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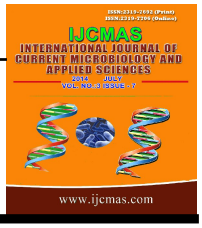


**Original Research Article**

# Estimation of Bio-Surfactant produced using *Bacillus Subtilis* CS14 in emulsion using Ultrasonic Probing technique

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**A B S T R A C T**

The aim of study is to estimate amount of biosurfactant produced by using Bacillus Subtilis CS14,a micro-organism. The addition of CS14 in broth, a mineral salt medium produces surfactant. Bio-surfactants are amphiphilic compounds. The production details of broth are given. Surface activity of supernatant gives structural information of surfactant. The study of surface activity and surface tension enables determination of CMC, critical micelle concentration. The surface activity of all concentration of broth in oil emulsion is studied by drop-weight method. The concentration at which Surface Tension increases or surface activity reduces is termed CMC. The CMC of Biosurfactant produced by Bacillus Subtilis CS14 is determined as 1 mg/l. As a graph of suitable physical quantity as a function of surfactant concentration helps in determination of CMC , assessment of surface activity by ultrasonic velocity measurement techniques is planned and study of collapsed foam in kerosene oil emulsions over different ranges of compositions for variation in ultrasonic velocity for characterization enabled us in finding linearity over a composition range. This graph helps us estimate the amount of biosurfactant produced by micro-organism and our method is unique in estimation of biosurfactant above CMC.

**Keywords**

Bio-surfactant, emulsion, ultrasonic, estimation, amphiphilic, surface activity, CMC, fermentation.

## Introduction

Surface active substances or surfactants are amphipathic molecule that tends to lower surface tension of fluids. Also, they have the ability to orient themselves at interface of two immiscible compounds. Surfactants, owing to their surface activity find many industrial applications such as detergents, emulsifiers, de-emulsifiers, dispersants, wetting agents, foam retardant, stabilizers, gelling agent etc., (Moo-Young et al., 2011). Bio-surfactants, as amphiphilic compounds contain hydrophobic and hydrophilic moieties; contain diverse group of structurally different surface active

molecules (Muthuswamy et al., 2008).A variety of microorganism like bacteria, fungi, yeast can produce bio-surfactant that are further classified as high molecular mass and low molecular mass surfactant. Bio-surfactants could also be classified according to their applications (Rahman and Gakpe, 2008; Reznik et al., 2010).The first category is proteins lipoproteins or complex mixture of polymers. The low molecular mass comprises glycolipids and lipopeptides (De-Souza et al., 2003).

The structure of bio-surfactant may include mycolic acid, glycolipids, polysaccharide lipid complex, lipoprotein, lipopeptides, phospholipids, microbial cell surface itself (Karanth et al., 1999).

Bio-surfactant may further be described as microbial metabolites and possess amphiphilic structures with hydrophobic moiety characterized by long chain fatty acid, alkyl hydroxyl fatty acid. Those with hydrophilic moiety are characterized as carbohydrate, amino acid, a cyclic peptide, phosphate, carboxylic acid or alcohol (Georgiou et al., 1992). They tend to interact at interface and affect adhesion and detachment of bacteria (Rodrigues et al., 2006). Previous studies looked for physicochemical properties, surface tension reduction and ability to form emulsion among potential bio-surfactant (Bordoloi and konwar, 2009).

The secondary metabolites help in survival of bio-surfactant producing microorganism. These facilitate nutrient transport microbe-host interaction and act as biocide agents. Lipopeptide has interesting surface activity and as antibiotic potential and therefore as bio-surfactant can be antibiotic, antiviral; anti-tumour agent, immune-modulators, specific toxins and enzyme inhibitors (Rodrigues et al., 2006).

*Bacillus Subtilis* ATCC 21332 produces cyclic lipo-peptide Surfactin, which is most powerful bio-surfactant that can be structurally described as follows: Seven amino acid ring structure, coupled to fatty acid chain via lactone linkages that lowers surface tension from 72mN/m to 27.9mN/m at concentration as low as 0.005% (Arima et al., 1968).

The application potential of bio-surfactant is wide ranging from agriculture to cosmetics; from food to petro-chemical and from textiles to petroleum production (Reiling et al., 1986; Ochsner et al., 1994) Enhanced oil recovery; remediation of organic and metal contaminated sites(Bodouret al., 2003).

