Cytotoxic effect of biosurfactant produced by *Lactobacillus acidophilus* and study its synergistic effect with certain antibiotics against *S. aureus* and *E. coli*

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ABSTRACT

The aim of this research was to extract a significant amount of biosurfactant on an industrial scale from a local isolation of Lactobacillus acidophillus. This assignment was assigned to a 7L laboratory bioreactor, and all production parameters were established using batch culture experiments. At 30°C and 6.5 pH, a bioreactor with a working volume of 3.5 L was run for 120 hours to achieve 32 g/L. The manufacturing medium's supernatant was employed to lower water's surface tension from 72 to 24 mN/m, resulting in a 57 percent emulsification activity. The product was extracted with a variety of solvents and purified on a silica gel column (3.5 x 30cm) using glass chromatography. The toxicity of BS against breast cancer (AMJ-13) cell line was found to be 23%. When BS was used with antibiotics to treat pathogenic clinical isolates, it was discovered that ampicillin and tetracycline have a synergistic effect against S. aureus and E. coli.

Keywords: Biosurfactant, bioreactor, emuslification, chromatography

Introduction

There are multiple features which make biosurfactants (microbially-produced surfactants)a promising alternative for chemically-synthesized ones in industrial applications, owing these features to their higher effectiveness and biodegradability at extreme temperature, Ph and salinity conditions, lower toxicity and environmental friendly. These molecules are biosynthesized by a number of microorganisms (21,22).

There's a big need for optimization of fermentation conditions in order to increase the product yield of biosurfactants as well as improving their specificity to overcome the barriers that limit industrial production and exploiting of microbial surfactants (26,28). The activity of BS depends on the surface active compound quantity until the concentration of critical micelle (CMC) is obtained. Based on the theories of classic surfactant aggregation, the molecules of surfactant are integrate into the structure of water, which called monomers in aqueous phase, and aggregate at the interface of air-liquid or liquid-liquid at concentrations less than CMC (14,37). Furthermore, various structures auto- assembled by BS when the concentration above the CMC, such as vesicles or lamella, depending on the concentration of BS and pH value (19,29). Glycolipids conquer a minor part of the molecular structure of the cell and commonly placed with lipids associated with external cell surfaces. Interestingly, glycolipids assist in molecular transfer across cell membrane as well as known to be involved in the biosynthesis of proteoglycans and glycoproteins (12,27). Biosurfactants can disturb the structure of

biological membranes due to their interaction with membranous proteins as well as phospholipids (43). One of the major problems of hydrophobic antibiotics is that the difficulty in their uptake bu cells. So RL are tested for enhancing the activity of such hydrophobic antimicrobials (16,25). The aim of the present work is to produce biosurfactant from *L. acidophillu*s in bioreactor and testing its synergistic effect with antibiotics against resistant clinical isolates.

Materials and Methods

Production of BS in Bioreactor

The bench-top LAMBDA MINIFOR laboratory bioreactor was used for BS production in optimized conditions. This model is easy to handle and all-important cultural conditions can be measured and controlled. The minimum working volume was 3.5 L of the 7 L capacity of the bioreactor vessel. The instrument is equipped with different sensitive probes for temperature, pH, air and agitation system The production medium was composed (MSM) as follows (g/l): (CaCl2 . 2H2O 0.1g, FeSO4. 7H2O 0.05g, K2HPO4 1.0g, KCl 1.1g, KH2PO4 0.5g, MgSO4. 7H2O0.6g, MnSO4 . 7H2O 0.03g , Na2MoO4. 2H2O 0.001g, NaCl5.0g, NH4NO3 1.0g, Peanut oil 2.0 % (w/v), pH was adjusted to 6.2) sterilized by Millipore filter (0.22µm), was added to the medium when warmed down after autoclaving (35). Seventy ml of the previous production medium (2%) that contain (0.5 OD) was used to inoculate the bioreactor containing same production medium with the daily addition of 5 ml of the remaining spent medium after sterilization with Millipore filter (0.22µm) as a good elicitor for BS production.

Biosurfactant and extraction of biosurfactant

Production of biosurfactant was carried out in optimum condition. The fermentation medium containing 50 ml of MSM with(pH 5) media were inoculated with 1 ml of selected isolate 1 x 10^8 bacteria / ml (O.D 600 nm). The cultures were incubated at 30°C for 96 hr, with anaerobic conditions. Then the cells were harvested by centrifugation at 8000 rpm for 20 minutes, thereafter were washed twice in distilled water and resuspended in 10 ml of phosphate buffer saline. The cells were then incubated at room temperature for 4 hrs. With gentle stirring, after 4 hrs. The bacterial suspension was centrifuged at 8000g for 20 min. The supernatant was taken for extraction. In a ratio of (2:1 v/v), equal amounts of chloroform and methanol were added. These combinations were shaken well to ensure appropriate mixing and then permitted to evaporate overnight. The presence of biosurfactant was confirmed by the appearance of a white coloured precipitate at the interface between the two liquids. The yields were determined gravimetrically in terms of g/L (36, 41).

