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Production and characterization of biosurfactant from *bacillus subtilis* MTCC441 and its evaluation to use as bioemulsifier for food bio - preservative

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ABSTRACT

Biosurfactants are amphiphilic compounds produced by various bacteria and fungi which reduce surface and interfacial tension. The Bacterial strain Bacillus subtilis MTCC 441 was used for the production of biosurfactant and biosurfactant activity was tested against different vegetable edible oils. The parameters for better growth of the bacterial strain was optimized and production of surfactant was carried out. The crude biosurfactant was extracted and the emulsification potency was assessed using different vegetable edible oils. Further, the rhamnolipid was detected from the extracted biosurfactant and was confirmed by Infrared spectroscopy. The results showed that strain showed high surfactant activity over the Gingelly oil, required mesophilic temperature and pH-7 for its better growth. The surfactant showed comparatively high emulsification index over Gingelly oil at the rate of 71%. The rhamnolipid was detected in the surfactant and IR spectrum showed a typical pattern of stretches for CH_2, CH_3 and C-O groups.

Key words: Biosurfactant, Bioemulsifier, Rhamnolipids, Biopreservatives.

INTRODUCTION

Surfactants are molecules that concentrate at interfaces and decrease surface and interfacial tension [1]. These compounds find applications in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization [2, 3] currently, almost all the surfactants being produced are chemically derived from petroleum [1]. How ever, naturally occurring surface-active compounds derived from microorganisms, also called biosurfactants, are attracting attention in recent years because they offer several advantages over chemical surfactants, such as low toxicity, inherent good biodegradability and ecological acceptability [4].

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi [5,6] from various substances including sugars, oils and wastes. However, carbohydrates and vegetable oils are among the most widely used substrates for research on biosurfactant production by B*acillus subtilis* strains. The amphiphiles that form micelles can be potentially used for surface chemical works are termed as surface active agents or surfactants. Soaps and detergents can be described as having similar characteristics as surfactants. All surfactants have two ends namely, 1) a hydrocarbon part which is less soluble in water (hydrophobic end). The hydrophobic part of the molecule is a long-chain of fatty acids, hydroxy fatty acids or α-alkyl-β-hydroxy fatty acids, 2) the water soluble end (hydrophilic) can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Additionally, the hydrophobic moiety is usually a C8 to C22 alkyl chain or alkyl aryl that may be linear or branched [7].

The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a number of industrial operations [8]. Biosurfactants reduce surface tension, Critical Micelle Concentration (CMC) and interfacial tension in both aqueous solutions and hydrocarbon mixtures [9,10]. In the present study, Otimised production of biosurfactant from *Baacillus subtilis* MTCC 441 and charecterised. The investigation has been made for its emulsification capacity as biopreservative in food.

In the current study, Production and extraction of biosurfactant was carried out using *Bacillus subtilis* MTCC44, the parameter optimization for the better growth of the strain was carried out. The extraction and emulsification potency of the surfactant was performed in order to use as bioemulsifier in food. Further, The rhamnolipid contents were assayed and confirmed by Infra red spectroscopy method.

MATERIALS AND METHODS

Microorganism and culturing conditions

The bacterial strain *Bacillus subtilis* MTCC441 was procured from Microbial Type Culture Collection (MTCC), India. The bacterial strain was enriched in Luria Bertani broth (Peptone – 1%, Sodium chloride – 1% and yeast extract – 3%, pH-7) incubated at 37°C for overnight. The culture was streaked in LB slants and stored at 4°C for further use.

Biosurfactant Production:

The inoculum was prepared using Luria Bertani (LB) broth and incubated overnight at 37◦C for over night with 100 rpm agitation. Production was carried out using production medium composed of Glucose – 1(g/l) KH_2PO_4 - 0.5 (g/l), K_2HPO_4-1(g/l), KCl - 0.1(g/l), $MgSO_4$ - 0.5(g/l), $FeSO_4$ – 8 (mg/l), $CaCl_2$ – 50 (mg/l) ,Urea – 6 (mg/l) with the addition of 1ml/l trace elements solution ($ZnSO_4$ - 4.4 mg/l, $MnSO_4$ - 3.3 mg/l, $CuSO_4$ - 0.1 mg/l) at pH 7. The production medium was seeded with 3% inoculum and incubated at 37°C for 48 hours with 150 rpm agitation. The cell free supernatant was used as crude surfactant.

Determination of Biosurfactant Activity

Biosurfactant activity of isolated bacteria was determined by oil spreading technique using five different vegetable oil namely Castor oil, Gingelly oil, Coconut oil, Mustard oil and Sunflower oil. Fifty ml of distilled water was taken in a large Petri plate and 100µl oil added over the surface of water. Further, 10µl of cell free supernatant was added over the oil and oil spreading zone was measured [11].

