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Physiochemical Characterization and Anti-colon Cancer Activity of Biosurfactant Produced from Marine *Pseudomonas* sp.

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ABSTRACT

Background: Biosurfactants are the surface-active molecules produced by the living micro-organisms. They have wide application in the food, cosmetic, pharmaceutical and petrochemical industry due to its physiological and functional properties. **Objectives:** In the present study, biosurfactant produced from *Pseudomonas* sp. was used for its physiochemical characterizations and anti-tumergenic property against colon cancer cell line (SW480). **Materials and Methods:** The biosurfactant was produced in Bushnell Haas Broth media and extracted by solvent extraction method. The physiochemical characterizations such as water solubility index, water activity, foaming index, oil holding capacity of extracted biosurfactant were performed. The degree of toxicity of biosurfactant was measured using a conversion assay of 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye against SW480 and mouse L292 fibroblastic cell line (non-tumergenic). Furthermore, acridine orange-EtBr (ethidium bromide) staining, Dichlorofluorescein Diacetate (DCF-DA) staining, wound healing assay were performed to determine the anti-colon cancer activity of biosurfactant. **Results:** The oil holding capacity, water activity (a_w), foaming capacity and water solubility index for biosurfactant were found to be 4.36 ± 0.05 g/g, 0.32 ± 0.03, 52.28 ± 0.10 % and 80.86 ± 0.20 % respectively. In the MTT assay, after 48 h of incubation, 84.7% of cell viability on L292 was observed at 250 µg/ml (maximum concentration) for biosurfactant. Whereas, only 50% cell viability was observed after 48 h of incubation on SW480 at 168.52 µg/ml biosurfactant concentration. **Conclusion:** The biosurfactant could be a bench-mark as a non-cytotoxic biosurfactant which may be used as a possible biological substance in the food industry as a stabilizing agent and in pharmaceutical industry as an anti-colon cancer agent.

Keywords: Biosurfactant, Physiochemical characterization, MTT assay, Anti-colon cancer activity.

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INTRODUCTION

Biosurfactants are biological surface-active molecule released by living organisms. Due to the functional and structural diversity, the marine derived biosurfactants have gained lots of interest. As these biosurfactants are highly effective and eco-friendly, it is obvious to turn for the microbial population for its potential exploration to satisfy the needs.[1] In the recent time, the use of biosurfactants in various industries including biomedical, industrial and environmental has highly been explored. Rapidly biodegradable, less toxic and environmentally favourable in nature allow their possible replacement to chemical surfactants. Although the various groups of biosurfactants characterized, glycolipid have more functional properties and they are explored for food industries, cosmetics, pharmaceutical and petroleum.[2] Many investigations on implementations of biosurfactant (BS) emphasize the usage of these molecules in bioremediation and environmental approaches to degrade the toxic pollutant. Nevertheless, this surface-active molecule has other potential applications because it has vital role in medicinal field, particularly acts as an anti-inflammatory, antimicrobial, antiadhesive and anticancer agents.[2] The BS molecule has been suggested as effective and safe option to antimicrobial agents and synthetic drugs.[3] The value of this molecule as antimicrobial agent in the field of pharmacy, medicine and different therapy because of their ability to disrupt the cell membrane leading to cell lysis by increasing the flow of the cell metabolites and the permeability of cell membrane. This is because of the alterations in the membrane physical structure caused by disruption of its protein conformation which modifies mechanisms of energy production and transport.[4] Recently, it has been shown that biosurfactants are active against cancer cells. For example, the surfactin (lipopeptide) biosurfactant has been shown to influence apoptosis in breast cancer cells.[5] Likewise, succinoyltrehalose lipids (STLs) and mannosylerythritol glycolipid (MELs) have been implicated in stunting and apoptosis of tumor cells.[6] Nevertheless, although biosurfactants are multipurpose, useful and valuable compounds for therapeutic purposes, some may pose a risk to humans and need to be carefully considered. *Pseudomonas aeruginosa* is known for the production of glycolipid biosurfactant, may be responsible for severe nosocomial infections but the glycolipid produce by this bacterial strain is very much useful for various therapeutic applications.[7] Recently few interesting results that has been reported for glycolipid biosurfactants is their capability to control various function of mammalian cancer cell and thus act as anticancer agents interfering with certain processes of cancer progression.[8] Basically, in this investigation, biopotential applications of biosurfactant (produced by a *Pseudomonas aeruginosa* ENO14) against SW480 colon cancer cell line was carried out to find its utility in the field of pharmaceutical industry.

