

Short Communication

In vitro synergistic effect of biosurfactant produced by *Bacillus subtilis* MTCC 441 against drug resistant *Staphylococcus aureus*

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ABSTRACT

Microorganisms are capable to produce a wide range of surface active metabolites which are known as biosurfactant. In the present study, the production of biosurfactant from *Bacillus subtilis* MTCC 441 and evaluation of its synergistic activities with known antibiotics against drug resistance *Staphylococcus aureus* were performed. Drops collapse grid method was developed for detection of biosurfactant production in the medium. The production of biosurfactant was done in previously defined Carvalho's medium. The extraction of biosurfactant was done in chloroform: methanol (2:1 v/v) solvent. The synergistic activity of crude biosurfactant was determined by disc diffusion and minimum inhibitory concentration assay. The *Staphylococcus aureus* was found resistant against ampicillin-sublactum (10 μ g), ampicillin (25 μ g), and cloxacillin (5 μ g), which showed no zone of inhibition but in the combination of crude biosurfactant, the zone of inhibition were measured as 14 mm, 14 mm and 19 mm, respectively. The crude biosurfactant showed good synergy with most of the antibiotics used in the study. The results demonstrated that biosurfactant possesses considerable potentiality to break the resistance of the pathogen tested. In future, it can be used for the development of effective drug against resistant bacteria.

INTRODUCTION

Biomolecules with amphiphilic nature are called as biosurfactant. Biosurfactant are produced by microorganisms, mostly by bacterial cells, as an extracellular metabolite which contains hydrophilic and hydrophobic moieties (Lin et al., 1998). There are various types of biosurfactants produced by microbial cells, e.g. rhamnolipids from *Pseudomonas aeruginosa*, surfactin from *Bacillus subtilis*, emulsan from *Acinetobacter calcoaceticus* and sophorolipids from *Candida bombicola* (Rodrigues et al., 2006). Biosurfactants have a special advantage over the chemical surfactants, such as lower toxicity, higher biodegradability, biocompatibility and digestibility, better environmental compatibility, higher foaming, high selectivity, effectiveness at extremes of pH, temperature, salinity and wide-spread applicability, and their unique structures which provide new properties that chemical surfactants may lack (Desai and Banat, 1997; Kosaric, 1992). The research on production of

biosurfactant and their applications have steadily increased during the past decade due to their broad range of functional properties and diverse synthetic capabilities of microbes. Biosurfactants has many applications in the areas such as food and food related industries (as emulsifiers, foaming, wetting, solubilizers, antiadhesive agents), biomedicine and therapeutics (Banat et al., 2000).

Some biosurfactants are a suitable alternative to synthesized medicines and may be used as safe and effective therapeutic agents (Singh and Cameotra, 2004). The antimicrobial activity of several biosurfactants has been reported against bacteria, fungi, algae and viruses (Cameotra and Makkar, 2004).

Lipopeptides, produced by *Bacillus* spp., have great biological activities such as antimicrobial, antitumor, antiadhesive and antiviral activities (Peypoux et al., 1999). The lipopeptide iturin from *Bacillus subtilis* showed potent antifungal activity (Besson et al., 1976). The various antimicrobial properties of surfactin, produced by *Bacillus subtilis*, have been reported in literature (Sabaté and Audisio, 2013). In this study, the synergistic effect of crude biosurfactant produced by *Bacillus subtilis* MTCC 441 was investigated against multidrug-resistant *Staphylococcus aureus*.

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MATERIALS AND METHODS

Microorganisms

Bacillus subtilis MTCC 441 and *Staphylococcus aureus* were used in this study. *Bacillus subtilis* MTCC 441 belongs to the Microbial Type Culture Collection (MTCC) of the laboratory of Institute of Microbial Technology, Chandigarh, India, and *Staphylococcus aureus* was obtained from the culture collection of Department of Microbiology, Ch. Charan Singh University, Meerut, India. The lyophilized culture (MTCC 441) was rehydrated using 0.1 % saline (NaCl and Tween 80) up to 4 hours duration and then subculture was done on the nutrient agar. The cultures were maintained on nutrient agar slant at 4 °C. Activation of the bacterial culture was carried out by inoculating culture from the slants into the nutrient broth and incubated for 16-18 hours at 35 °C.

