International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (2), pp. 001-010, February, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Investigating the effects of various additives on surface activity and emulsification index of biosurfactant resulting from broth media of *Bacillus subtilis* PTCC 1023

Golamreza Dehghan Noudeh[1,3]*, Ali Dehghan Noodeh[2], Mohammad Hassan Moshafi[3], Masoud Ahmadi Afzadi[1], Abbas Pardakhti[3] and Maryam Salandari[3]

[1]Pharmaceutics Research Centre, Kerman University of Medical Sciences, Kerman, Iran.
[2]Department of Life Sciences, Anglia Ruskin University, East Road, Cambridge, CB1 1PT, UK.
[3]Department of Pharmaceutics, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Accepted 24 August, 2017

Surfactants are amphipathic molecules which reduce surface tension and are widely used in pharmaceutical, cosmetic and food industries. In the present study, the production of biosurfactant by *Bacillus subtilis* PTCC 1023 was studied. *B. subtilis* was grown in the nutrient broth medium and biosurfactant production was evaluated by measuring the surface tension and emulsification index (E_{24}) each 24 h. The bacterium's biosurfactant production was investigated in different status with variable factors such as incubation time, temperature, aeration rate and presence of several additives. Then the best fermentation condition was investigated for maximum biosurfactant production and finally biosurfactant identity was investigated using some chemical and spectroscopy methods. The maximum biosurfactant production by *B. subtilis* PTCC 1023 was obtained when it was grown in brain-heart broth medium containing $FeSO_4$ (4×10^{-3} M), $MnSO_4$ (1.3×10^{-3} M), starch (4%) and castor oil (4%) which incubated in a 300 rpm rotary shaker at 30°C for 24 h. Lipopepetide natures in this biosurfactant was confirmed by biochemical and spectroscopic methods.

Key words: *Bacillus subtilis*, biosurfactant, emulsification index, surface tension.

INTRODUCTION

Surfactants are amphiphilic molecules which tend to lower the interfacial tension. Biosurfactants are amphiphilic compounds produced on living surfaces and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively. Biosurfactants are complex molecules comprising different structures that include peptides, glycolipids, glycopeptides, fatty acids and phospholipids. The most commonly isolated and widely studied group of surfactants produced by microorganism is glycolipids (Lin et al., 1998; Mercade and Manresa, 1994).

*Corresponding author. E-mail: gholamreza_dehghan@yahoo.com. Tel: 98-341-3205014. Fax: 98-341-3205215.

These surface-active compounds have applications in industry, agriculture, mining, and oil recovery, with functional properties such as wetting agents, foaming agents and as emulsifiers in pharmaceutical and cosmetic pro-ducts. There are various types of surfactants produced microbially or by enzymatic methods important for household, cosmetic and industrial purposes. They have advantages over chemical surfactants such as low toxicity, inherent good biodegradability (Makkar and Cameotra, 1997).

Unlike chemically synthesized surfactants which are classified according to the nature of their polar groups, biosurfactants are categorized primarily by their chemical composition and microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptide anions or cations; mono, di, or polysaccharides; and a hydrophobic moiety consisting of unsaturated or saturated fatty acids. Accordingly, the

major class of biosurfactant includes 1-Glycolipids (Rhamnolipids, Trehalolipids and Sophorolipids), 2-Lipopeptide and Lipoprotein, 3-fatty acids, phospholipids, and neutral lipids 4-polymeric biosurfactants and 5-particulate biosurfactants (Gautam et al., 2006).

Recent advances in the field of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant – producing microbes and assessing their potential. The kinds of methods include haemolysis, axisymmetric drop shape analysis (ADSA-P), rapid drop-collapsing method, colorimetry method and thin-layer chromatography (Desai and Banat, 1997; Vasileva et al., 2005).

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, haemolysis activity, stabilization or destabilization of emulsions, and hydrophilic-lipophilic balance (HLB). The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion by comparing it with surfactants with known HLB values and properties (Desai and Banat, 1997).

There are many advantages of the biosurfactants as compared to their chemically synthesized counterpart. Unlike synthetic surfactants, microbially produced compounds are easily biodegradable and thus particularly suited for environmental applications such as bioremediation and dispersion of oil spills. The chemical diversity of naturally produced amphiphiles offer a wider selection of surface-active agents with properties closely related to specific applications. In addition, microbial processes from raw materials, which are available in large quantities, can produce biosurfactants. Biosurfactants can also be produced from industrial wastes an area of particular interest for bulk production and can be efficiently used in handling industrial emulsions, control of oil spills, biodegradetion and detoxification of industrial effluents and in bioremediation of contaminated soil (Nitschke et al., 2004; Bognolo, 1999).

The gram-positive soil bacterium *Bacillus subtilis* ATCC 6633 produces subtilin, a representative of lantibiotics containing the unusual thioether amino acids meso–lanthionine and 3–methyl–lanthionine (Chatterjee et al., 2005; Stein, 2005). Lantibiotic activity is based on the formation of voltage–dependent pores that cause efflux of ions and metabolites, and finally, the collapse of the membrane potential (Breukink et al., 1999; Hasper et al., 2004).

Genes involved in lantibiotic biosynthesis, immunity, and regulations are allocated within large gene clusters. Subtilin is ribosomally synthesized as a prepeptide and posttranslationally modified to its biologically active form. Gram–positive lantibiotic producer needs efficient self–protection mechanisms to obviate the lethal activity of their products, a phenomenon that is referred to as immunity (Saris et al., 1996). The widespread application of lantibiotics as an alternative for chemical reagents in food preservation spurred research activities to increase the moderate productivity of lantibiotic producing organisms (Cheigh et al., 2005).

Subtilin is an effective bactericidal agent against gram–positive bacteria including strains of *Lactococcus, Streptococcus, Staphylococcus, Micrococcus, Pediococcus, Lactobacillus, Listeria* and *Mycobacterium*. Gram–positive spores like *Bacillus* and *Clostridium* spp. are particulary susceptible to subtilin, with spores being more sensitive than vegetative cells. Additionally, researches showed the antimicrobial activity of subtilin in the control respiratory tract infections caused by *Staphylococcus aureus* in model animals. Also, it is a natural peptide that effectively inhibits fungi (Gautam and Tyagi, 2006; Zhang et al., 2008). Additionally subtilin has been used as a biopreservative. Although the main subtilin application in foods as natural agent preservative, researches have verified its potential use for therapeutic purposes, particularly in the treatment of atopic dermatitis, stomach ulcers and colon infections for patients with immune deficiencies. Also, studied is an application of bacteriocin subtilin as an efficient alternative to antibiotics for the treatment of staphylococcal mastistis during lactation in women (Delves et al., 1996; Valenta et al., 1996).