MATERIALS AND METHODS

Culture condition and production of biosurfactant

Pseudomonas aeruginosa ENO-14 was already isolated, characterized and stored in cryoinstant (Tarsons Cryo vial) a specialized preservation system for microbiological cultures in our laboratory. The sequence data of the strain in Genbank databases have the Accession number

MH271625. The culture was revived from Glycerol stock on Luria-Bertani agar plates. The inoculum was prepared on Luria Bertani Broth by incubating at 37°C for 16h with an agitated speed of 200 rpm and was then transferred to Bushnell-Haas Broth (BHB) medium supplemented with 1% glucose as a carbon source for biosurfactant production. The media was then incubated at 37°C for 168 h in 150 rpm.

Recovery of biosurfactant

The culture broth from BHB medium was centrifuged at 10000 rpm for 15 min at 4°C after 168 h of incubation. The cell free broth was acidified to pH 2 by adding 6N HCl and incubated overnight for biosurfactant precipitation at 4°C. Then equal volume of ethyl acetate was added to the precipitated broth in separating funnel. The mixture was then vigorously shaken for few minutes and allowed to set for phase separation. The organic clear phase (upper phase) was collected in a glass beaker and one spatula of anhydrous sodium sulphate was added into it to remove the remaining water molecule. Finally, the clear solvent was evaporated by using rotary evaporator and the honey colour concentrated product was considered as partially purified biosurfactant, named as ENO14BS. The biosurfactant was then lyophilized for further use.

Oil absorption capacity

Oil absorption capacity (OAC) was estimated as discussed by Abbey and Ibeh.[9] Five mL of oil (Sun flower oil) was mixed with 0.5g of biosurfactant. Then the mixture was kept undisturbed for 30 min and centrifuged at 4000 rpm for 10 min. The wet residue was measured in

OAC. OAC (g/g) = (Weight of wet sample residue - Weight of dry sample)/ Weight of dry sample.

Foaming capacity and water activity

Five ml sample of ENO14BS (1mg/ml) was added with 40 mL of distilled water at 25°C in a 200 mL glass cylinder. The mixture was shaken for few minutes for the foam formation. Volume of foam after 30s was measured. The foaming capacity was determined as follows: FC (%) = [(Volume after whipping -Volume before whipping) / Volume before whipping] *100.

Water activity of ENO14BS was determined by an electronic dew point water activity meter (Aqualab Series 4TE, Washington, USA) at room temperature (30 ± 2°C).

Determination of water solubility index (WSI)

WSI of ENO14BS was measured as described by Anderson.[10] 0.2g of ENO14BS was added in 5 ml of distilled water and continuously stirred for 20 min in a water bath at 30°C to obtain a homogenous solution. Finally, the solution was centrifuged at 4000 rpm for 15 min and the supernatant was transferred in a glass Petri plate and dried at 110°C for 3 h. WSI was determined based on the following formula,

WSI = (Dry weight of solid in supernatant × Weight of dry sample) × 100.

Cell lines and culture conditions

DMEM cell culture media were bought from Invitrogen, USA; antibiotic-antimycotic solutions and fetal bovine serum (FBS) were purchased from Himedia, India. The cell line L292 were collected from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM media supplemented with L-glutamine, 10 % FBS and streptomycin-penicillin solution to a final concentration of 100 µg/ml streptomycin and 100 I.U./ml penicillin at 37°C, 5 % CO_2 in 95 % humidified air. In the tissue culture flask, cells were preserved by routine sub culturing technique. The medium of the culture was altered every 48 h and the cells were passaged when they attained confluence.