Production and isolation of biosurfactant from *Bacillus subtilis* MTCC 441

Production medium and culture condition

A previously defined medium described by Carvalho (2005) was used for the production of biosurfactant from *Bacillus subtilis* MTCC 441 which consisted of (per liter of distilled water): glucose, 40.0g; (NH₄)₂SO₄, 8.5g; NaNO₃, 8.5g; K₂HPO₄, 13.6g; KH₂PO₄, 4.0g; MgSO₄·7H₂O, 0.5g; and in this medium 10 ml of the solution consisting of (per liter of distilled water): CaCl₂, 0.42g; FeSO₄·7H₂O, 2.29g; MnCl₂·4H₂O, 0.10g; ZnCl₂, 0.17g; CuCl₂, 0.03g; CoCl₂·6H₂O, 0.06g and Na₂MoO₄·2H₂O, 0.06g was added. The broth pH was adjusted to 7. The cells of *Bacillus subtilis* MTCC 441 from their mid-log phase were inoculated in Carvalho's production medium with the inoculum size of 10⁷-10⁸ per ml (as per McFarland standard) and incubated at 35 °C and 160 rpm for 5 days in a rotatory shaker incubator.

Extraction of biosurfactant

After an incubation period of 5 days, the cells were removed from the production medium by centrifugation at 2000 rpm for 10 min. The supernatant was again filtered with Whatman No. 1 filter paper to obtain clear broth solution. The cell free broth was used for the detection of biosurfactant. The pH of cell-free broth adjusted to 2.0 using 6N HCl and keeping it at 4 °C for overnight (Cooper and Goldenberg, 1987; Peypoux *et al.*, 1999). Precipitated material from cell free broth was collected by centrifugation using refrigerated centrifuge at 11,000 rpm for 20 minutes. The precipitate was air dried at room temperature and weighed for quantification. The extraction of biosurfactant was done in of chloroform-methanol (2:1 v/v) solution. 500 mg of the dry product was added in 50 ml of solvent and incubated in a rotatory shaker at 250 rpm and 30 °C for 15 minutes (Fernandes *et al.*, 2007). The mixture was filtrated using a 0.45 µm filter paper to remove debris and extra solvent evaporated by rotavapour. The solid product dissolved in DMSO (dimethyl sulfoxide) with the final concentration of 20 mg/ml and used as crude biosurfactant for the antibacterial activity.

Detection of biosurfactant

The presence of biosurfactant in producing medium was determined by the drop collapse method (Jain *et al.*, 1991) with a slight modification. This assay is based on the drop collapse as well as change in optical distortion of the grid that is caused by surfactants. Five wells of microtiter plate were filled by 100 µL of distilled water. A drop of mustard oil was put into each of the wells except one which used as a control for optical distortion of the grid. After this, 20 µL of distilled water (as a negative control), 20 µL of cell free broth, and 20 µL of SDS (as a positive control) were added in wells labelled as c, d, and e, respectively. The presence or absence of oil drop and optical distortion of the grid were observed to confirm the activity of biosurfactant.