In the present study, the production of biosurfactant by *B. subtilis* PTCC 1023 was studied. *B. subtilis* was grown in the nutrient broth medium and biosurfactant production and the biosurfactant was characterized.

MATERIALS AND METHODS

The bacterium, *B. subtilis* PTCC 1023, was purchased from the Persian Type culture Collection, Tehran, Iran. All medium were prepared based on the manufacturer protocols (Merck). In order to activate the lyophilized microbe, the ampule containing microbe was wiped with a disinfectant solution in 90% ethanol and the tip was broken just above the cotton wool on the microbe. The cotton wool was removed and the microbe was suspended in a few drops of normal saline.

In order to make stock culture, the strain was streaked on the surface of nutrient agar plates and they were then moved to a refrigerator after incubation at 37°C for 48 h. This stock could be used for several weeks. The strain was streaked on the surface of nutrient agar plates and was then incubated at 37°C for 24 h. *B. subtilis* PTCC 1023 was inoculated on blood agar plates containing 5% v/v blood and incubated at 37°C for 48 h. Haemolytic activity was detected when a definite clear zone was seen around colonies (Cobb, 1919).

B. subtilis PTCC 1023 was grown in 250 mL conical flasks, each containing 50 ml nutrient broth medium. The flasks were then incubated in a 200 rpm shaker incubator at 37°C. Samples were examined to analyze the surface activity, emulsification index and biomass every 24 h. This process lasted up to 120 h (Makkar and Cameotra, 1997; Desai and Banat, 1997; Kim et al., 1997). Microbial cells were removed from medium by centrifugation (8000 rpm, 4°C and 20 min) and biomass was measured by washing the pelleted cells with normal saline and recentrifugation in 8000 rpm for 20 min. The cells were dried overnight at 65°C and biomass was measured (Corvey et al., 2003).

Biosurfactant concentration is expressed in terms of critical Micellar dilution (CMD). This was estimated by measuring the surface tension in different concentrations using a duNouy Tensiometer. The amount of 20 ml supernatant was put into a glass

beaker (50- mL) and placed onto the tensiometer platform. In order to measure the surface tension (mN m^{-1}), a platinum plate was then slowly allowed to touch the liquid-air interface. The platinum plate was rinsed three times with water, three times with acetone between each measurement, and was then allowed to dry. All measurements were made on cell-free broth. CMD^{-1} (critical micelle dilution)$^{-1}$ and CMD^{-2} (critical micelle dilution)$^{-2}$ measurements were performed by measuring the surface tension of 10- and 100-times diluted cell-free broth (Carrillo et al., 1996). Negative controls contained sterile culture medium plus *B. subtilis* PTCC 1023 (an inoculum), at zero time.

Emulsifier activity was measured by adding 5 mL of mineral oil (liquid paraffin) to 5 mL of supernatant in a graduated tube and vortexed at high speed (3000 rpm) for 2 min. The emulsion stability was determined after 24 h. The emulsification index (E_{24}) was calculated by measuring the formed emulsion layer (Cooper et al., 1987). In order to measure the foam forming activity, *B. subtilis* PTCC 1023 was grown in 250-mL conical flasks, each containing 50 mL of nutrient broth (MERCK at pH 7.4). The flasks were incubated in a 200 rpm shaker incubator at 37°C. Samples were examined each 24 h. Then 5 mL of supernatant in a graduated tube was vortexed at high speed (3000 rpm) for 1 min. Foam activity was detected as duration of foam stability, and foam height was determined in a graduated cylinder (Razafindralambo et al., 1998; Heerklotz et al., 2001).

Two different media were used: Nutrient broth medium (Merck) and brain – heart broth medium (Merck). *B. subtilis* PTCC 1023 was grown in 250 mL conical flasks, each containing 50 mL of the culture media. Nutrient broth and brain – heart broth media were separately added to each flask. There were three conical flasks for each culture. The flasks were then incubated in a 200 rpm shaker incubator at 37°C. In order to select the best medium for biosurfactant production the samples were then examined to analyse the emulsification index and foaming activity (Peypoux et al., 1999; Cooper et al., 1981).

The selected strain was grown in brain – heart broth medium in 250-ml conical flasks. Different salts including KH_2PO_4 (4×10^{-2} M), $FeSO_4$ (4×10^{-3} M), $MgSO_4$ (4×10^{-3} M) and $MnSO_4$ (1.3×10^{-3} M) were added individually to each conical flask in order to get maximum production of biosurfactant. Three conical flasks were incubated in a 200 rpm shaker incubator at 37°C for 24 h for each salt. Samples were then examined to analyze the emulsification index and thereby to select the best salt for biosurfactant production (Peypoux et al., 1999; Cooper et al., 1981).

The selected strain was grown in brain – heart broth in 250-ml conical flasks. Starch (2 g), almond oil (2 mL), olive oil (2 mL) and castor oil (2 mL) was separately added to each flask**.** There were three conical flasks for each additive and they were then incubated in a 200 rpm shaker incubator at 37°C for 24 h. As mentioned previously, samples were then examined to analyse the emulsification index to select the best additives for biosurfactant production (Peypoux et al., 1999).

According to previous studies, *B. subitils* strains can grow and produce biosurfactants at temperatures of 30 to 45°C. Four temperatures were chosen and microbes were incubated at these temperatures, to obtain the best temperature for biosurfactant production by *B. subtilis* PTCC 1023. The strains were inoculated into 50 ml of brain – heart broth and were incubated at 200 rpm for 24 h at 30, 35, 40 and 45°C separately. After the incubation time cultures were taken out from the shaker incubator in order to evaluate E_{24} of the emulsion (Carrillo et al., 1996; Paik et al., 1998).

The production of biosurfactant of *B. subtilis* has been reported under aerobic conditions; therefore the aeration rate plays an important role in biosurfactant production (H. Paik et al., 1998).

According to different studies, five rates were chosen to obtain the best condition for cultivation. The strains were inoculated into Erlenmeyer flasks containing 50 mL of brain – heart infusion broth medium and were incubated at 37°C for 24 h with four different rates of 200, 225, 250,275 and 300 rpm. The medium were removed from shaker after the incubation time and biomass were removed by centrifugation, the supernatant was then evaluated by emulsion index in order to gain the optimum cultivation time (Carrillo et al., 1996; Delves et al., 1996).

