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Full Length Research Paper

Antimicrobial activity of a biosurfactant produced by *Bacillus licheniformis* strain M104 grown on whey

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The aim of this study is to investigate the antimicrobial effect of the lipopeptide biosurfactant produced by *Bacillus licheniformis* strain M104 grown on whey. The biosurfactant was investigated for potential antimicrobial activity by using disc-diffusion method against several Gram positive bacteria (*Bacillus subtilis*, *Bacillus thuringiensis* (2 strains), *Bacillus cereus*, *Staphylococcus aureus* (2 strains), and *Listeria monocytogenes*), Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* (2 strains), *Salmonella typhimurium*, *Proteous vulgaris* and *Klebsiella pneumoniae*) and a yeast (*Candida albicans*). The biosurfactant showed profoundly distinct antibacterial activity toward tested bacteria and displayed an antifungal activity against tested yeast. Maximum antimicrobial activity of the biosurfactant was shown against *S. aureus*. The biosurfactant had a broad inhibition effect on intracellular components of *S. aureus*. The antimicrobial effect of lipopeptide biosurfactant produced by *B. licheniformis* was found to be time and concentration dependent. When biosurfactant was added to *S. aureus* medium in a concentration of (48 µg/ml), the maximum reduction of acid soluble phosphorous (53.06%), total lipid (90.47%) total proteins (53.43%), ribonucleic acid (RNA) (83.29%) and deoxyribonucleic acid (DNA) (48.50%) were recorded after 12 h of incubation period. From these results, it can be concluded that biosurfactants are a suitable alternative in potential applications of medical fields.

Key words: Biosurfactant, *Bacillus licheniformis*, cheese whey, antimicrobial activity, *Staphylococcus aureus*.

INTRODUCTION

Biosurfactants are amphiphilic compounds produced by microorganisms with pronounced surface and emulsifying activities (Singh et al., 2007). Microbial surfactants comprise a diverse group of surface-active molecules which are categorized by their chemical composition and microbial origin. They include glycolipids, lipopeptides, polysaccharide–protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids (van Hamme et al., 2006). Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation (Singh and Cameotra, 2004). These compounds can be synthesized by microorganisms growing on water-immiscible hydrocarbons as well as on water-soluble compounds (Mukherjee et al., 2006).

The antimicrobial activity of several biosurfactants has been reported in the literature for many different applications (Cameotra and Makkar, 2004). Some biosurfactants are known to have therapeutic applications as antibiotics and antiviral compounds. Among the many classes of biosurfactants, lipopeptides represent a class of microbial surfactant with remarkable biological activities, such as antimicrobial, antitumor, antiviral and antiadhesive activities (Khire and Khan, 1994; Banat, 1995; Peypoux et al., 1999). These properties make them relevant molecules for applications in combating many diseases and as therapeutic agents.

However, the main factor that restricts the widespread use of biosurfactants is their production cost when compared to their synthetic counterparts (Mukherjee et al., 2006). The cost can be reduced by strain

improvement, optimizing medium composition by statistical methods or by using alternative inexpensive substrates. The choice of inexpensive raw materials is important to the overall economy of the process as they account for 50% of the final production cost and also reduce the expenses with waste treatment (Makkar and Cameotra, 1999).

Whey is a liquid by-product of cheese production containing the water soluble components. It is composed of high levels of lactose (75% of dry matter) and 12 to 14% protein (Joshi et al., 2008). In addition, organic acids, minerals, and vitamins are present. Whey disposal represents a major pollution problem especially for countries depending on dairy economy. Only half of the cheese whey produced annually is recycled into useful products such as food ingredients and animal feed and the rest is regarded as a pollutant (Joshi et al., 2008). These studies showed that whey wastes might be comparatively used as better substrates for biosurfactant production at a commercial scale and might be efficient in dairy wastewater management.

The aim of the present study was to investigate the antimicrobial effect of the biosurfactant produced by *Bacillus licheniformis* strain M104 grown on whey. Moreover, the effect of the produced biosurfactant on intracellular components of a pathogenic bacterial strain, *Staphylococcus aureus* ATCC 25928 was studied.