In vitro Antibacterial and synergistic activity of crude biosurfactant

Disk Diffusion assay

The enhancement of the activity of antibiotic drugs and antimicrobial activity of the biosurfactants was evaluated using the disc diffusion Kirby-Bauer method (Bauer *et al.*, 1966). Sterile paper disks (6 mm in diameter, Whatman No. 1) were impregnated with crude biosurfactant (50 µg) and twelve antibiotics (50 µg) for antibacterial activity. The stock solutions of antibiotics were freshly prepared in saline water or DMSO. For synergistic activity of biosurfactant with antibiotics, single paper disk was impregnated with crude biosurfactant (50 µg) as well as antibiotic (50 µg). In order to produce an appropriate inoculum, an overnight culture of *Staphylococcus aureus* in nutrient broth was standardized to an opacity equivalent to 0.5 on the McFarland Scale (10⁸ CFU/ml). 200 µL of standardized suspension of broth culture of *S. aureus* was spread over Nutrient agar plate with the help of sterile spreader. The previously prepared paper disks were put in each Petri plate along with solvent disk. All the 12 antibacterial drugs were examined in different plates. All plates were incubated at 35 °C for 24 hours. The inhibition zones were measured with the help of HiAntibiotic Zone Scale™ of HiMedia. All the experiments were carried out in triplicate.

MIC determination

A broth dilution technique was employed to determine the MIC (minimum inhibitory concentration) of crude biosurfactant and antibiotic (NCCLS, 2012). The concentration of DMSO was maintained less than 4% in the final test volume. Various concentrations (1000... 7.8 µg/ml) of test compounds were dispensed into wells, then inoculated with the test organism with approx. 2.5 × 10⁶ cells/ml and incubated at 37 °C for 24 h. The MIC values were determined as the lowest concentration resulting in no growth. All the experiments were done in triplicate in separate time.

FIC index

The fractional inhibitory concentration (FIC) index is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. Synergy

and antagonism were defined by FIC indices of ≤ 0.5 and > 4 , respectively. An FIC index result of > 0.5 but ≤ 4 was considered indifferent (Odds *et al.*, 2003). Both, crude biosurfactant and antibiotic were serially diluted in growth medium upto the concentration of 1.95 $\mu\text{g/ml}$. The MIC values in combination with different antibiotics were determined by the broth micro dilution method in 96 well plates.

RESULTS AND DISCUSSION

Biosurfactant production and detection

Approximately 500 mg dry weight of the dark brown color product was obtained from 1500 ml of production medium. The presence of biosurfactant in cell free broth was detected in microtitre well plate. The distilled water formed a flat surface in the well of Microtiter plate which showed the negligible optical distortion. The drop of mustard oil in distilled water was became beaded and forms a concave lens which distorts the image of the grid (negative control). In another well, after addition of cell free broth, the drop of oil is collapsed and partial distortion of the grid image occurred which indicate the presence of biosurfactant in cell free broth (Fig. 1). Similar result was obtained with Sodium Dodecyl Sulfate (positive control).

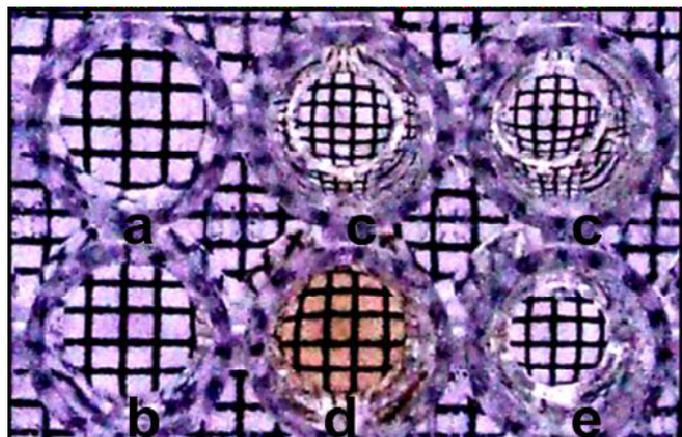


Fig. 1: Drop collapse grid test; a. Blank, b. Distilled water, c. Oil drop in distilled water (negative control), d. Oil drop in cell free broth, e. Oil drop in SDS (positive control).