Cytotoxicity assay of biosurfactant

The degree of toxicity of ENO14BS was measured using a conversion assay of 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye against SW480 (human colon cancer) and L292 cell line as per the protocol described by Patowary *et al.*[11] with necessary modifications. The MTT study determines proliferation and cytotoxicity using colorimeter, based on the metabolic activity of living cells in the reduction of tetrazolium salts.[12] 96-well flat-bottomed plates (Corning Inc. USA) were seeded at approximately 6 x 10^3 cells per well with SW480 and L292 actively growing in complete DMEM and incubated at 37°C, 5% CO_2. Five different concentrations of the column purified ENO14BS (50, 100, 150, 200 and 250 µg/ml) made in serum-free DMEM were used to treat cultured cells. ENO14BS was added in wells in triplicates and it was incubated for 0 h, 24 h and 48 h. A control, i.e., only DMEM (without the addition of ENO14BS) was also used for comparison. 20 µL of 5 mg/mL MTT was added to each well after the incubation period and incubated at 37°C. After 6 h, the liquid content of each well was decanted and 100 µL of dimethyl sulfoxide (DMSO) was administrated to solubilise the purple formazan crystals. The absorbance of the plate was measured after a brief incubation in a UV–Vis well plate reader at 570 nm. The cell viability in percentage was estimated basis on the following formula

Cell viability (%) = $N_t/N_c \times 100$ where, N_t indicates the optical density of the biosurfactant treated cells, whereas N_c denotes optical density of the untreated control cells.

Live-dead cell viability staining

About 1 x 10^5 cells of SW480 were seeded in 24-well plates (Corning Inc. USA) with the complete medium and incubated overnight, at 37°C with 5% CO_2. The concentration of IC_{50} of ENO14BS was prepared and added to the allotted wells. After incubation (24h), the culture media was removed and phosphate buffer saline was used to wash the wells twice. At this stage, live cell imaging was performed. Stock solutions of acridine orange and ethidium bromide (1 mg/ml) were made in DMSO for viability staining. Preferred working stock solution of 10µg/ml was made in phosphate buffer saline and it was used immediately. 200 µL of a 1:1 mixture of the dye solution was administrated to the wells and examined using inverted fluorescence microscope (Nikon Eclipse Ti, New York) by a blue excitation filter.[13] All the images were taken with 20x objective and it was then further processed with Nikon Br 4.0 software. In all the cases, control cells were observed for making comparative remarks.

Dichlorofluorescein Diacetate (DCF-DA) Staining

The degree of reactive oxygen species was calculated by staining with DCF-DA. SW480 cells were treated with ENO14BS for 24 h. After the treatment, the cells were rinsed with phosphate buffer saline (pH 7.4). Then the cells were stained with DCF-DA (10 µM) for 30 min at 37°C, covered with aluminium foil. Finally, the cells washed with PBS and observed under a fluorescence microscope (Nikon Instruments Inc., NY, USA).

Wound Healing Assay

Approximately 1x10^6 SW480 cells were seeded in each wells of 6-well plate and allowed to grow to full confluency. Then, the medium was removed, scratches/wound were made and washed with 1X PBS to remove the loose cells. The images of the wounds centre were recorded at that time as 0 h and then the cells were treated with and without ENO14BS (conc. 100µg/ml). Images were taken at regular time interval after the treatment using Nikon Eclipse inverted fluorescence microscope.

Statistical Analysis

All the experiments were performed in triplicates and the values were given as mean ± standard deviation (SD) using Microsoft Excel 2010 (Microsoft, USA).