MATERIALS AND METHODS

Isolation and screening of biosurfactant- producing bacteria

Soil samples were screened for biosurfactant-producing isolates by using the modified procedure described by Bodour et al. (2003). A sample (5 g) of each soil sample was placed into a 250 ml flask containing 50 ml of tap water and incubated at 30 ± 2°C on a shaker at 150 rpm for 21 days. On days 3, 7, 14, and 21, a sample from each soil slurry was serially diluted, plated on peptone-yeast-glucose (PYG) agar and incubated for 3 days. After incubation, morphologically different bacteria were selected for biosurfactant screening (approximately 10 to 15 isolates per sampling time). Developed colonies on the plates were then repeatedly sub-cultured on (PYG) agar medium to obtain pure isolates, and then maintained on slants of the same medium. Purified culture slants were maintained at 4°C and were transferred at regular intervals. Purification of subcultures, if needed, was carried out by streaking for several consecutive times. Homogeneity of the isolates was confirmed by Gram staining.

Isolated colonies were inoculated into 50 ml minimal salt medium (MSM) containing 0.5% (v/v) crude oil as the sole carbon and energy source. The broth cultures were incubated with shaking (150 rpm) for 7 days at 30 ± 2°C. The cell suspensions were then tested for the presence of surfactant by using the qualitative drop-collapse method (Youssef et al., 2004). Briefly, the drop-collapse technique was carried out by adding 100 µl culture supernatant to wells of a 96-well microliter plate lid, then 5 µl of crude oil was added to the surface of the culture supernatant. Biosurfactant-producing culture gave flat drops. Aliquots from a culture of each strain were analyzed on two separate plates.

Production media and culture conditions

The seed culture was prepared by transferring a loopful from a fresh culture of *B. licheniformis* strain M104 into 50 ml of peptone yeast glucose medium (PYG) containing (g/l): Peptone, 5; yeast extract, 5; glucose, 15 (Rocha et al., 1992). The flasks were incubated with shaking at 150 rpm at 30 ± 2°C for 48 h.

Erlenmeyer flasks (500 ml volume) containing 100 ml of the modified minimal salt medium of Deziel et al. (1996) modified by Ramadan et al. (2011) was used. It contained the following constituents (g/l): $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02; KH_2PO_4, 3; K_2HPO_4, 3; urea, 2 and $FeCl_3$, 0.05, whey was added as the sole carbon source in a concentration (10 g/l). Initial pH was adjusted to 7.0 and then inoculated with 10% of the seed culture (Sepahy et al., 2005). The inoculated flasks were incubated on a rotary shaker (150 rpm) for 7 days at 30 ± 2°C.

Precipitation and extraction of biosurfactant

The preparation of biosurfactant was described by Cao et al. (2007). Briefly, the bacterial cells were removed by centrifugation at 10,000 rpm at 4°C for 10 min. The cell free supernatant was adjusted to pH 2 using 6 N HCl and kept for 24 h at 4°C. The acid precipitate was collected by centrifugation at 15,000 × g for 30 min and resuspended in distilled water and adjusted to pH 7.0 to make most of precipitate dissolved and recentrifuged again and lyophilized. For the extraction of biosurfactant compounds, 50 ml of chloroform-methanol (2:1 v/v) was added to 500 mg of the dry product and incubated in a rotatory shaker at 250 rpm, 30°C for 15 min. The mixture was filtrated using a 0.45 µm Millipore membrane (Thaniyavarn et al., 2006). The filtrate was lyophilized, weighed for quantification and then used for antimicrobial activity tests.

Antimicrobial assay

The antimicrobial activity of the produced biosurfactant was studied against different gram positive, gram negative bacteria and yeast (Table 1).The identified strains were obtained from microbial culture collection (MIRCIN) at Faculty of Agriculture, Ain Shams University. The antimicrobial activity was evaluated by agar disc diffusion method (Bauer et al., 1966). Sterile discs (0.6 cm) soaked with the biosurfactant solution in methanol (50 µg/ml) was assayed on the surface of an nutrient agar and malt extract media for bacteria and yeast, respectively inoculated with the tested microorganism. After incubation period for 24 h at 37 ± 2° and for 48 h at 25 ± 2°C for bacteria and yeast, respectively, the diameter of inhibition zones was measured (Bradshaw, 1992). Negative controls were prepared using the same solvents as employed to obtain the extract. As positive controls, ofloxacin (5 µg, Oxoid) was used for Gram-positive bacteria, cefaperazone–sulbactam (10 µg, Oxoid) for Gram-negative bacteria and amphotericin B (30 µg, Sigma) for *Candida albicans* To ensure that the results were reproducible, the average of three independent measurements was taken.