The bacterial cells were removed from the liquid culture by centrifugation (15000 g, 25 min, 4°C). The crude biosurfactant was isolated by adding 6 N HCl to the supernatant. A flocculated precipitate was formed at pH 2.0 that was collected by centrifugation (15000 g for 20 min at 4°C). The precipitate was dried under vaccum in dessicator and kept overnight at 4°C. The crude product was re-suspended in dichloromethane. After stirring overnight, the suspension was filtered with Whatman filter paper No 1, to remove the coarse impurities. The filtrate was extracted twice with equal volumes of distilled water (pH 8.0) while stirring for 20 min. After this period, it was left 3 h in a separating funnel to allow the two phases to separate. The aqueous phases containing the biosurfactant were collected and acidified to pH 2.0 by 6 N HCl. The biosurfactant, which precipitated in the form of white to yellowish crystal, was recovered using centrifugation (15000 g, 15 min, 4°C) and dried under vacuum in dessicator, kept overnight at 4°C (Makkar and Cameotra, 1997; Desai and Banat, 1997; Cooper et al., 1981; Feignier et al., 1995) . The biosurfactant was hydrolysed with 6 M HCl at 110°C for 20 h and the lipid moiety was subsequently separated by extraction with chloroform. Several drops of bromine water were then added to the extract (Jenny et al., 1991; Wagner and lang, 1988).

Ninhydrin and biuret reactions were used in order to identify the amino acids. The ninhydrin reagent was added to the sample. A deep blue colour developed after the heating of amino acid or peptide with ninhydrin. The biuret reagent was then added to the sample. A positive result was then indicated by a violet or pink ring, due to the reaction of peptide bond proteins or short-chain polypeptides, respectively.

Such a result would not occur in the presence of free amino acids (Dehghan–Noudeh et al., 2005). The biosurfactant was obtained from *B. subtilis* PTCC 1023 and its exact structure confirmed by infrared spectroscopy. IR spectra were collected between 400 and 4000 wave numbers (cm^{-1}). The sample was dissolved in dichloromethane and UV spectra were obtained between 200 to 450 nm, using a UV-Visible Spectrophotometer, 2100 - Shimadzu (Arima et al., 1968; Ferre et al., 1997).

RESULTS AND DISCUSSION

Screening for production of biosurfactants

The screening of biosurfactant-producing microorganisms is generally carried out using monitoring parameters that estimate surface activity, such as surface tension, ability to emulsify oils, foam stability, foam height and haemolytic capacity. In the present study, these parameters were evaluated as potential predictors of surfactant-producing bacteria. *B. subtilis* PTCC 1023 isolated from nutrient agar cultures was tested by haemolytic method and showed haemolytic activity (Dehghan–Noudeh et al., 2005) (Figure 1).

Cell growth time course and biosurfactant production by *B. subtilis* PTCC 1023

Figure 2 shows the growth course time of *B. subtilis*

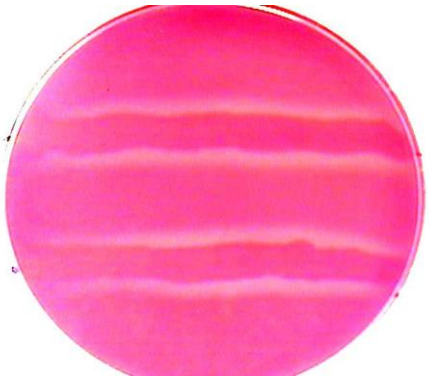

Figure 1. Haemolysis caused by *Bacillus subtilis* PTCC 1023 on blood agar.

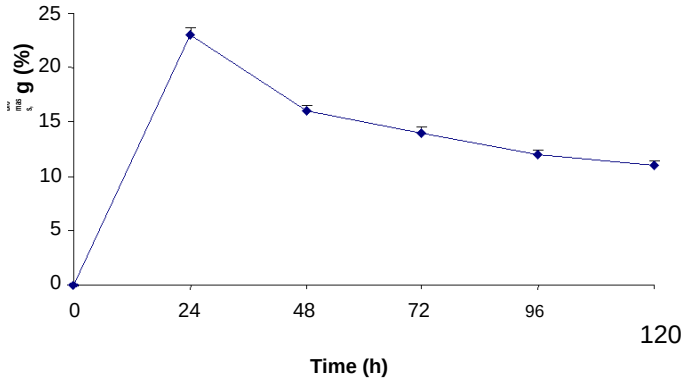

Figure 2. Cell growth and biosurfactant production by *Bacillus subtilis* PTCC 1023 against time (200 rpm, 37°C) (Mean±SD).

PTCC 1023 on nutrient broth medium. Biomass was reached in its maximum level after 24 h of incubation and followed by decreasing the growth until 120 h of fermentation. As shown in Figure 2, the maximum yield of the biomass was obtained at 24 h after incubation. Reduction of supernatant surface tension was considered as selection criteria for biosurfactant production by microorganism in liquid medium. Therefore, *B. subtilis* PTCC 1023 was cultured in nutrient broth and the surface tension was considered for bioemulsifier production. Reduction of surface tension was within 24 h of the microorganism growth (Table 1). CMD^{-1} and CMD^{-2} measurements were carried out by measuring the surface tension of 10- and 100-times diluted cell-free broth. Maximum of bioemulsifier production was achieved within 24 h of incubation and CMD values (Figure 3) showed minimum at this point. According to the growth curve of microorganism, surface tension profile of supernatant and CMD values, it could be expected at the minimum surface tension, biosurfactant production was maximum. Hence, this point was chosen as optimum for biosurfactant production by the microorganism for further experiments.

Emulsification index measurement of supernatant

Figure 4 and Table 2 show emulsification index of *B. subtilis* PTCC 1023 supernatant in nutrient broth. As expected, while the incubation time increased, the improvement in emulsion index was seen and it continued till 24 h of incubation followed by a reduction as it reached minimum after 120 h.

Results also showed that there was a rational correlation between surface activity (Figure 3) and emulsification index (Figure 4), because while the supernatant surface tension was at its lowest point, emulsification index rose to its highest level. Emulsification index values followed a similar pattern as surface tension was lowering. The best incubation time for biosurfactant production was 24 h, since emulsification index was an important parameter of surface active agents in producing a stable emulsion.

Figures 5, 6 and Table 3 show foam stability and foam height of *B. stubtilis* PTCC 1023 supernatant in nutrient broth; as it was expected, increasing incubation time caused an improvement in stability and height of foam and this increase continued till 24 h of incubation followed by a decrease during the time and it reached its minimum after 120 h.