RESULTS

Oil absorption capacity

The oil holding capacity (OHC) or oil absorption capacity (OAC) is one of the very important parameters to measure as oil helps to retain the flavour. The oil holding capacity for ENO14BS was found to be 4.36 ±0.05 g/g (Table 1). This result clearly indicates that permeable structure of ENO14BS might have bind to the fatty acids chain of oil. Therefore, biosurfactants could be highly useful in oil absorbed food products to increase the flavour retention and shelf-life of the product.

Water activity and foaming capacity

Water activity (a_w), an intrinsic property, measures free water available in a food system. Based on its values the shelf life of a particular food system is determined. Limited water activity of a food article makes it resistant to microbial growth and deteriorative biochemical reactions while higher water activity reduces its shelf life. In the current study, we found the water activity (a_w) of ENO14BS of 0.32 ± 0.03 and foaming capacity of 52.8 ± 0.1 % (Table 1). Lower water activity recorded for the rhamnolipid reiterates their longer shelf stability

Water solubility index (WSI)

The WSI of the biosurfactant was found to be 80.86 ± 0.2 % and may be due to the intensive hydrogen bonding of rhamnolipid with water (Table 1). These results showed a potential choice for utilizing biosurfactant as hydrocolloids in foods as bio thickener and stabilizer agent.

Cytotoxicity study of the biosurfactant

To evaluate the anti-cancer activity of ENO14BS, we treated the colon cancer (SW480) cell lines at increasing doses of ENO14BS for 0 h, 24 h and 48 h followed by an MTT conversion assay. As Figure 1, incubation of colon cancer cells (SW 480) with ENO14BS for 24 and 48 h exhibited decrease in number of viable cells with variation of inoculums dose. At the beginning of (0^{th} h) the treatment, the cell viability was 95.6% with 250 µg/ml of ENO14BS (highest conc.), whereas a cell viability of 100% was reached in case of control, even after 48h. The IC50 value of ENO14BS after 48 h of incubation on SW480 was 168.52 µg/ml. After 24-h of treatment, ENO14BS had an IC_{50} value of 182.66 µg/ml on SW480 cells.

Simultaneously we exposed L292 cell line to better understand the toxicity of ENO14BS on non-tumorigenic cell line. As revealed in Figure 2, ENO14BS exhibited negligible toxicity up to 250 µg/ml (highest conc.). While 100% of the cell viability was reached in control. After 48 h of incubation, 84.7 % of cell viability on L292 was observed at 250 µg/ml (highest conc.) for ENO14BS.

These results clearly indicate that ENO14BS more effectively inhibits the proliferation of tumor cells over non-tumorigenic L292 cells and may serve as a reference for non-toxic biosurfactant that can be used as a potent biological molecule in the field of biological interfaces. This result also supports the probable utilization of this biosurfactant which gain safety standards for living creatures.

Biosurfactant induce apoptosis in SW480 cells

In the present study to recognize the anti-cancer activity of ENO14BS, we exposed the SW480 cells with DMSO (mock) and ENO14BS for 24 h. Then, acridine orange/ethidium bromide staining was performed to verify the dead and living cells by fluorescent microscopy. This experiment makes it possible to differentiate the process of cell death by necrosis or apoptosis. Acridine orange can penetrate virtually all cells. Therefore, viable and non-living cells will be dyed by acridine orange which will cause green fluorescence of the cells. Ethidium bromide can only penetrate cells whose membrane integrity has been altered, making them fluorescent orange. The Living cells are stained green, as Figure 3A. In late apoptotic cells, significant disruption to membrane makes it possible for ethidium bromide to enter cells and thus late apoptotic cells seem orange (Figure 3B). Necrotic cells are also stained orange, but their nuclear morphology makes it possible to differentiate them from late apoptotic cells. The late apoptotic cells nuclei appear in condensed and often fragmented form. But the necrotic cells have similar nuclear morphology with the viable cells, without condensed chromatin. Chromatin condensation, nuclear marginalization was also observed cells treated with ENO14BS, which are all signatures of apoptotic cell death. Ao-Eb staining in SW480 cells gave green fluorescence in control cells whereas ENO14BS biosurfactant (250 µg/ml) treated cells emitted green as well as different shades of orange, red or yellow fluorescence (non green). There was a significant increase of non-green cells in the treated group compared to the untreated control.