MIC determination

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of biosurfactants at which no visible growth could be observed after incubation for the required time (Ericsson and Sherris, 1971). MIC was determined for *S. aureus* ATCC 25928 by the serial agar dilution method as described by Copper (1972).

Effect of biosurfactant on growth and intracellular components of *S. aureus* ATCC 25928

Erlenmeyer flasks (500 ml volume) containing 100 ml of the nutrient broth medium were inoculated with a suspension of *S.aureus* ATCC

Table 1. Antimicrobial activity of the biosurfactant produced by *B. licheniformis* strain M104.

Microorganisms	Gram positive bacteria	Zone of inhibition diameter (mm)
B. subtilis	ATCC 6633	16 ± 1.41
B. thuringiensis var. kurstaki	ATCC 19266	20 ± 3.54
B. thuringiensis	ATCC 10792	17.5 ± 2.50
B. cereus	ATCC 9634	12 ± 0.00
S. aureus	ATCC 25928	25 ± 3.54
Methicillin-resistant *S.aureus* (MRSA)	ATCC 25928	11 ± 0.00
L. monocytogenes	ATCC 19115	-
	Gram negative bacteria	
P. aeruginosa	ATCC 10145	17 ± 7.78
E. coli	ATCC 11775	19 ± 3.54
E. coli	ATCC 11246	18 ± 0.71
S. typhimurium	ATCC 14028	12 ± 0.00
P. vulgaris	ATCC 13315	10 ± 0.71
K. pneumoniae	ATCC 10031	-
	Yeast	
C. albicans	ATCC 70014	12.5 ± 2.12

Values are means ± S.D (mm) of three separate experiments, - = no inhibition zone.

25928 cells ($O.D_{600}$ = 10^7 cells / ml). Different concentrations of biosurfactant (0, 6, 12, 24, 36 and 48 µg / ml) were added. The inoculated flasks were incubated on a rotary shaker (150 rpm) for 12 h at 37 ± 2°C. Ten ml samples were withdrawn at 2 h intervals (after 0, 2, 4, 6, 8, 10 and 12 h) to elucidate effect of biosurfactant on the bacterial growth and intracellular components.

Cell growth measurement

Bacterial growth was determined by measuring the absorbance at 600 nm (OD_{600}) by a spectrophotometer UV-VIS Double Beam PC, Labomed INC (Kim et al., 1999).

Fractionation and quantitative estimation of intracellular components

Acid - soluble phosphorous compounds

Ten ml samples of biosurfactant – treated and untreated cultures (control) were withdrawn at 2 hours intervals (after 2, 4, 6, 8, 10 and 12 h). Cells were collected by centrifugation, washed twice with ice-cold saline (0.9% NaCl sol.) and extracted twice with 2.5 ml of 5% ice-cold trichloroacetic acid (TCA). The suspension was finally centrifuged at 6000 rpm for 10 min and the combined TCA extracts were used for the determination of acid- soluble phosphorous according to the method described by Toribarn et al. (1956).

Total lipids

The residue after removal of the acid-soluble phosphorous was extracted 3 times with 5 ml a mixture of chloroform: methanol (2:1, v/v) as described by Bligh and Dyer (1959). The combined extracts were used for determination of total lipids according to Knight et al. (1972).

Total proteins

The dilapidated cells were incubated with 2 ml 1N KOH at 37°C for 20 h. One ml of the product was saved for protein determination as described by Lowry et al. (1951) using bovine serum albumin as standard.

Ribonucleic acid (RNA)

The remaining portion of the samples (1 ml) after hydrolysis by 1 N KOH was subjected to extraction of RNA and DNA fractions. 0.4 ml of 6 N HCl was added to each sample then the solution was completed with the same volume of 10% TCA. After centrifugation, the supernatant was used for RNA determination as described by Merchant and Kahn (1969).

Deoxyribonucleic acid (DNA)

The residue after extraction of RNA was hydrolyzed with 5 ml of 5% TCA at 90°C for 30 min, cooled and centrifuged at 6000 rpm. The residue was washed once with 2 ml of 5% TCA and the super-natants were combined to form DNA fraction, then the DNA content was measured as described by Dische and Chargaff (1955).

Statistical analysis

Results are presented as mean value ± standard deviation (SD). The Microsoft Excel 2003 and SAS 9.1.3 statistical program were used for data analysis.