Optimizing biosurfactant production by *B. subtilis* PTCC 1023

Figure 7 showed, emulsification index of *B. subtilis* PTCC 1023 supernatant in two different medium including nutrient broth medium and brain heart broth medium. In the present study, brain heart broth medium caused improvement in emulsion index. Hence this medium was chosen for biosurfactant production by the microorganism for further experiments.

As shown in Figure 8, incubation of *B. subtilis* PTCC 1023 in brain heart broth medium at 300 rpm caused improvement in emulsification index. Hence it can be concluded that incubation of the microorganism in a 300 rpm shaker incubator has positive affects on biosurfactant production yield.

In Figure 9, incubation of *B. subtilis* PTCC 1023 at 30°C caused improvement in emulsification index. As there was a rational correlation between emulsification index and biosurfactant production, it is concluded that the highest amount of biosurfactant is produced at 30°C.

When *B. subtilis* PTCC 1023 was grown in brain- heart broth medium, the production of the bioemulsifier was relatively poor and it was improved by addition of $FeSO_4$ and $MnSO_4$ salts. Cations of Fe^{2+} and Mn^{2+} caused enhancement of the yield. $FeSO_4$ caused a larger emulsification index in comparison with $MnSO_4$. Also, from emulsification index studies, it can be concluded that when Fe^{2+} and Mn^{2+} was added to brain heart broth medium, the best yield bioemulsifier was obtained (Figure 10).

The cell – surface hydrophobicity of *B. subtilis* PTCC

Table 1. Surface tension studies, (Critical micelle dilution)$^{-1}$; CMD^{-1}; and (Critical micelle dilution)$^{-2}$; CMD^{-2}; results for supernatant of *B. subtilis* PTCC 1023, grown in nutrient broth (37˚C, 200 rpm).

Time (h)	Surface tension (mN/m) Mean±SD	CMD^{-1} (mN/m) Mean±SD	CMD^{-2} (mN/m) Mean±SD
0	63.15± 0.12	65.60±0.35	66.57±0.17
24	45.25±0.55	49.42±0.40	52.67±0.41
48	48.25±0.47	50.62± 0.33	55.57±0.39
72	51.27±0.34	54.50±0.35	56.55±0.46
96	56.30±0.33	59.65±0.40	61.05±0.35
120	59.50± 0.31	62.75±0.44	64.52±0.17

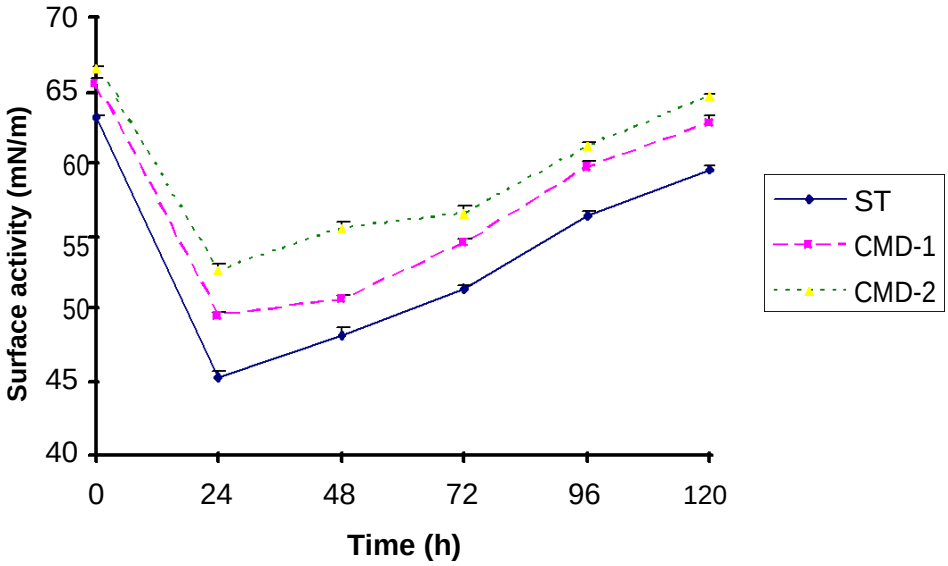

Figure 3. Surface activity profile of *B. subtilis* PTCC 1023 against time (37˚C, 200 rpm) (Mean±SD).

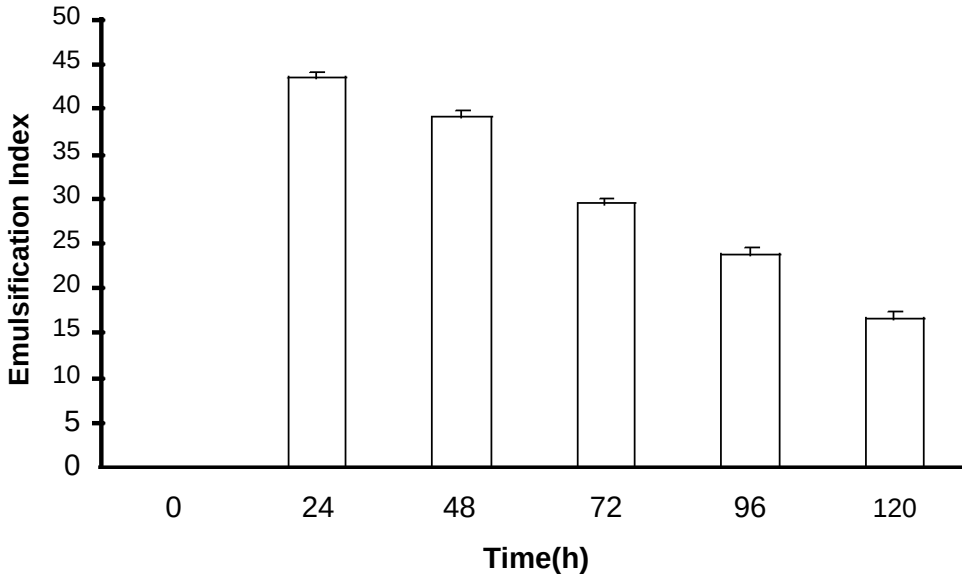

Figure 4. Emulsification index; E_{24}, graph of *B. subtilis* PTCC 1023 against different incubation time (37˚C, 200 rpm) (Mean±SD).

Table 2. Emulsification index; E_{24}; results for supernatant of *B. subtilis* PTCC 1023 grown in nutrient broth (37°C, 200 rpm).

