H. J. Bhosale et al. / Journal of Pharmacy Research 2014,8(2),139-143

Research Article
ISSN: 0974-6943

Available online through
http://jprsolutions.info

Evaluation of antimicrobial activity and radical scavenging potential of lipopeptide biosurfactant from *Klebsiella pneumoniae* MSO-32

H. J. Bhosale*., T. A. Kadam., S. Phulari
School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded (M.S)-431606, India.

Received on:15-12-2013; Revised on: 08-01-2014; Accepted on:20-01-2014

ABSTRACT

Objective: In the present study we evaluated the antimicrobial and antiradical potential of a lipopeptide biosurfactant isolated from a soil bacterium, *Klebsiella pneumoniae* MSO-32 strain. **Methods:** Biosurfactant was produced by shake flask fermentation and purified by column chromatography. Its antimicrobial activity was estimated against gram-positive and gram-negative bacteria using agar well diffusion method. MIC and MBC values were determined to find efficacy of biosurfactant against test bacteria. The effect of biosurfactant on cell wall integrity of sensitive strain *M. luteus* was determined in terms of leakage of UV absorbing intracellular components. Antiradical potential and metal chelation capacity of biosurfactant was determined by using DPPH and hydroxyl radical scavenging assay and iron chelation activity respectively. **Results:** Chemically, biosurfactant was lipopeptide in nature and exhibited notable antimicrobial activity against all test bacteria. *M. luteus* displayed highest sensitivity with MIC at 10µg/ml and MBC at 30µg/ml. The biosurfactant caused gradual leakage of proteins, DNA and RNA from treated cells and showed concentration dependent scavenging effect on DPPH, hydroxyl radical and ferrous ion. **Conclusion:** The biosurfactant of *K. pneumoniae* MSO-32 exhibited promising antimicrobial and antiradical potential.

Key words: Oxidative stress, surface active molecules, antimicrobial action, antioxidant potential, lipopeptide biosurfactants, *K. pneumoniae*

INTRODUCTION

Infectious diseases and diseases linked with oxidative stress are the world's leading cause of deaths. About 10% of deaths are caused each year by HIV, tuberculosis and malaria. New pathogens continue to emerge as demonstrated by the SARS epidemic in 2003 and the Swine Flue pandemic in 2009[1]. Unfortunately, the intensive use and misuse of the antimicrobials caused emergence of resistant strains[2] which further complicated the treatment of infectious diseases in immunocompromised patients. Oxidative stress is a deleterious process generated due to high levels of reactive oxygen species (ROS). It is a major cause for chronic and degenerative ailments including cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging and cardiovascular and neurodegenerative diseases[3]. ROS are produced in the body, primarily as a result of aerobic metabolism. Antioxidants are the compounds that help to regulate ROS generated ill effects and are reported to possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities[4]. However, these systems are inadequate to prevent excess oxidative stress derived from high metabolic demands or radiations and chemicals. The synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are known to induce carcinogenic effects in living cell. The knowledge regarding emergence of new diseases and multiple drug resistance to human pathogenic bacteria and fungi, toxic nature of ROS and harmful side effects associated with available synthetic drugs, have opened a new vista for the search of new antimicrobial and antiradical agents from natural sources.

Biosurfactants are structurally diverse group of surface active molecules produced by a number of microorganisms. These are chemically categorized as glycolipids, lipopeptides, lipopolysaccharides, phospholipids and particulate biosurfactants. Apart from the regular roles in oil recovery, bioremediation and emulsification, several biosurfactants are known to exhibit antibacterial, antifungal and antiviral activities. The role of biosurfactants as antitumor, antioxidant and moisturizing agent is also revealed recently[5-7]. The unsaturated lipids such as squalene are reported to function as ROS scavengers to prevent the harmful chain reaction of lipid peroxidation[8], hence other lipid containing compounds might be expected to have potential as novel antioxidants. In, the present study we report, the radical

***Corresponding author.**
H. J. Bhosale
School of Life Sciences,
Swami Ramanand Teerth Marathwada University,
Nanded (M.S),-431606, India.

scavenging potential and antimicrobial activity of lipopeptide biosurfactant isolated from *K. pneumoniae* MSO-32.

