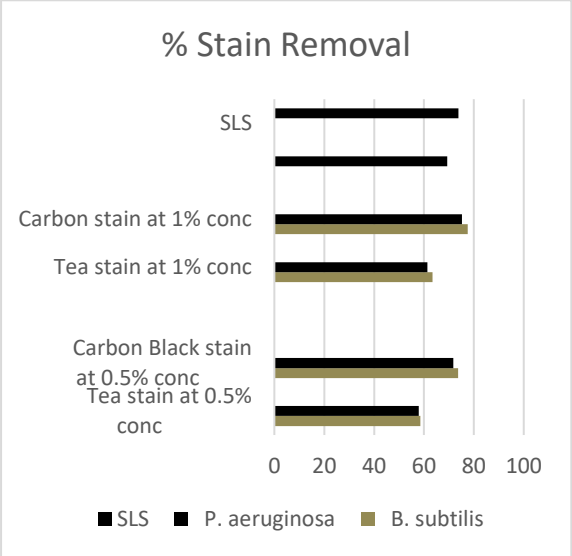

Fig. 9 Stain removal percentage

Evaluation of cleaning activity of produced crude biosurfactant and formulated detergent performed as per procedure. This step will help to reduce the usage of chemical based surfactant that is threat to ecosystem and human beings. This study validates wide applications of crude biosurfactant in detergency industry by cost effective synthesis. The fig.9 shows the graph of % stain removal from Carbon stained and Tea stained cloth when washed with detergent formulation. Crude biosurfactant made from *Bacillus subtilis* and rapeseed cake showed the best stain removal and also nearly similar to SLS. All crude biosurfactants, i.e made from different microorganisms at different concentrations have stain removal activity of above 60%. Tea stain removal for surfactant synthesized ranges from 58-70%. % Stain removal of carbon stain was seen highest for surfactant made from *Bacillus subtilis* & rapeseed cake i.e 77.5%, higher than SLS which was found to be 73.8%.

The bio surfactant thus produced can be used as a good substitute for chemical based surfactant for application in laundry detergent formulations.

5. Conclusion

Synthesis of lipoprotein biosurfactant was successfully studied for cost effectiveness, waste utilization, and making environment friendly synthesis. The study is conclusive in making use of vegetable de-oiled cake with microorganisms at optimized parameters for production of crude biosurfactant. The crude biosurfactant showed very efficient being comparable with SLS in all analytical studies. This study has tried to overcome the hurdles of synthesis of lipoprotein based biosurfactant and enhance the scope of applications.

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