Time (h)	0	24	48	72	96	120
Emulsification index (E_{24}) Mean ±SD	0±0.0	43.48±0.50	39.13±0.64	29.5±0.50	23.8±0.72	16.66±0.76

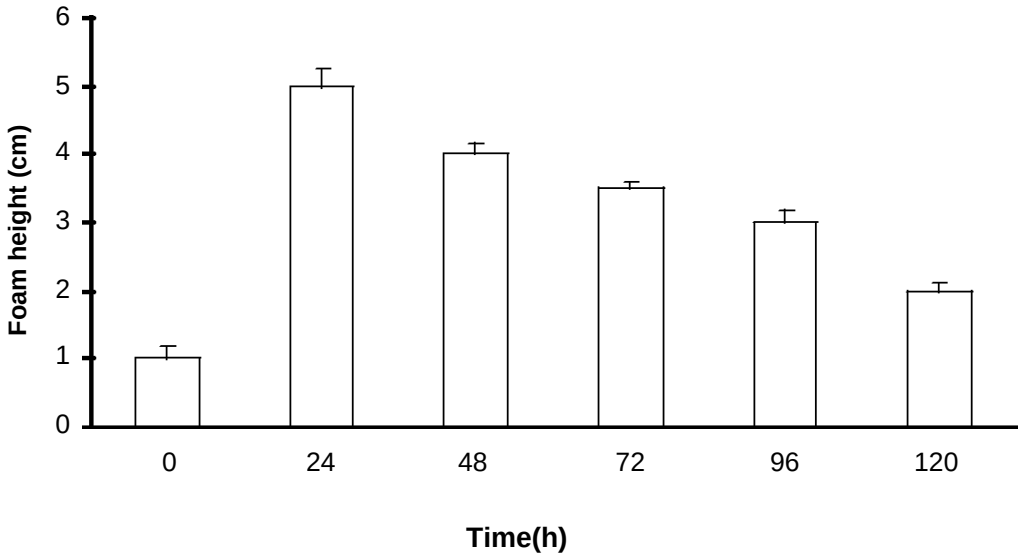

Figure 5. Foam height (cm); results for supernatant of *B. subtilis* PTCC 1023 grown in nutrient broth against time (37˚C, 200 rpm) (Mean±SD).

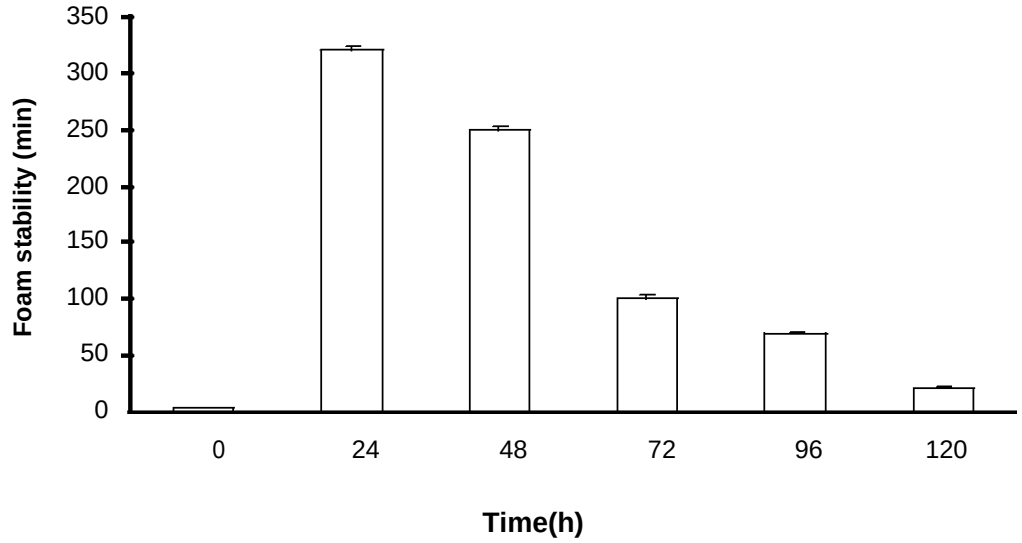

Figure 6. Foam stability (min); results for supernatant of *B. subtilis* PTCC 1023 grown in nutrient broth against time (37˚C, 200 rpm) (Mean ± SD).

Table 3. Foam stability (min) and foam height (cm); results for supernatant of *B. subtilis* PTCC 1023 grown in nutrient broth (37°C, 200 rpm).

Time (h)	0	24	48	72	96	120
Foam stability (min) Mean ± SD	3±0.30	320±0.82	250±0.47	100±0.62	70±0.40	20±0.23
Foam height (cm) Mean ± SD	1±0.20	5±0.26	4±0.17	3.5±0.10	3±0.17	2±0.10

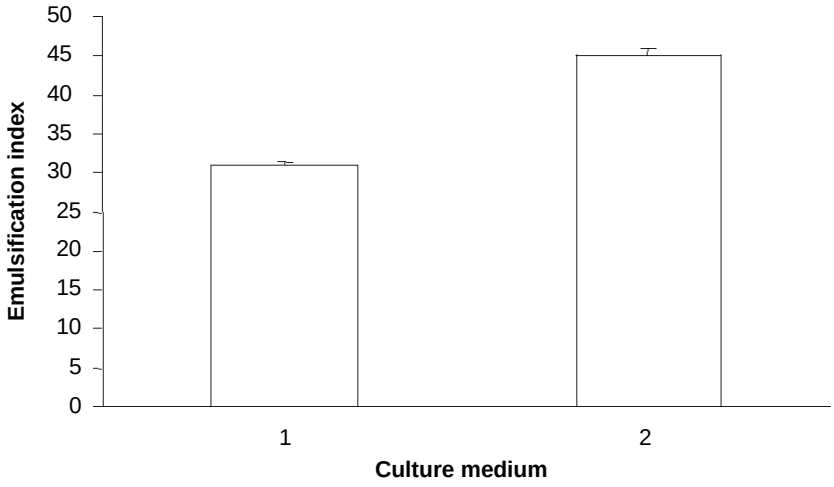

Figure 7. Emulsification index of biosurfactant studies from growth of *B. subtilis* PTCC 1023 in different media after 24 h of fermentation (37˚C, 200 rpm). (1) nutrient broth (2) brain-heart broth (Mean±SD).