Optimization of Growth:

Bacterial growth was optimized using different parameters like pH, temperature and different kinds of vegetable oils as carbon sources. The growth was measured photometrically at *A590*.

Extraction of Biosurfactant.

The production was carried out with optimized condition for 48 hours and the bacterial cells were removed by centrifugation at 10.000 rpm for 20 min under cooling condition. In order to precipitate the lipids and proteins, the concentrated HCl was added with the supernatant to bring final pH of 2.0 and kept for overnight at 4°C. [12]. Resulted Grey white precipitation was collected by centrifugation at 10.000 rpm for 20 min at 4°C. For further extraction of biosurfactant compounds, 10mL of chloroform methanol (2 : 1v/v) was added to precipitated pellet and incubated in a rotatory shaker at 30°C for 15 minutes with 250 rpm agitation. The content was centrifugation at 10.000 rpm for 20 min under cooling condition and the supernatant was evaporated by air drying. The remaining residue was dispensed in sodium phosphate buffer (pH 7.0) and stored at 4°C.

Determination of Emulsification Activity

The emulsification potential was carried out using modified method of Cameotra et al. 2004 [13]. The extracted surfactant (0.5 mL) was added to a screw capped tube containing 7.5ml of Tris-Mg buffer (20mM Tris HCl (pH 7.0) and 10mM $MgSO_4$) and 0.1mL of edible vegetable oil. After a vigorous vortex, the tubes were allowed to stand for one hour. Absorbance was measured at 540nm. Emulsification activity (EA) was calculated.

Detection of rhamnolipids

The detection of rhamnolipids were based on the hemolysis of erythrocytes by rhamnolipids [14]. Ten µl of extracted surfactant was spotted on paper filter discs (6.0 mm, Whatman AA) and then put onto agar plates containing 5% sheep blood. The blood agar plate was incubated at room temperature for 2 days and then the zones of hemolysis was observed.

Infrared spectroscopy (IR) analysis

The biosurfactant was extracted from the supernatant fluid (2 ml) with chloroform (2 ml), dried with Na_2SO_4 and evaporated on a rotary evaporator. The IR spectra were recorded on the Bruker IFS113vFTIR-spectrometer, in the 4000 - 400 cm^{-1} spectral region at a resolution 2 cm^{-1} using a 0.23 mm KBr liquid cell.

RESULTS AND DISCUSSION

The bacterial strain *Bacillus subtilis* MTCC441 was subjected for the production biosurfactant and activity was screened by oil displacement method. The results suggested that the extract possessed biosurfactant activity and showed the oil spreading zone about maxium 2.1cm diameter. The formation clear oil displacement zone confirmed the presence of biosurfactant activity in the obtained extract. Among the different vegetable oils tested for oil displacement method, Gingelly oil was spreaded more than Mustard oil and Sunflower oil (Table.1).

Table.1 Oil displacement activity of biosurfactant

Vegetable oil	Range of Biosurfactant activity
Castor oil	Partial
Mustard oil	Low
Coconut oil	Partial
Gingelly oil	High
Sunflower oil	Low

After confirmation of biosurfactant production, the parameter optimization study for the better growth of the strain was carried out for various parameters. The result revealed that the strain *Bacillus subtilis* MTCC441 showed well growth at pH 6-7 and temperature 37°C Fig.1&2). Further more, the different edible oils were used as a carbon source for the growth of the strain. The results showed that the sunflower oil followed by gingelly oil were utilized well by the strain for the growth and other oils showed more or less similar growth rate (Fig.2).