Biosurfactant purification

The BS components were separated from the crude mixture as follows. Heat activated silica gel 60 slurry in chloroform was decanted onto aglass chromatography columns (3.5×30) cm. One gram of crude extracted biosurfactants was dissolved in 10 ml of methanol and loaded in column until majority of the solvent is absorbed. Then the column was eluted with gradient of chloroform and methanol ranging from 50: 3 (250 ml) (250 ml chloroform + 15 ml methanol), 50:5 (200 ml) (200 ml chloroform + 20 ml methanol), 50:50 (100 ml) (50 ml chloroform + 50 ml methanol), and methanol alone (100 ml). The eluted extract was flow rate 20 ml / hr. and 3 ml from each fraction were collected. All eluted fractions were collected and tested for their surface tension and emulsification activity, then each fraction which contain the (biosurfactant) tested for further experiments (34).

Critical Micelle Concentration (CMC)

For CMC analysis, produced BS were subjected to serial twofold dilutions then measuring the surface tension. CMC is the minimum concentration that reduces the surface tension to the lowest level (7). For measuring critical micelle concentration (CMC) of biosurfactant after purification, concentrations range (2-200 mg/L) was prepared at room temperature (23). The CMC as well as the surface tension at the point of CMC were specified from the cut-point in the surface tension of these solutions and recorded (31)

Dry weight cell determination

After cultivation for 4 days, the fermentation broth was centrifuged at 8,000 g for 20 min to remove bacterial cells. 10 ml of the supernatant was taken to detect emulsification index (E_{24}) and surface tension. The collected bacterial cells allowed to dry in oven to obtain the dry weight, which is reported in terms of g/L (6).

Cytotoxicity Assays

For determining the cytotoxic effect of BS, the assay of MTT cell viability was done with the aid of (96_well) plates. The cell lines were seeded at a density of 1× 104 cells per well. Cells were treated with BS after 24 hours or when they had formed a confluent monolayer. After 72 hours of treatment, the vitality of the cells was determined by removing the medium, adding 28 liters of a 2 mg/mL MTT solution, and incubating the cells for 2.5 hours at 37 °C. After removing the MTT solution, the crystals remained in wells were solubilized through the addition of 130 µL of DMSO followed by incubation for at 37 °C for 15 min in oscillatory shaker (1). On a microplate reader, the absorbency at 492 nm (test wavelength) was determined, and the assay was repeated three times. The following equation was used to compute the rate of cell growth inhibition (the percentage of cytotoxicity) :-

Cytotoxicity = A_B/A ×100

Where A and B are the control and test optical densities, respectively.

Synergistic effect with antibiotics

The purified BS was tested for synergistic effect with some antibiotics against selected pathogenic isolates *S. aureus* and *E. coli* in well-plate method after combination, such as ampicillin, gentamycin, azithromycin, tetracycline, Ciprofloxacin. CMC of BS was mixed with concentration of antibiotic used in this experiment and placed in wells of Muller Hinton agar cultured with pathogenic agents.

Results and Discussion

Production of BS in bioreactor

After 120 h of incubation at 30ºC with steady favorite conditions, the surface tension of solution reached its minimum value (22.5 mN/m) with emulsification activity 57% and biomass 11 g/l. The BS concentration was 32 g/l. The dry cell weight was nearly constant after 24 hrs of production till the end of the experiment, while there was a continual increasing in BS production as well as emulsification index, whereas the surface tension decreased gradually. Noh *et al*., (24) obtained 23.6 g/L of RL from *P. aeruginosa* USM-AR2.

Purification of extracted biosurfactants using chromatography technique

In order to obtain a purified biosurfactant, silica gel column chromatography was used, by loading the column with crude biosurfactant which was dissolved in chloroform. All eluted fractions were collected, then the emulsification activity and surface tension for each one was measured. The results were revealed that the presence of 2 peak of BS produce in synthetic MSM media in which the first one appeared between (65- 72) fraction number in elution3 using Chloroform: Methanol (50: 50) (100 ml), while the second one between (79-83) result in elution 4 using methanol (100 ml). Results also indicated that the first peak gave the higher emulsification activity (E_{24}%= 53) and lower the surface tension to 27 mN/m in synthetic MSM.