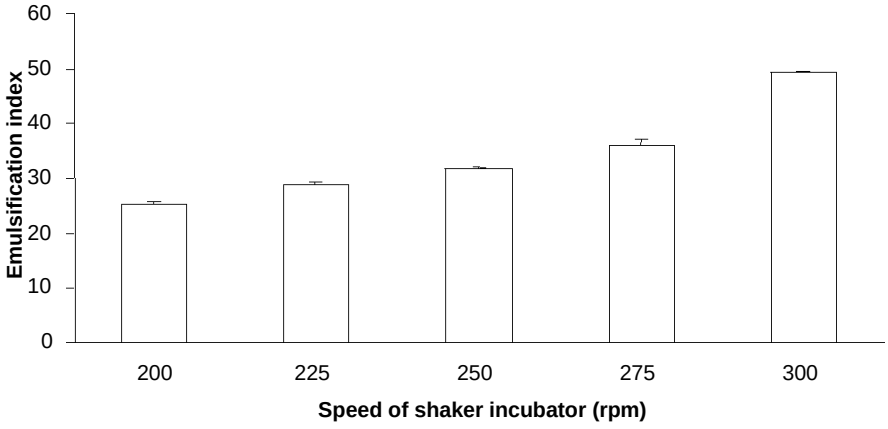

Figure 8. Emulsification index (E_{24}) of *Bacillus subtilis* PTCC 1023 at different aeration rates (37˚C, 24 h) (Mean±SD).

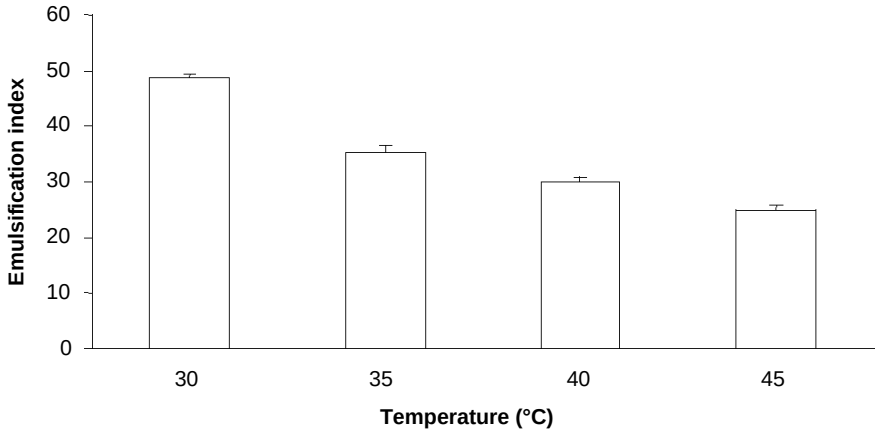

Figure 9. Emulsification index (E_{24}) of *Bacillus subtilis* PTCC 1023 at different temperatures (200 rpm, 24 h) (Mean ± SD).

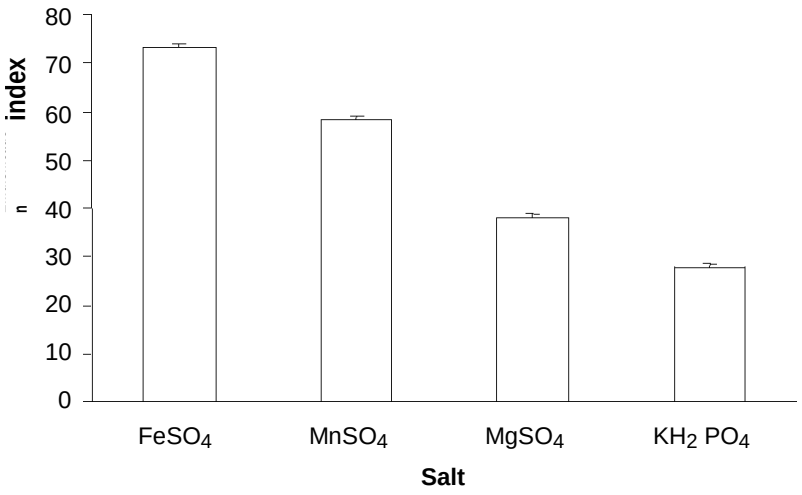

Figure 10. Emulsification index (E_{24}) of *B. subtilis* PTCC 1023 when different salts were applied at (300 rpm, 24 h, 30˚C) (Mean±SD).

Table 4. Cell-surface hydrophobicity (%) and emulsion index (E_{24}; %); results for supernatant of *B. subtilis* PTCC 1023 grown in brain-heart broth with cations (37ºC, 200 rpm).

Brain heart broth medium with salts	Hydrophobicity (%) Mean ± SD	Emulsion index (E_{24} ;%) Mean ± SD
$FeSO_4$	23.77±0.37	73.3±0.51
$MnSO_4$	33.19±0.28	58.33±0.47
$MgSO_4$	52.54±0.66	38.0±0.82
$KH_2 PO_4$	71.24±0.74	28.0±0.41

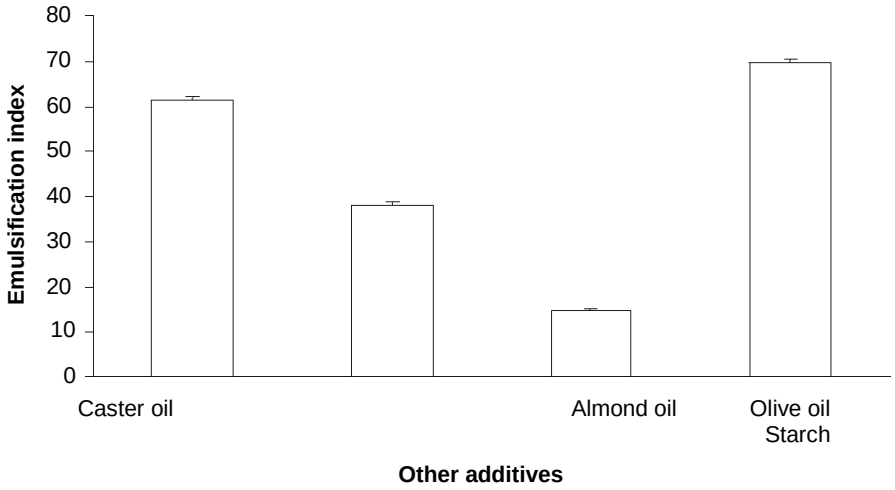

Figure 11. Emulsification index (E_{24}) of *B. subtilis* PTCC 1023 when different hydrocarbons were applied (300 rpm, 24 h, 30˚C) (Mean±SD).

1023 was studied by addition of iron, magnesium, potassium and manganese salts to brain – heart broth medium. The hydrophobicity of *B. subtilis* PTCC 1023 was shown in Table 4.