RESULTS AND DISCUSSION

Ten bacterial strains were isolated from oil-contaminated soil samples with different degrees of oil contamination

collected from different plots around oil wells of Western desert of Egypt. Depending on qualitative drop-collapse method of Youssef et al. (2004), *B. licheniformis* strain M104 was the most efficient biosurfactant-producing bacterial strain and was selected for further studies.

Biosurfactant production depends on the type of carbon source present in the medium (Davis et al., 1999; Adamczak and Bednarsk, 2000). In this study, whey was used for biosurfactant production. Whey from dairy industries has been reported previously as a cheap source for good microbial growth and biosurfactant production (Patel and Desai, 1997; Dubey and Juwarkar, 2001, 2004). These studies showed that whey wastes might be comparatively better substrates for biosurfactant production at the commercial scale than synthetic media. Furthermore, the potential use of dairy wastewaters provides a stratagem for the management of efficient dairy wastewater.

One useful property of many biosurfactants that has not been reviewed extensively is their antimicrobial activity (antibacterial, antifungal and antiviral). Other medically relevant uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as therapeutic agents (Singh and Cameotra, 2004).The lipopeptide biosurfactant produced by *B. licheniformis* strain M104 exhibited interesting antimicrobial activities. The results listed in Table 1 show that all tested microorganisms were sensitive to the biosurfactant except a gram-positive bacterium (*Listeria monocytogenes*) and a gram-negative bacterium (*Klebsiella pneumoniae*) which showed resistant to biosurfactant.

Several lipopeptide biosurfactants produced by *B. licheniformis* have been shown to have antimicrobial activity (Jenny et al., 1991; Fiechter, 1992; Yakimov et al., 1995). *S. aureus* ATCC 25928 was found to be the most sensitive strain to biosurfactant. These results were in accordance with Singh and Cameotra (2004) who reported that lipopeptide produced by *B. subtilis* was active against several microorganisms, especially *S. aureus.*

Management of *S. aureus* infections is a major problem due to wide spread resistance to beta-lactams and glycopeptides (Heinemann, 1999). Moreover, the produced biosurfactant showed an antifungal activity against *C. albicans*. Similiarly, Thimon et al. (1995) reported that the biosurfactant produced by *B. subtilis* have an antifungal effect on yeast cells. Nevertheless, the lipopeptide surfactants produced by *Bacillus* genus present a great potential for biotechnological and biopharmaceutical applications due their biological properties (Singh and Cameotra, 2004).

Sheppard et al. (1991) showed that various interesting biological properties of lipopeptide biosurfactants were presumed to be the result of interactions with the membranes of target cells. One explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused deterioration in the integrity of cell membrane and also breakdown in the nutrition cyle (Hingley et al., 1986). Another explanation is the amphiphilic structures of biosurfactants, insertion of fatty acids components of biosurfactants into a cell membrane caused an increase in the size of the membrane and significant ultra structural changes in the cells such as ability of the cell to interiorize plasma membrane. Alternatively, it is possible that insertion of the shorter acyl tails into the cell membrane causes a disruption between cytoskeletal elements and the plasma membrane, allowing the membrane to lift away from the cytoplasmic contents (Desai and Banat, 1997). However, the ways in which the biosurfactants affect the membrane integrity differ. For example, Thimon et al. (1995) suggested that the lipopeptide biosurfactant is thought to disrupt plasma membranes of cells by the accumulation of intra membranous particles in the cells and increasing the electrical conductance of the membrane. On the other hand, Carrillo et al. (2003) reported that the lipopeptide biosurfactant has been shown to increase membrane permeability though interaction with cell membrane phospholipids.

The minimum concentration (MIC) of the biosurfactant produced by *B. licheniformis* strain M104 was found to be 6 µg/ml. It is worthy to note that the produced lipopeptide biosurfactant had a good activity against bacteria in comparison to rhamnolipid produced by *P. aeruginosa* (32 µg/ml, Abalos et al., 2001 and 8 µg/ml, Benincasa et al., 2004). Five concentrations of the biosurfactant (6, 12, 24, 36 and 48 µg/ml) were selected, as multiplication of MIC, for studying their influences on cell growth and intracellular components of the most sensitive bacteria, *S. aureus*. The antimicrobial effect of lipopeptide biosurfactant is found to be time and concentration dependent, therefore, producing maximum inhibitory effect on cell growth and intracellular components at 48 µg/ml after 12 h in comparison with the control cells.