Fig.1 Effect of temperature on growth of bacterial strain

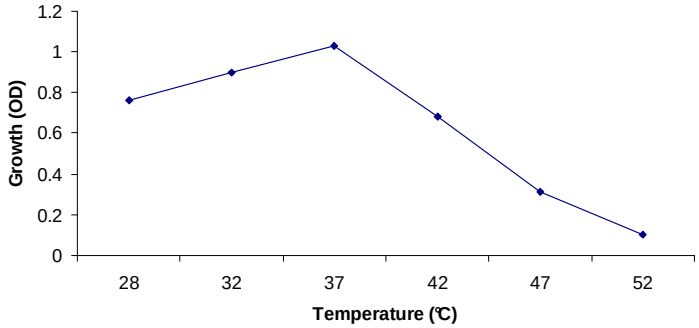


Fig.2 Effect pH temperature on growth of bacterial strain

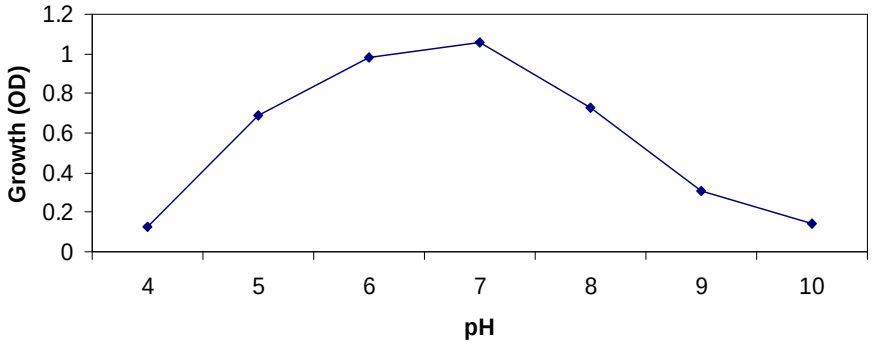
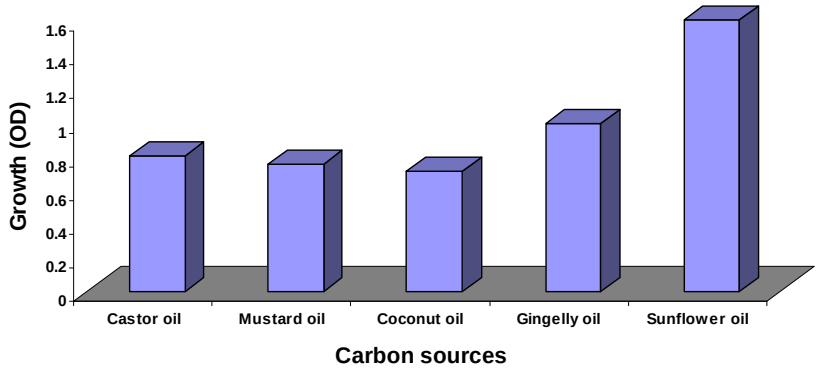


Fig.3 Utilization of vegetable oils as carbon source



Emulsification

The production of biosurfactant was carried out using optimized parameters and extraction of surfactant was made. The efficiency as bioemulsifier was tested and bioemulsification index (EI) was estimated. It showed that surfactant produced from gingelly oil showed highest emulsification index of 71% and other oils showed moderate indices (Table.2).

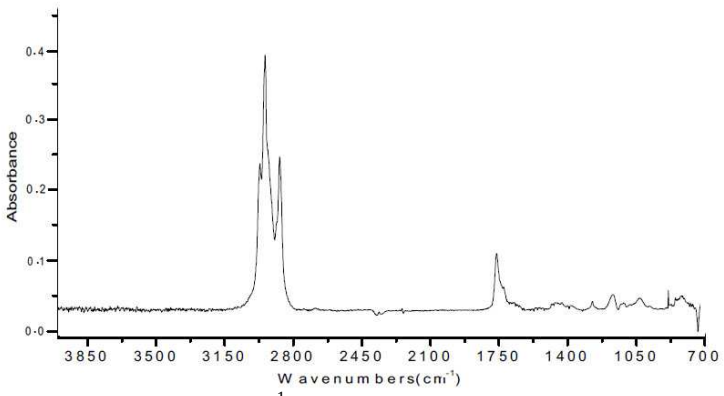
Table.2 Emulsification potency of Biosurfactant

S.No.	Oil source	Emulsification Index
1	Castor oil	59.37
2	Mustard oil	65.62
3	Coconut oil	56.25
4	Gingelly oil	71.87
5	Sunflower oil	56.25

The emulsification indices are differing based on the nature of the surfactant compound and source of the organisms. The emulsification activity of the biosurfactants produced from marine *Bacillus* spp. were tested with different hydrocarbons showed that the highest index was showed corn oil followed by kerosene and sunflower oil [15]. Similarly, The *Bacillus subtilis* and *Pseudomonas aeruginosa* had the ability of emulsifying various vegetable oils, kerosene, petrol and diesel [16].

Finally, the extraction of rhamnolipids from the produced surfactant was carried out and analysed by IR analysis. IR spectrum showed characteristic bands in the region 3000-2700 cm^{-1} indicated C-H tretching bands of CH_2 and CH_3 groups.

Fig.4 IR spectrum of surfactant for rhamnolipids analysis



The deformation vibrations at 1467 and 1379 cm^{-1} also confirm the presence of alkyl groups. Carbonyl stretching band was found at 1745 cm^{-1} which is characteristic for ester compounds. The ester carbonyl group was also proved from the band at 1250 cm^{-1} which corresponds to C-O deformation vibrations. The IR analysis results are positively correlated with previous work of Christova et al. 2004 [17]. Among the different classes of biosurfactants rhamnolipid and surfactin are best studied biosurfactants. Rhamnolipid is one of the type of glycolipids, in which one or two molecules of rhamnose are linked to one or two molecules of hydroxydecanoic acid while the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acid is involved in ester formation **[**18**]**.

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