METHODS

Biosurfactant (BS) producer strain

The soil isolate MSO-32 was screened as biosurfactant producer strain by emulsification assay[9], hemolytic assay[10], lipase activity[11] and E_{24} test[12]. The strain was characterized as *Klebsiella pneumoniae* on the basis of biochemical and cultural characterization and 16S rRNA sequencing. The 856 base pair sequence of MSO-32 was submitted to NCBI as *K. pneumoniae* MSO-32 with the accession number KC710339.

Production and purification of biosurfactant

The culture of MSO-32 (10^5cells/ml) was inoculated (1%) into 1L flask containing 500ml mineral salt medium[13] with 5% soybean oil as carbon source and incubated at 30°C for 96h under shaking (200 rpm). After incubation, biosurfactant was extracted by acid precipitation and purified as described by Seghal et al.[14] . The active fraction was confirmed by emulsification assay, checked for purity by thin layer chromatography (TLC), lyophilized and surface tension of purified biosurfactant was measured with tensiometer by duNouy procedure at 28°C.

Compositional analysis

The chemical composition of biosurfactant was determined by TLC for proteins, carbohydrates and lipids. The protein content of the purified BS was determined by Lowry method[15] using bovine serum albumin as standard, carbohydrate by phenol-sulfuric acid method using glucose as standard[16] whereas lipid fraction was determined by gravimetric estimation[17]. Functional group characterization was carried out by obtaining spectra of BS on Fourier-transformed infrared (FTIR) spectrophotometer (Perkin Elmer paragon 1000) over the range of 400-4000cm^{-1}.

Antimicrobial assay

The column purified active fraction and crude biosurfactant were tested for antimicrobial activity using agar well diffusion method[14]. The test pathogens were *E. coli, Proteus vulgaris, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus epidermidis, Enterobacter cloacae, Bacillus cereus* and *Staphylococcus aureus* obtained from clinical laboratories. Briefly, 0.1 ml of pathogen inoculums (˜10^6 CFU/ml) was inoculated on the surface of Muller Hinton agar. Wells were made and 50µl of biosurfactant (1mg/ml in methanol) was added in wells. Plates were incubated at 30°C for 24h. The extent of growth inhibition was determined by measuring the diameter of zone of inhibition around the wells. The assay was performed in triplicate with methanol as control and the mean values were represented. Student's t test was performed to determine the statistically significant differences in the zone of inhibition produced by purified active fraction and the crude biosurfactant.

Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC)

The agar disc diffusion assay and broth microdilution susceptibility assay were used for determination of MIC and MBC, respectively for different test bacteria[18].

Detection of leaky substances

Leakage of UV-absorbing cellular components from *M. luteus* (10^5 CFU/ml) was measured by growing the strain in Luria Bertani broth upon treatment with biosurfactant (10µg/ml). The control set was prepared with detergent Triton X-100 (0.1%). The cultures were withdrawn at 20 min interval and used for studying leakage of intracellular components. The cells were harvested at 10,000 rpm for 30 min in cold. The concentration of DNA and RNA in cell free supernatant was measured over a period of 3h using diphenylamine[19] and orcinol reagent[20] respectively. The calf thymus DNA and standard RNA (HiMedia) were used as reference. Leakage of proteins was determined by using the Lowry's method[16] with bovine serum albumin as a standard.

Radical scavenging and metal chelation activity

The radical scavenging potential and metal chelation activity of biosurfactant was found by 1,1- Diphenyl-2-picryl hydrazyl (DPPH) scavenging assay[7], hydroxyl radical scavenging capacity[21] and iron chelating assay[22] by using ascorbic acid, BHT and EDTA as standards respectively.

RESULTS

Purification and chemical composition of biosurfactant

The crude biosurfactant obtained by acid precipitation was purified on silica gel column. The bioactive fraction reduced surface tension of pure water upto 28 mN/m and showed a single spot on TLC (Rf, 0.63 cm). Chemically, biosurfactant was 2% carbohydrate, 63% lipid and 35% protein. The protein and lipid fractions were also detected after TLC analysis when reacted with ninhydrin and iodine vapors, respectively. The FTIR spectrum exhibited absorption bands at 3364 and 3226 cm^{-1} indicating the presence of N-H stretch and OH group which is a characteristic of carbon containing compounds with amino groups. The presence of long alkyl chains was confirmed by peaks at 2944 and 2989 cm^{-1}. The peaks observed at 1632 cm^{-1} and 1551 cm^{-1}