Critical Micelle Concentration (CMC)

The BS produced by *L.acidophillus* was able to reduce the surface tension of distilled water to 24 mN/m when the CMC value is 64 mg/L. This means that only 80 mg/L of BS is needed to reduce the surface tension to the minimum level. These values were different with Samadi *et al*., (33) who found that 20 mg of BS was able to reduce the surface tension to 22 mN/m. However; the synthetic surfactants generally had a higher CMC values that BS. Sodium dodecyl sulfate show CMC of 220 mg/L. The results have shown the effectiveness of BS produced by *L.acidophillus* isolate

Cytotoxicity effect

The cytotoxicity of BS in concentration of 25 µg/mL was 22% on AMJ-13 cell line (Figure 1 and 2), this concentration is considered as acceptable for animal cell line because of low toxicity which gives a strong indication that BS could be used as antimicrobial therapy. Also, the higher concentrations (50 and 100 µg/mL) are less than 50% toxicity, which gives an indication about the high difference between the toxic dose and effective dose for antimicrobial therapy.

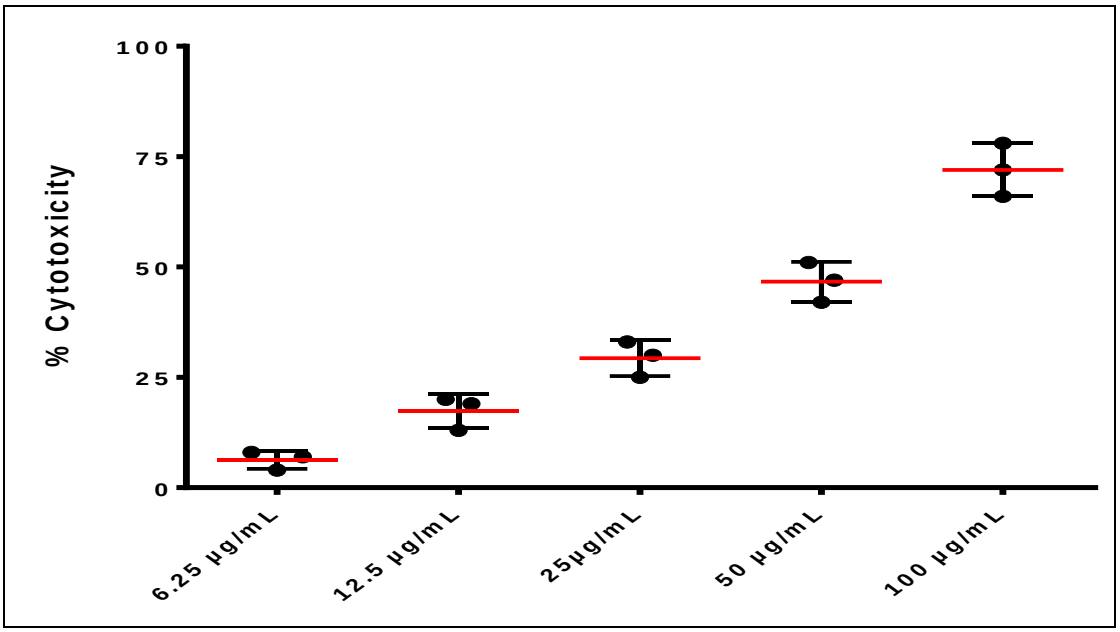

Figure (1) Cytotoxic effect of biosurfactant in AMJ-13 cells. IC50=23.81 µg/ml

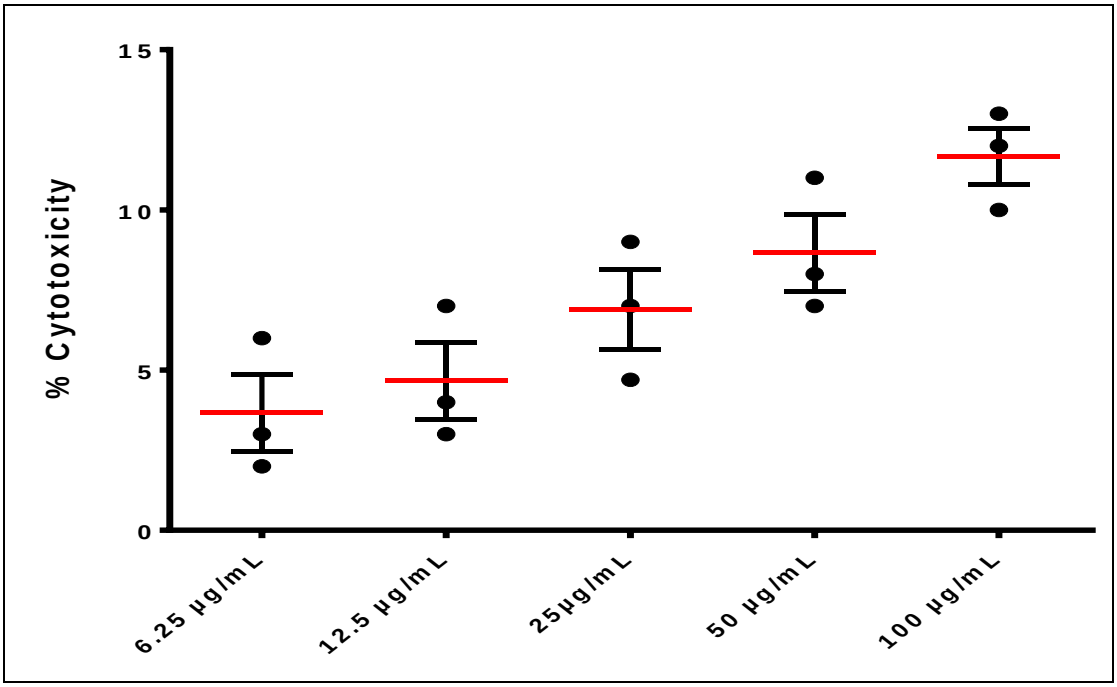

Figure (2) Cytotoxic effect of biosurfactant in HBL cells. IC50=213.55 µg/ml

Synergistic effect of BS with antibiotics

The requirement for new antimicrobial agents to beat bacterial antibiotic-resistance leads to investigation for modern antimicrobial strategies. Thus, the combination of different antimicrobial compounds in order to increase their efficacy is considered as a magnificent