Jones et al. (1996) reported that antimicrobial agents that affect the growth and multiplication of certain types of cells may interact with various targets (chemo-receptors) in the sensitive cell. Theoretically, these targets might be numerous but with decreasing the concentrations of the antimicrobial agent; the number of targets also decreased.

Effect of the biosurfactant on the *S. aureus* ATCC 25928 cells including growth rate, acid soluble phosphorous, total lipids, total proteins, RNA and DNA were studied. The results were presented in (Figures 1 to 6) and show that the maximum reduction of acid soluble phosphorous (53.06%), total lipid (90.47%) total proteins (53.43%), RNA (83.29 %) and DNA (48.50%) were reached after 12 h at 48 µg/ml of biosurfactant.

The biosurfactant exerts a significant decrease in the total lipid content of *S. aureus* ATCC 25928 cells after 12 h as compared to the normal control cells (Figure 3). This

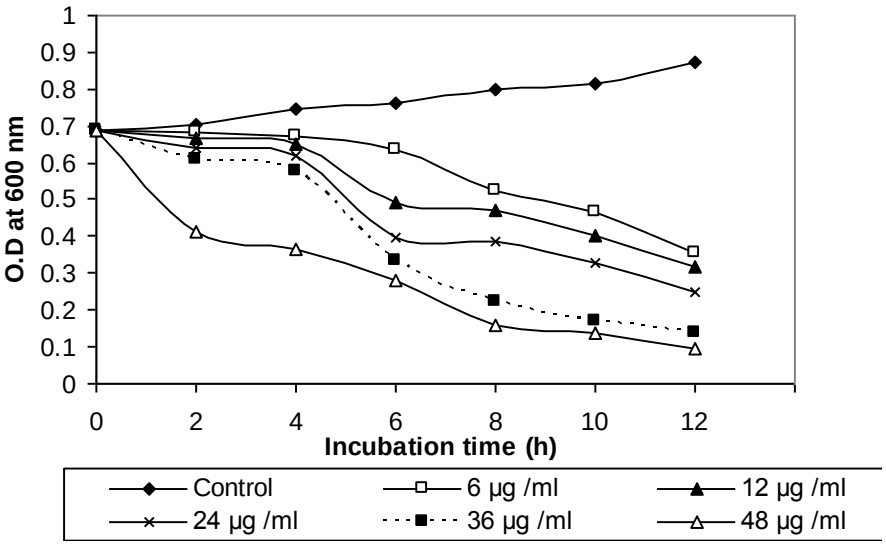

Figure 1. Effect of biosurfactant on the growth rate of *S. aureus* cells.

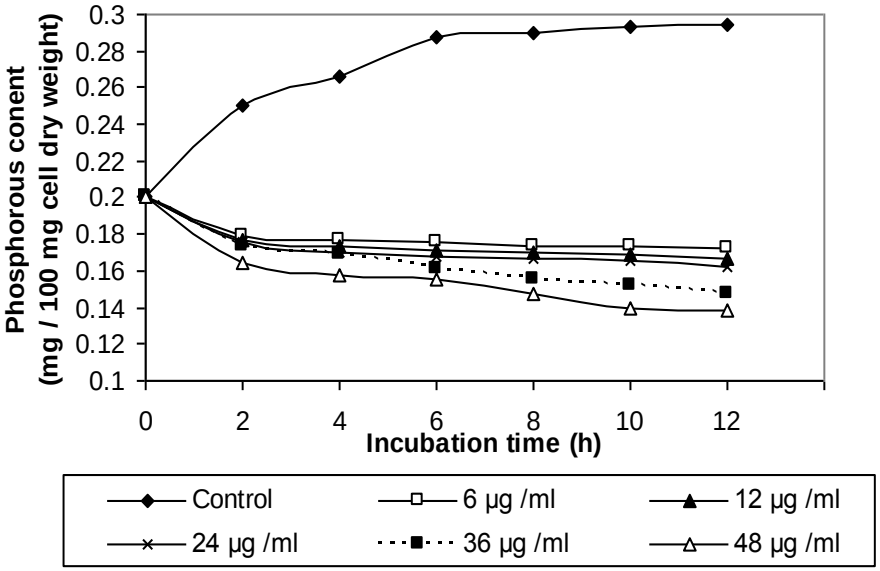

Figure 2. Effect of biosurfactant on the cellular acid soluble phosphorous of *S. aureus* cells.

decrease may be due to the effect exerted by the biosurfactant on the permeability of cell membrane or on the level of double layer of bacterial membrane (Novo et al., 2000). Moreover, Vander (1985) reported that the alternation in fatty acid contents may be due to disturbance in membrane permeability result from direct interaction of the biosurfactant with lipids which cause inhibition of membrane bounded enzyme and leakage of intracellular components. In the same regard, Cameotra and Makkar (2004) reported that the antimicrobial biosurfactant may disturb the membrane structure through interaction with phospholipids as well as membrane proteins.