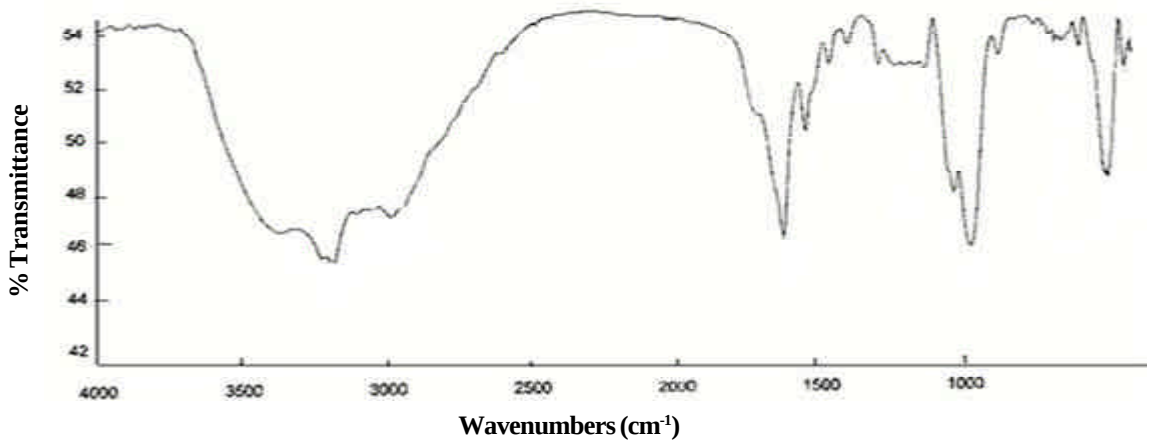

Fig.1. FTIR spectrum of purified active fraction of BS isolated from *K. pneumoniae* MSO-32

represented peptide and C=O bonds respectively (Fig. 1). This further confirmed the lipopeptide nature of biosurfactant.

Antimicrobial assay

The biosurfactant was able to inhibit all test organisms. *M. luteus* displayed highest sensitivity followed by *Staph. epidermidis* and *Staph. aureus*. The zones produced by column purified bioactive fraction were significantly larger than those produced by crude biosurfactant (Table 1). The lowest value of MIC was found to be 10µg/ml whereas the lowest MBC was observed at 30µg/ml. These concentrations affected the growth of *M. luteus* and *Staph. epidermidis*. However, the MIC and MBC values for other test organisms ranged from 20-40µg/ml and 40-80µg/ml respectively.

Table 1: Antimicrobial activity of biosurfactant isolated from *K. pneumoniae* MSO-32

Test pathogen	Zone of inhibition (mm)		MIC (µg/ml)	MBC (µg/ml)
	Crude BS	Purified BS		
Escherichia coli	8.2±1.4	11.5±0.6*	20	40
Pseudomonas aeruginosa	6.0±1.0	10.2±0.75**	40	80
Proteus vulgaris	7.5±2.0	10.3±0.5*	30	60
Micrococcus luteus	14.0±0.5	19.2±1.0**	10	30
Staphylococcus aureus	13.6±1.0	15.3±1.0**	20	40
Staph. epidermidis	10.2±0.5	17.4±1.0**	10	30
Enterobacter cloacae	6.5±0.5	10.7±1.0*	30	50
Bacillus cereus	9.5±1.0	12.5±1.0*	30	60

The values of zone diameter represent the mean ± SD of three independent readings.
*Statistically significant difference with respect to crude biosurfactant, **p < 0.01 and * p < 0.05*

Detection of leaky substances

The treatment of *M. luteus* cells with biosurfactant (10µg/ml) resulted in a gradual leakage of DNA, RNA and Proteins (Fig. 2) from bacterial cells. The leakage was increased according to the treated time with a plateau around 120min.