## Materials and Methods

### Microorganism

Bio-surfactant producing *Bacillus sp.* CS 14 strain was obtained from Department of Bio-technology, Sri Sankara Arts and Science College, Kanchipuram, Tamilnadu, India. The bacteria were confirmed by all identification methods like morphological, physiological and biochemical characterization.

### Culture media

For bio-surfactant production, cultivation media, comprising of Mineral Salt Medium (MSM) is used and the following composition (g/l) was utilized. $Na_2HPO_4$(2.2) $KH_2PO_4$(1.4), MgSO4.7$H_2O$(0.6); $FeSO_4.7H_2O$(0.01), NaCl(0.05), $CaCl_2$(0.02), yeast extract (0.02) and 0.1ml of trace element solution containing (g/l):(2.32)g $ZnSO_4.7H_2O$; 1.78g $MnSO_4.4H_2O$, 0.56g $H_3BO_3$, 1.0g $CuSO_4.5H_2O$, 0.39 $Na_2MoO_4.2H_2O$, 0.42g $CoCl_2.6H_2O$, 1.0g EDTA, 0.004g $NiCl_2.6H_2O$ and 0.66g KI. pH of medium was adjusted to 6.8 ± 0.2 (Bodouret al., 2003).

## Bio-surfactant Production

Bio Surfactant production was carried out using a lab scale fermentor (New Brunswick Scientific, BIOFLO-110). After the inoculum was prepared, the fermentor vessel was sterilized, DO (Dissolved Oxygen) probe was polarized and the pH probe was calibrated. 2.5 litre of the production medium was prepared and 2.450litres added to the fermentor vessel. All the ports were thoroughly checked and were cotton plugged except one port to avoid medium loss due to pressure raise during autoclaving. The vessel along with the medium was sterilized at 121°C/15 lbs. The vessel was allowed to cool and inoculum was poured in sterilized condition using ethanol cleaning and with flame for sterile environment. The program was set according to bio-surfactant producing organism *Bacillus sp.* CS14 as 30°C, 200 rpm, 2 vvm, pH 6.8 cascaded to agitation. The fermentor was run for three days and 5 ml samples were retrieved and bio-surfactant production estimated.

The amount of biomass (g/l) and bio-surfactant (g/l) produced were estimated according to procedure described (Davis et al., 2001). The samples retrieved from the fermentor were centrifuged in pre-weighed centrifuge tubes at 10,000 rpm for 15 minutes. After the centrifugation the cell free supernatant was loaded in large glass chromatography column and remaining biomass pellet was allowed to air dry and was weighed to calculate the amount of biomass. The cell free supernatant in the chromatography column was applied with compressed air from base of column using a Millipore pump to produce foam slowly. The foam was collected in beaker and the collapsed foam was filtered with 0.22µm filter. The filtrate was tested quantitatively for bio-surfactant production as described by Ramesh et al. (2010).

## Determination of critical Micelle concentration (CMC) value:

Surface activity of supernatant was calculated using formula.

> Surface activity = (Surface Tension of Uninoculated medium) - (Surface tension of supernatant)

The surface activity of all concentration is determined by drop weight method(Ramesh et al., 2010).The concentration at which surface tension begins to increase or surface activity begins to reduce was considered CMC.

## Alternative methods for finding CMC

Surfactants are amphiphilic materials containing both a polar long chain hydrocarbon 'tail' and polar usually ionic 'head' groups. In polar solvent, example water, this dual character of the amphiphile leads to self association or micellization: the surfactant molecule arranged themselves into organized molecular assemblies known as micelles. Depending on chemical structure of surfactant, its micelle can be cationic, anionic, ampholitic (zwitterionic) or non ionic. The concentration (actually an arbitrary concentration with narrow range) above which micelles form is called critical micelle concentration (CMC). Above the Cmc monomers and micelles exist in dynamical equilibrium. Micelles are small colloidal particles. When micelles form, surfactant behaves as micro-heterogeneous medium. The value of CMC can be determined by change in physicochemical properties of surfactant solution as the surfactant concentration increases. Experimentally, CMC is found by plotting a graph of suitable physical property as a function of surfactant concentration (Dominguez et al., 1997).