A decreasing in total protein content of *S. aureus* ATCC 25928 cells after treatment with biosurfactant may be due to the inhibition of protein synthesis (Figure 4). It may bind to the 30S ribosome subunit and thus prevent the association of aminoacyl-tRNA with the bacterial ribosome as reported by Schnappinger and Hillen (1996). Also, Singh et al. (2002) suggested that the antimicrobial biosurfactant prevent the protein synthesis by inhibition of the peptidyltransferase in binding mainly the 23S rRNA in the 50S subunit of the bacterial ribosome. In the present study, the decrease in the lipids content has a relation

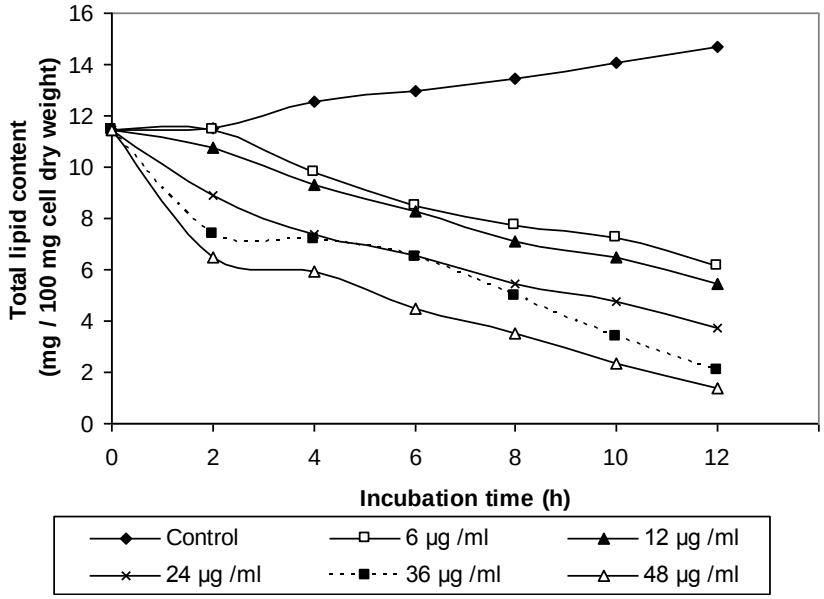

Figure 3. Effect of biosurfactant on the cellular total lipids content of *S. aureus* cells.

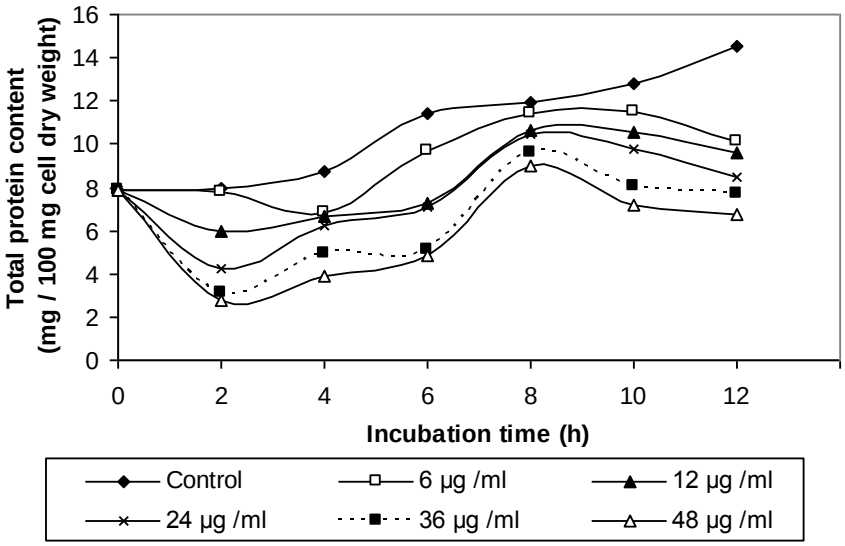

Figure 4. Effect of biosurfactant on cellular total proteins content of *S. aureus* cells.

with the disturbance in the proteins content due to the treatment of the *S. aureus* ATCC 25928 cells with the biosurfactant. Price et al. (2001) attributed this decrease to the property of expanding membrane protein causing conformational changes of lipid and protein content, this in turn may affect the growth rate of bacterial strain.