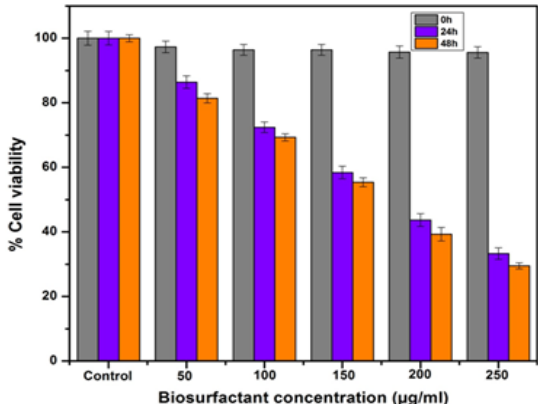

Figure 1: Cytotoxicity of different doses of biosurfactant upon treating the SW480 cell line in terms of the cell viability percentage. All the experiments were carried out in triplicate and the values were presented as mean ± SD (*n*=3).

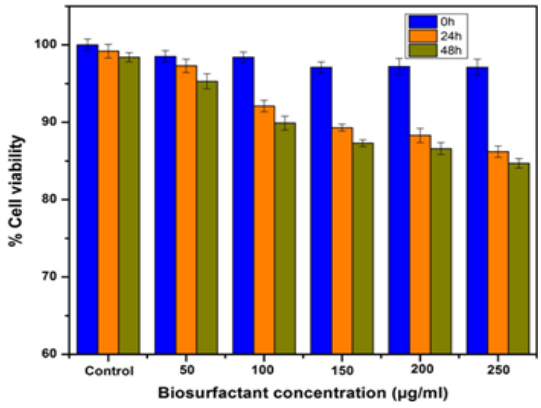

Figure 2: Cytotoxicity of different doses of biosurfactant upon treating the L292 cell line in terms of the cell viability percentage. All the experiments were carried out in triplicate and the values were presented as mean ± SD (*n*=3).

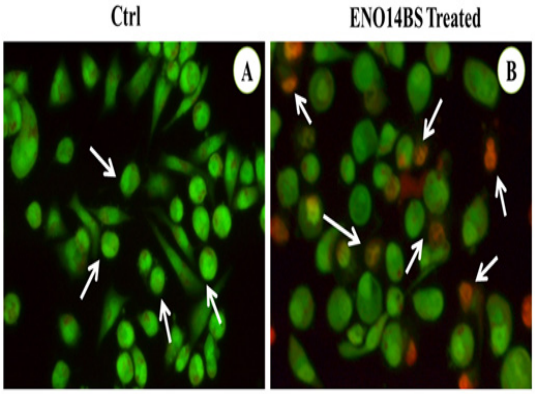

Figure 3: Live-dead cells staining: Fluorescence microscopy.

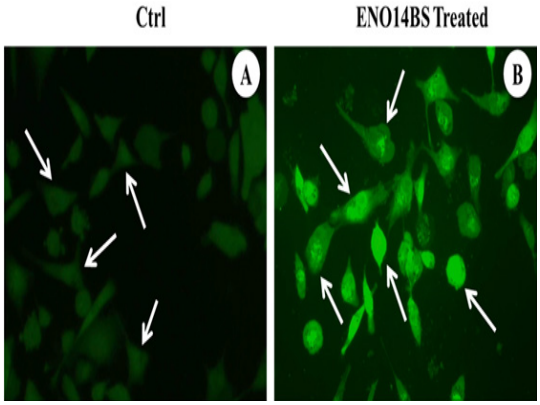

Figure 4: ROS generation: DCFDA staining.

Table 1: Physiochemical properties of Biosurfactant.