**Alternative method for Assessment of surface activity using Ultrasonic velocity measurement techniques**

For the emulsifications conditions, the kerosene oil: collapsed foam (presents 5g/l concentration of crude bio-surfactant) was taken in increasing ranges such as 20:10 to 100 ratios. The emulsions were sonicated using Ultra Turraxsonicator for five minutes. After sonication, the samples were subjected to ultrasonic velocity measurements. A sample cell made of brass with X-cut transducers was employed with a base frequency of 1MHz placed at distance of 2.6 cm. A pulse generator, a function generator and a synchroscope were employed to generate and monitor ultrasonic wave. The output of pulse generation was 20V and accuracy in ultrasonic velocity ±0.1m/s. All the assays were conducted in triplicate (Hodate et al., 1997).

## Results and Discussion

Bacillus is a genus of gram positive, rod-shaped bacteria and a member of division Firmicutes. Bacillus includes both free living and pathogenic species. Many bacillus species are able to secrete large quantities of industrial bio-products. *Bacillus sp.* is one of the best understood prokaryotes in terms of molecular biology and cell biology. Research on *Bacillus sp*. is of primary concern in the study of fermentation process (Madigan and Martinko, 2003). A bio-surfactant producing *Bacillus sp*. CS14 strain obtained from Department of Biotechnology of Sri Sankara Arts & Science College is shown in figure 1.

**Surface activity of bio-surfactant using drop weight method**

Quantitative measurement of bio-surfactant production by drop weight method is explained (Ramesh et al., 2010). The production of bio-surfactant by *Bacillus sp*. CS14 showed surface activity of 51.38mN/m(figure 2). It was able to reduce the surface tension of mineral salt media from 88.8mN/m to 37.42mN/m.

**Table.1** Assessment of surface activity by Ultrasonic velocity measurement technique

| Kerosene oil (ml) | collapsed foam (ml) (5g/l concentration of crude biosurfactant) | Ultrasonic velocity (m/s) |
|---|---|---|
| 20 | 10 | 1265.8±0.10 |
| 20 | 20 | 1276.0±0.20 |
| 20 | 30 | 1298.8±0.20 |
| 20 | 40 | 1292.0±0.20 |
| 20 | 50 | 1310.4±0.20 |
| 20 | 60 | 1322.0±0.23 |
| 20 | 70 | 1332.5±0.20 |
| 20 | 80 | 1340.2±0.30 |
| 20 | 90 | 1352.2±0.26 |
| 20 | 100 | 1370.8±0.41 |

**Figure.1** Colony morphology of *Bacillus* sp. CS14

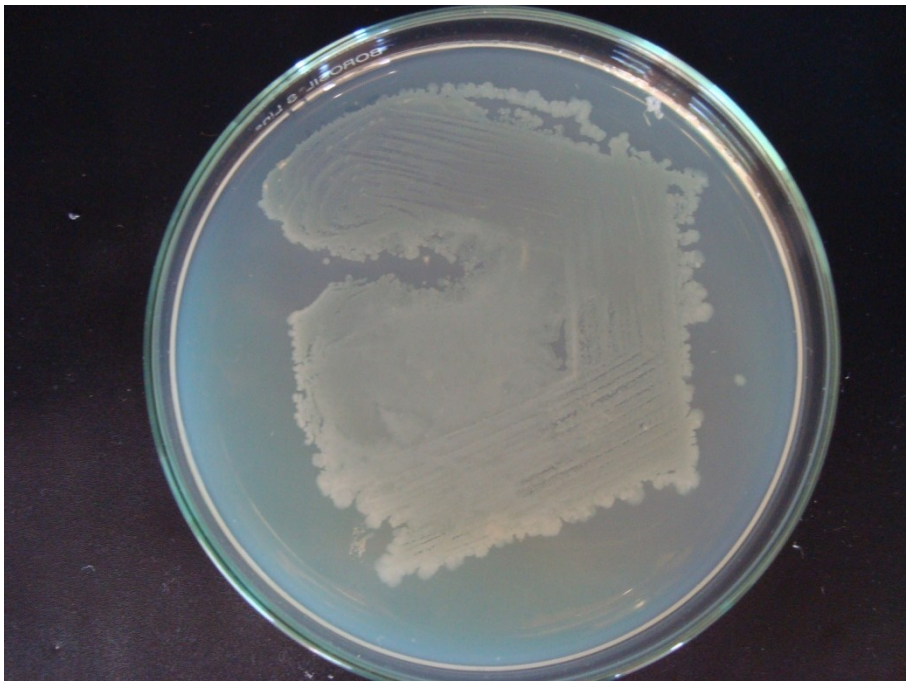
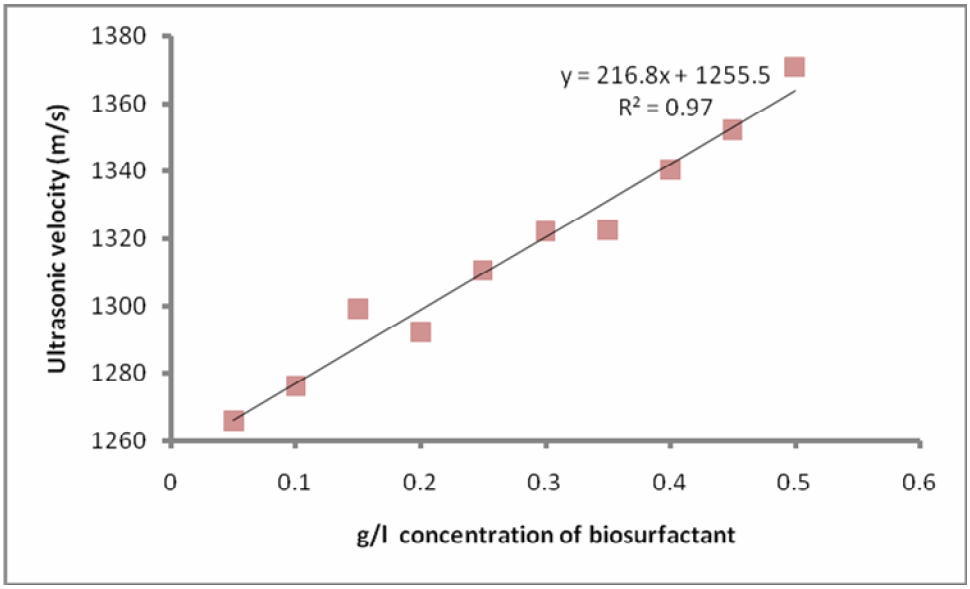