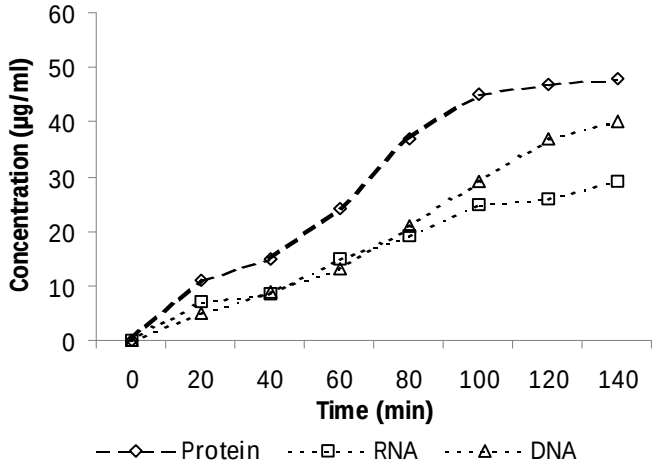

Figure 2: Leakage of intracellular substances from *M. luteus* cells upon treatment with lipopeptide biosurfactant (10µg/ml) of MSO-32.

Radical Scavenging and Metal Chelation Activity

The DPPH and hydroxyl radical scavenging activity ($p<0.05$) and iron chelation effect ($p<0.01$) of biosurfactant was concentration dependent and significantly increased with increasing concentration from 0.1 to 10 mg/ml (Fig. 3a-3c).

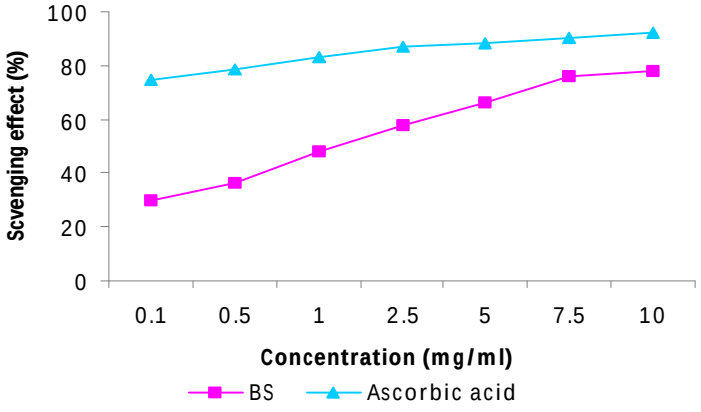

Fig. 3a

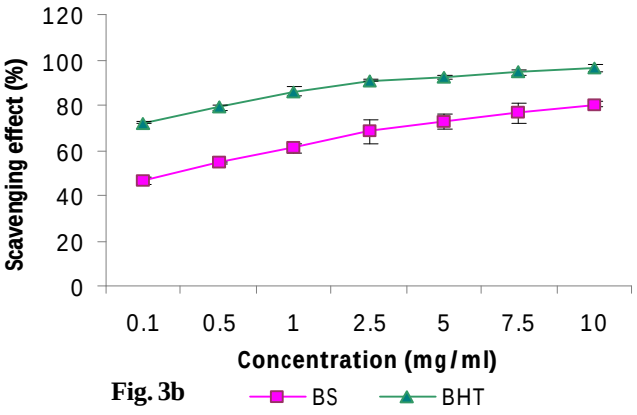

Fig. 3b

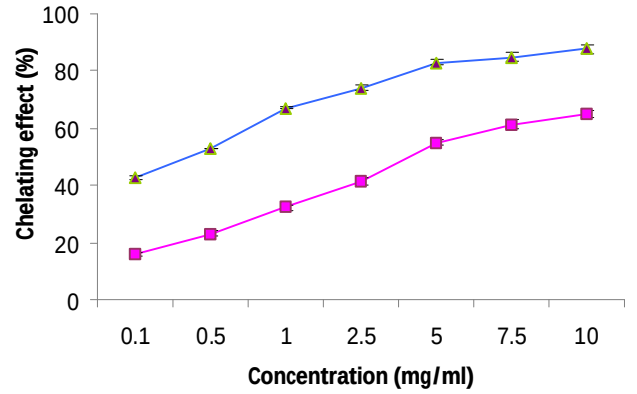

Fig. 3c

Fig.3. Radical scavenging activity of biosurfactant; a, DPPH scavenging effect; b, Hydroxyl ion scavenging effect and c, Ferrous ion chelating effect. Each value is mean ± standard deviation (n=3)

DISCUSSION

The present work was designed for evaluating the antimicrobial and antiradical potential of lipopeptide biosurfactant isolated from *K. pneumoniae* MSO-32 in the midst of searching novel therapeutic agents. The column purified active fraction of biosurfactant showed a powerful surface tension reducing ability and displayed pronounced antimicrobial action. The statistically significant variation in the size of inhibition zones for crude and purified biosurfactant was observed. This could be explained by their differences in degrees of purity. The bioactive fraction of MSO-32 was lipopeptide in nature as it showed the presence of peptide and lipid moiety during TLC and FTIR analysis. Other lipopeptide biosurfactants reported in the literature have also yielded similar IR absorption spectra[23,18].