Analysis	Properties
Oil absorption capacity (g/g)	4.36 ± 0.05
Water activity (a_w)	0.32 ± 0.03
Foaming capacity (%)	52.8 ± 0.10
Water solubility Index (%)	80.86 ± 0.20

All the analysis was carried out in triplicate. The values were presented as mean ± SD (*n*=3).

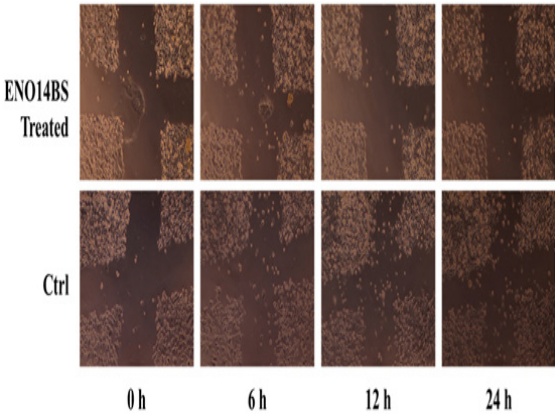

Figure 5: Wound Healing Assay.

Generation of ROS (reactive oxygen species) generally linked with apoptosis cell. To determine the degree of ROS formation in SW480 cells with or without the ENO14BS, we applied an oxidation-sensitive DCF-DA fluorescent dye. Fluorescent microscopy images showed less ROS generation in untreated control (Figure 4A) compared with the treated group (Figure 4B). Therefore, increase level ROS intermediates may have done a role in apoptotic induction, evidenced by DCF-DA staining.

In the case of wound healing assay, it was observed that control cells were able to grow, migrate and close the wound made in about 24 h. However, treatment with ENO14BS (100µg/ml) resulted in the inhibition of wound closure even at 6 h, suggesting that ENO14BS treated sample significantly inhibited cell migration compared to control (Figure 5). There are many methods to show the anti-metastatic activity of a compound. Wound healing or wound closure assay is a simple and straightforward method that gives a preliminary idea about the anti-metastatic potential. Together, these results indicate that ENO14BS biosurfactant can effectively inhibit *in vitro* cell migration in SW480 cells.

DISCUSSION

The unflagging engrossing biosurfactant, representing ecological substitute to their synthetic equivalent has gained enormous attention in 21st century and their potential applicability in pharmaceutical, food and petrochemical industries is being explored and expanding. Due to the functional and structural diversity, the marine derived biosurfactants have gained lots of interest. As these biosurfactants are highly effective and eco-friendly, it is obvious to turn for the microbial population for its potential exploration to satisfy the needs. In our investigation, the biosurfactant showed remarkable physiochemical property and excellent anti-colon cancer activity. These results could be very much useful for the food and pharmaceutical industry. Previously, it has been reported that this surface-active molecule has many potential applications in the field of biomedicine.[14] The biosurfactants molecule has also been discussed as effective and safe substitutes to anti-microbial agents and synthetic drugs.[3] In our study, the biosurfactant was found to be non-toxic to normal cell line whereas it can effectively destroy the colon cancer cell. According to ISO report, the cell viability greater than 80 % may be regarded as non-toxic.[15] Therefore the biosurfactant could be useful as a stabilizing agent in food industry and anti-colon cancer agent in pharmaceutical industry.

CONCLUSION

Based on the overall application demonstrated in this study, it has been considered that biosurfactants are the green alternative for synthetic surfactants. Their recognized functional properties and biological activities have inspired this molecule to be used in the pharmaceutical and food industries.

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CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest

ABBREVIATIONS

MTT: 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide; **DCF-DA:** Dichlorofluorescein Diacetate; **EtBr:** Ethidium bromide; **BS:** Biosurfactant; **STLs:** succinoyltrehalose lipids; **MELs:** mannosylerythritol glycolipid; **BHB:** Bushnell-Haas Broth; **OAC:** Oil absorption capacity; **HCl:** Hydrochloric acid; **WSI:** Water solubility index; **FBS:** fetal bovine serum; **NCCS:** National Centre for Cell Science; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl sulfoxide.

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