**Figure.2** Biosurfactant Production by *Bacillus* sp. CS14 using a lab scale Fermentor

**Figure.3** Surface activity measurement showing linearity graph




According to Mulligan (2004) and Gnanamani (2010), a good surfactant must reduce surface tension of water from 72 to 35mN/m and should show a surface activity of atleast 37 mN/m. *Bacillus sp*. CS14 showed a surface activity of 51.38mN/m and can be categorized as a very good bio-surfactant producer. That's why this strain was selected for the present investigation of bio-surfactant production.

The bio-surfactant concentration in collapsed foam can be measured using $CMC^{-1}$as described by Huang et al.(2009) and Carillo et al. (1996). The surface activity was found to be present until the dilution reaches 1:10 after which starts to decreases. The $CMC^{-1}$ (Rashedi et al., 2005) of the bio-surfactant produced by *Bacillus subtilis* was determined as 1mg/l.

**Ultrasonic Velocity Measurement**

For emulsification condition, the kerosene oil: collapsed foam (presents 5g/l concentration of crude bio-surfactant) was taken at increasing ranges such as 20:10 to 100 ratios. These emulsions were sonicated using Ultra Turraxsonicator for 5 minutes. After sonication, the samples were assessed for surface activity.

For Ultrasonic measurement, a sample cell made of brass with two quartz X-cut transducers with a base frequency of 1MHz placed were employed at a distance of 2.6 cm. A pulse generator, a function generator and synchroscope were employed to generate and monitor ultrasonic wave. The output of the pulse generation was 20 volt and accuracy in ultrasonic velocity was ±0.1m/s. The results are shown in table 1.

A remarkably linear graph(figure 3) for different composition range of kerosene-oil: collapsed foam emulsion was obtained. Thus ultrasonic studies proved to have immense potential for characterization of emulsion and promises to be an effective tool for quantifying concentration of bio-surfactant present in collapsed foam. These results correlated with the current method of estimation of bio-surfactant concentration in collapsed foam broth. The drop weight method

shows CMC at 1mg/l but the advantage of ultrasonic tool is that it can detect surface activity even above CMC of bio-surfactant.

The present study demonstrates application potential of ultrasonic interference methods in real time estimation of bio-surfactant during fermentation by using broth samples directly even after reaching CMC. The method presents vital evidence to prove versatility of ultrasonic tool. A standard graph obtained in present study may not be directly useful for all microbial strains. Individual standard graph may be generated for these strains. To top it all, as of now, this is first report of estimation of bio-surfactant above CMC using ultrasonic velocity measurements.

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