As shown in Figure 11, applying starch to the medium caused improvement in emulsification index. Adding olive oil had a negative effect on emulsified layer. Effect of almond oil was not significant but caster oil caused major

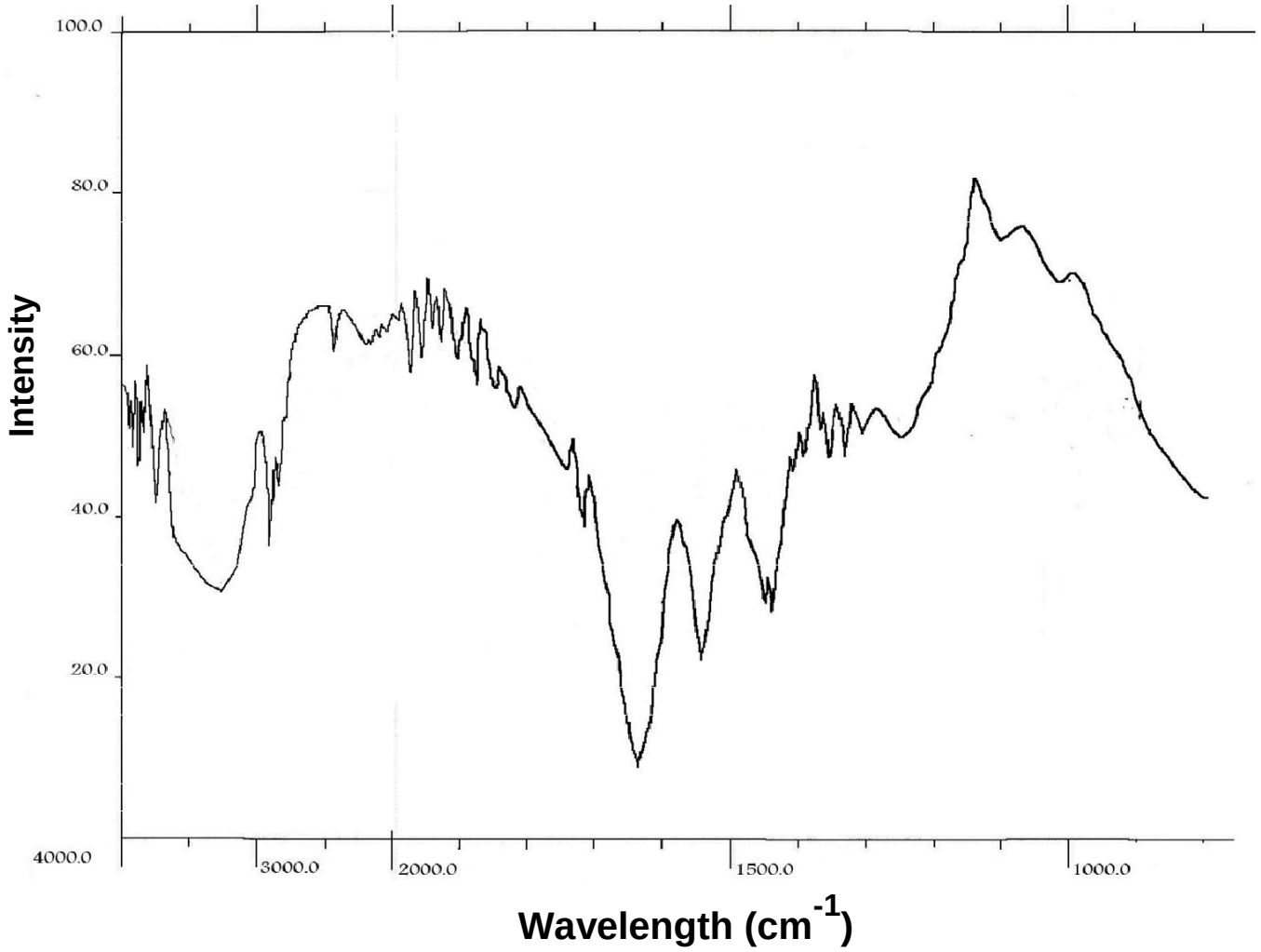

Figure 12. IR spectrum of produced biosurfactant.

increase on the emulsification index. Negative control was always used to confirm the results. Hence it can be suggested that the best conditions for biosurfactant production is the brain heart broth containing starch, $FeSO_4$, $MnSO_4$ and caster oil incubated in a 300 rpm shaker incubator at 30°C for 24 h.

Characterization of bioemulsifier

Bromine water reaction was negative; indicating that the fatty acid chain was saturated. Ninhydrin reaction was negative, indicating that the peptide has a blocked N-terminal. Biuret reaction was positive indication for polypeptides, a finding which is verified by other reports. The results indicated that the biosurfactant had a lipopeptide structure.

The IR spectrum of the sample (Figure 12); shows the bands 2966, 1456, and 1371 cm^{-1} reflected aliphatic chains (-CH3,-CH2-) of the sample. The absorption region at 1740 - 1680 cm^{-1} was due to lactone carbonyl absorption. It also showed absorption bands of peptides at 3312, 1647 and 1539 cm^{-1} resulting from the N-H stretching mode, stretching mode of the C=O bond, and the deformation mode (combined C-N stretching mode) of the NH bond, respectively.

Presence of peptide bonds was clearly demonstrated from UV spectrum at 237.8 nm, which indicated the product was lipopeptide. These results indicated that the product contained aliphatic hydrocarbons as well as a peptide-like moiety (Figure 13).

ACKNOWLEDGEMENT

This work was taken from a pharmacy student thesis in Kerman University of Medical Sciences (Thesis No.526). This research was supported by a grant (No. 87/112) from Vice President for Research, Kerman University of Medical Sciences.

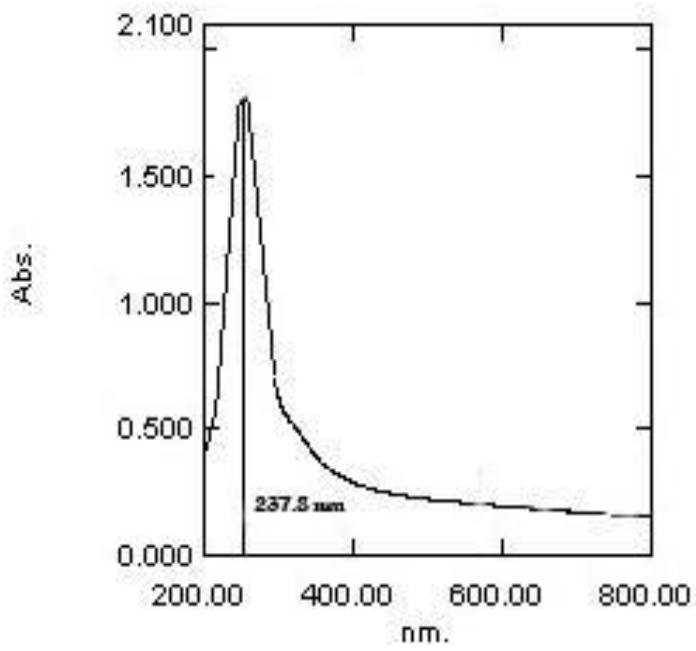

Figure 13. UV spectrum of produced biosurfactant.