The biosurfactant also exhibited a decrease in the total RNA and DNA contents of *S. aureus* ATCC 25928 cells. This may be due to the loss of intracellular components through the damaged cell wall (Figures 5 and 6). Moreover, Volk et al. (1996) reported that the bactericidal agent forms complexes with guanine residues in helical DNA. It also prevents RNA polymerase on the DNA template. At higher concentrations, DNA replication also is inhibited. It may also be caused by selectively inhibiting an enzyme (DNA gyrase) needed for the replication of DNA (Drlica and Hooper, 2003). The bactericidal agent binds to the ß subunit of the DNA-dependent RNA polymerase and inhibits the initiation of transcription by preventing the synthesis of RNA larger than dinucleotides (Chaisson, 2003).

From the previous data the biosurfactant can penetrate

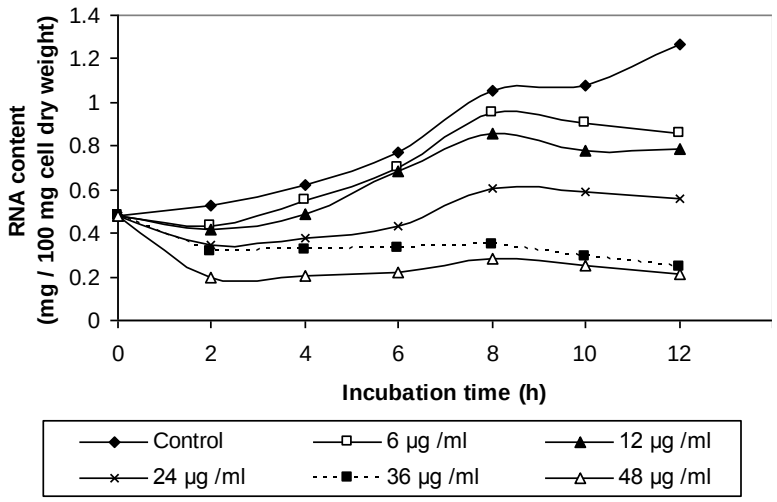

Figure 5. Effect of biosurfactant on the cellular total RNA content of *S. aureus* cells.

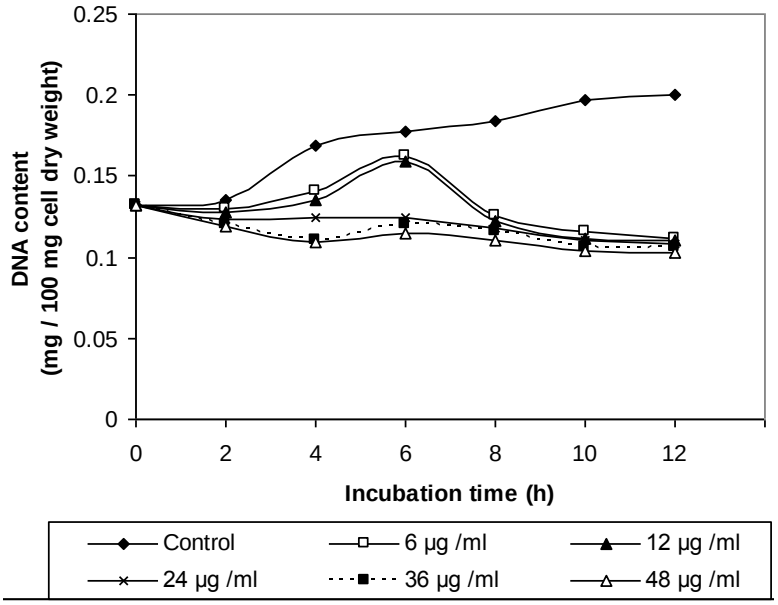

Figure 6. Effect of biosurfactant on the cellular total DNA content of *S. aureus* cells.

the cell wall structure to reach its main target cell membrane. In conclusion, results of the present study demonstrated that the biosurfactant produced by *B. licheniformis* strain M104 present a great potential for biotechnological and biopharmaceutical applications due their biological properties.

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