solution for the problem. A synergistic association is when two or more agents act to enhance efficacy compared with single ones (11). Probably, the biosurfactant interfere with bacterial membranes and forming pores that permit the entrance of antibiotics into the cells, which increase the antimicrobial agent activity, owing to the amphiphilic nature of the surfactants, which destabilize lipid packing of biological membranes, altering integrity and penetrate these coats through hydrophobic interactions, that lead ultimately to an increase of the antibiotic impact (15). The best combination effect was found when clinical isolates were resistant to both mono-BS and ampicillin, but sensitive when these two antimicrobial agents were combined together in their CMC (Table 1 and Figure 3). A similar study conducted to demonstrate that concentrations of BS lower than CMC could increase the quantity of anionic phospholipids in membranes of *B. subtilis* 168 that lead to more sensitivity to sulfonates antimicrobials (39).

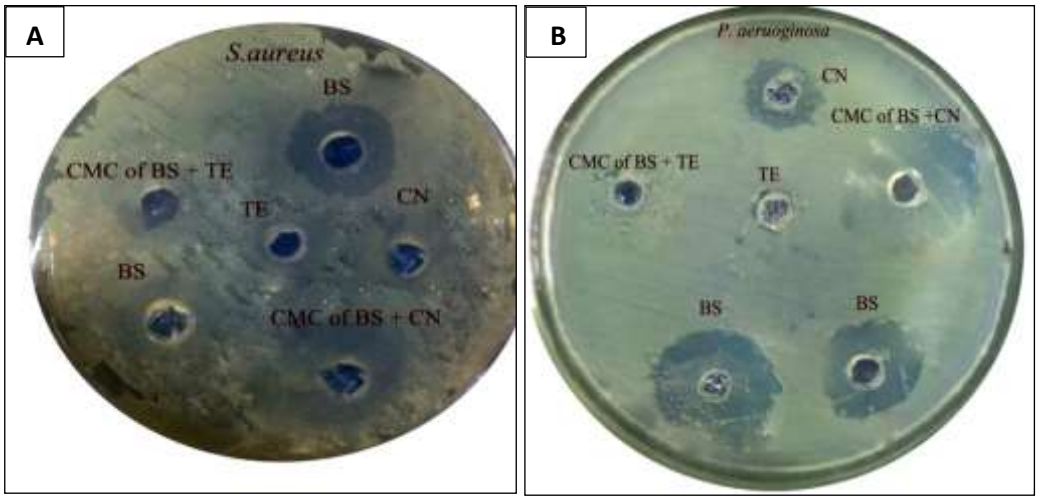

Figure (3): Combination effect of biosurfactant produced from MSM with antibiotics against clinical isolates (A) *S.aureus* , (B) *P.aeroginosa* : (CN) Gentamicin, (TE) Tetracycline

Table (1): Synergistic effect of biosurfactant produced by *L.acidophillus* with different antibiotics

Antibiotic	Inhibition zone (mm) (Ant.)	Inhibition zone (mm) (Bios.)	Inhibition zone (mm) (Bios. + Ant.)	Inhibition zone (mm) (Ant.)	Inhibition zone (mm) (Bios.)	Inhibition zone (mm) (Bios. + Ant.)
	S. aureus			***E.coli***		
Ampicillin (25µg/ml)	10	14	26	16	15	25
Azithromycin (15µg/ml)	11	16	22	--	14	12
Ciprofloxacin (15µg/ml)	25	21	27	22	23	31
Gentamycin (30µg/ml)	11	25	28	--	26	21
Tetracycline (30µg/ml)	24	26	29	12	20	29

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