Antibacterial activity

The antibacterial activity of crude biosurfactant along with its synergistic effect is described in Table 1. The results showed that inhibition zones of antibiotics were measured from 0 to 24 mm against *Staphylococcus aureus*. It was observed that *S. aureus* showed complete resistance to ampicillin-sulbactam, ampicillin, and cloxacillin with no inhibition zone and very less susceptible to many other drugs (see Table. 1). The biosurfactant separately showed the zone of inhibition of 13 mm. In previous study, the pure surfactin showed the zone of inhibition with a mean value of 14.6 mm against *S. aureus* (Fernandes *et al.*, 2007). After the addition of biosurfactant with antibiotics, the range of inhibition zones was increased and measured from 14 mm to 36

mm (Fig. 2). It clearly showed that in combination of biosurfactant, the activity of antibiotics increased.



Fig. 2: Plates showing the effect of biosurfactant; a. Control (disk of solvent), b. Biosurfactant, c. Antibiotic, d. Antibiotic with biosurfactant.

MIC determination

The MIC value of crude biosurfactant against *S. aureus* was found at 250 $\mu\text{g/ml}$. The chloramphenicol, tetracycline, ciprofloxacin, and streptomycin were found most potential antibiotics against *S. aureus* with MIC value of 62.5 $\mu\text{g/ml}$. While, antibiotics like ampicillin-sulbactam, ampicillin, cloxacillin, cefadroxil, amphotericin, and kanamycin are less effective with a MIC value of 500 $\mu\text{g/ml}$.

Synergistic studies

The *in vitro* synergistic activity of crude biosurfactant in combination with different antibiotics was determined for drug resistant *S. aureus* (Table 1). The results showed that the MIC value in combination with crude biosurfactant reduces many folds which indicated the good synergistic activity. All the antibiotics except ciprofloxacin (FICI=0.627) and co-trimoxazole (FICI=1.0) showed synergistic behavior with crude biosurfactant (FICI value < 5). The results supported that biosurfactant possesses considerable antimicrobial activity and good synergistic activity with known antibiotics.

CONCLUSIONS

The present investigation suggested that the biosurfactant produced by *B. subtilis* have the property of enhancing the activity of antibiotic as well as antibacterial activity in itself. Biosurfactant showed a considerable antibacterial effect against drug resistance strain of *S. aureus*. The combined effect of both, crude biosurfactant and antibiotics, changed the susceptibility behavior of *S. aureus*. After addition of biosurfactant, antibiotics showed 5% to more than 100% enhancement in their antibacterial. Finally, biosurfactant is a suitable alternative to synthetic drugs as antimicrobial agents and may be used as safe and effective therapeutic agents. Further research should be done in order to investigate the chemical structure and cellular toxicity of these compounds.

Table.1: *In vitro* synergistic activity of crude biosurfactant against *Staphylococcus aureus*.

Antibiotic	Zone of Inhibition ± S.D. (in mm)		MIC (in µg/ml)		FICI
	A	A+B	A	A+B	
Chloramphenicol	24±0.57	30±1.15	62.5	15.62	0.312 (Syn)
Ampicillin-Sulbactam	-	14±1.5	500	62.50	0.375 (Syn)
Ampicillin	-	14±0.57	500	62.50	0.375 (Syn)
Tetracycline	24±1.15	36±1.0	62.5	07.81	0.156 (Syn)
Ciprofloxacin	20±0.57	25±0.57	62.5	31.75	0.627 (Ind)
Streptomycin	24±0.57	29±1.0	62.5	15.62	0.312 (Syn)
Gentamycin	13±1.15	20±1.0	250	31.75	0.254 (Syn)
Cloxacillin	-	19±0.57	500	31.75	0.190 (Syn)
Co-trimoxazole	12±0.57	16±1.50	250	62.50	1.000 (Ind)
Cefadroxil	10±0.57	17±1.52	500	62.50	0.375 (Syn)
Amphotericin	10±1.15	17±0.57	500	62.50	0.375 (Syn)
Kanamycin	10±1.0	22±1.0	500	31.75	0.190 (Syn)

A= 50µg Antibiotic; B=50µg crude biosurfactant. Syn= synergy; Ind= indifferent. The MIC value of crude biosurfactant was 250 µg/ml.(-) means no inhibition.

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