REFERENCES

Arima K, Kakinuma A, Tamura G (1968). Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Res. Commun., 31: 488-494.

Bognolo G (1999). Biosurfactant as emulsifying agents for hydrocarbons, Colloids and surfaces. Physicochem. Eng. Aspects., 152: 41-52.

Breukink E, Wiedemann I, Van Kraaij C, Kuipers OP, Sahl H, De Kruijff B (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science., 286: 2361-2364.

Carrillo PG, Mardaraz C, Pitta Alvarez SI, Giulietti AM (1996). Isolation and selection of biosurfactant producing bacteria. World J. Microbiol. Biotechnol., 12: 82-84.

Chatterjee C, Paul M, Xie L, Van der Donk WA (2005). Biosynthesis and mode of action of lantibiotics. Chem. Rev., 105: 633-684.

Cheigh CI, Park H, Choi HJ, Pyun YR (2005). Enhanced nisin production by increasing genes involved in nisin Z biosynthesis in *Lactococcus lactis* subsp. *lactis* A164. Biotechnol. Lett., 27: 155-160.

Cobb WM (1919). A culture medium for the maintenance of stok cultures of bacteria. J. Bacteriol., 4: 603-608.

Cooper DG, Goldenberg BG (1987). Surface – active agents from two *Bacillus* species. Appl. Environ. Microbiol., 53: 224-229.

Cooper DG, Macdonald CR, Duff SFB, Kosaric N (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous production removal and metal cation additions. Appl. Environ. Microbiol., 42: 408-412.

Corvey C, Stein T, Dusterhus S, Karas M, Entian KD (2003). Activation of subtilin precursors by *Bacillus subtilis* extracellular serine proteases subtilisin (AprE), Wpr A and Vpr. Biochem. Bioph. Res. Co., 304: 48-54.

Dehghan – noudeh G, Housaindokht M, Fazzly Bazzaz BS (2005). Isolation, characterization and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by *Bacillus subtilis* ATCC 6633. Microbiol. Society Korea., 43: 272-276.

Delves – Broughton J, Blackburn P, Evans RJ, Hugenholtz J (1996). Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek., 69(2): 193-202.

Desai JD, Banat IM (1997). Microbial production of surfactant and their commercial potential. Microbiol. Mole. Biol., 61: 47-64.

Feignier C, Besson F, Michel G (1995). Studies on lipopeptide biosynthesis by *Bacillus subtilis*: isolation and characterization of iturin$^-$, surfactin$^+$ mutants. Appl. Environ. Microbiol., 5(1): 545-556.

Ferre G, Besson F, Buchet R (1997). Conformational studies of the cyclic L, D-lipopeptide surfactin by fourier transform infarared spectroscopy. Spectrochim Acta Part A., 53: 623-635.

Gautam KK, Tyagi VK (2006). Microbial surfactants (review). J. Oleo. Sci., 55(4): 155-166.

Paik HS, Chakicherla A, Hansen JN (1998). Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. J. Biol. Chem., 273(36): 23134-23142.

Hasper HE, De Kruijff B, Breukink E (2004). Assembly and stability of nisin-lipid II pores. Biochemistry, 43: 11567-11575.

Heerklotz H, Seelig J (2001). Detergent – like action of the antibiotic peptide surfactin on lipid memberanes. Biophys J., 81: 1547-1554.

Jenny K, Kappeli O, Fiechter A (1991). Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. Appl. Microbiol. Biotechnol., 36: 5-13.

Kim HS, Yoon BD, Lee CH, Oh HM, Katsuragi T, Tani Y (1997). Production and properties of a lipopeptide biosurfactant from Bacillus subtilis C9. J. Ferment Bioeng., 84: 41-46.

Lin SC, Lin KG, LO CC, Lin YM (1998). Enhanced biosurfactant production by a Bacillus licheniformis mutant. Enzyme Microb. Technol., 23: 267-273.

Lin TC, Young CC, HO MJ (2005). Characterization of floating activity of indigenous diesel-assimilating bacterial isolates. J Biosci. Bioeng., 99(5): 466-472.

Makkar RS, Cameotra SS (1997). Utilization of molasses for biosurfactant production by two Bacillus strains at thermophilic conditions. JAOCS, 74: 887-889.

Mercade ME, Manresa MA (1994). The use of agroindustrial by-products for biosurfactant production. J. Am. Oil Chem. Soc., 71: 61-64.

Nitschke M, Ferraz C, Pastore M (2004). Selection of microorganisms for biosurfactant production using agroindustrial wastes. Braz. J. Microbiol., 35: 81-85.

Peypoux F, Bonmatin JM, Wallach J (1999). Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol., 51: 553-563.

Razafindralambo H, Popineau Y, Deleu M (1998). Foaming properties of lipopeptides produced by *Bacillus subtilis*: effect of lipid and peptide structural attributes. J. Agric. Food. Chem., 416: 911-916.

Saris PE, Immonen T, Reis M, Sahl HG (1996). Immunity to lantibiotics. Antonie Van Leeuwenhoek., 69: 151-159.

Stein T (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol., 56: 845-857.

Valenta C, Bernkop – Schnurch A, Rigler HP (1996). The antistaphylococcal effect of nisin in a suitable vehicle: a potential therapy for atopic dermatitis in man. J. Pharm. Pharmacol., 48(9): 988-991.

Vasileva – Tonkova E, Gesheva V (2005). Glycolipids produced by *Antarctic nocardioides* sp. during growth on n-paraffin. Process Biochem., 40: 2387-2391.

Wagner F, lang S (1988). Nonionic and anionic biosurfactants: microbial production, structures and physicochemical properties. Paris: ASPA, 2: 71-80.

Zhang B, Xie C, Yang X (2008). A novel small antifungal peptide from *Bacillus* strain B-TL2 isolated from tobacco stems. Peptides., 29: 350-355.