The isolated biosurfactant showed activity against both Gram-positive and Gram-negative bacterial strains. Most of the lipopeptide BS reported earlier showed activity against Gram-positive bacteria[24,25]. However, antimicrobial action of biosurfactant against both Gram-positive and Gram-negative bacteria including multidrug resistant pathogenic bacterial strains has also been reported[18]. The antimicrobial effect of biosurfactant is expected because of the adhering property of biosurfactants to the cell surfaces and ability to form direct interactions with membrane target cell. This causes deterioration in the integrity of cell membrane and also breakdown in the nutrition cycle[26]. The Gram-positive bacteria *M. luteus*, and *Staph. epidermidis* demonstrated more sensitivity (10µg/ml) towards MSO-32 than other test bacteria (20-40µg/ml). Thus this study demonstrated the significance of biosurfactant as a potential drug candidate in humans.

The treatment resulted in a gradual leakage of protein, DNA and RNA from bacterial cells. The release of protein in supernatant could be due to the effect exerted by the biosurfactant on permeability of cell membrane. Studies reported that the agents known to damage the cell wall or membrane would cause changes in the permeability as well as membrane potential[27]. Further, direct interaction of the BS with membrane lipids disturbs permeability of membrane causing inhibition of membrane bound enzymes and leakage of intracellular components[28]. The increase in the RNA and DNA contents of *M. luteus* cells could be due to loss of intracellular components through damaged cell wall that release in the supernatant periodically.

The radical scavenging potential of biosurfactant was investigated with DPPH scavenging hydroxyl radical scavenging and iron chelation assays. The results indicate that biosurfactant showed maximum scavenging effect on DPPH in the range of 76-78% at 10 mg/ml which was comparable with ascorbic acid (90-92.33%). This indicates the capacity of biosurfactant to donate hydrogen and this was confirmed by the presence of free hydroxyl moieties in FTIR spectra. Hydroxyl radicals are the major active oxygen species causing lipid peroxidation in enormous biological damage. The isolated biosurfactant was found to scavenge OH radical significantly and in dose dependent manner and thus may protect the cells from biological damage. The transition metal iron is capable of generating free radicals from peroxides and may be implicated in human cardiovascular disease[29] as well as in proliferation of neoplastic cells during tumor formation. The iron chelating effect shown by biosurfactant and EDTA was concentration dependent. The lower scavenging and chelating effect of lipopeptide BS compared to ascorbic acid, BHT and EDTA can be caused by lower contents of hydroxyl groups than those present in standards.

CONCLUSION

Considering the need for safe, novel and potent therapeutic agents in the drug market, the present study revealed the significance of lipopeptide biosurfactant isolated from *K. pneumoniae* MSO-32 as a promising antimicrobial and antiradical agent. The producer strain can be exploited to develop new surface active agents with important biological activity.

ACKNOWLEDGEMENTS

The authors would like to thank the SRTM University, Nanded for providing the facilities needed to conduct this research.

REFERENCES

1. Alakija Wole (2000) Essentials of Community Health, Primary Health Care and health Management. Medisuccess Publication Benin.
2. Clark DR, Eradicating infectious disease: can we and should we? Front. Immunol. 2, 2011, 1-3.
3. Pham-Huy LA, He H and Pham-Huyc C (2008). Free Radicals, Antioxidants in Disease and Health. *International Journal of Biomedical Science*, **4**(2): 89- 96.
4. Sala A, Recio MD, Giner RM, et al. Anti-inflammatory and antioxidant properties of *Helichrysum italicum*. J. Pharm. Pharmacol. 54, 2002, 365–371.
5. Berg D, Youdim MB, Riederer P, Redox imbalance. *Cell Tissue Res.* 318, 2004, 201-213.
6. Kohno Y, Egawa Y, Itoh S, Nagaoka S, Takahashi M, Mukai K, Kinetic Study of Quenching Reaction of Singlet Oxygen and Scavenging Reaction of Free Radical by Squalene in n-Butanol, *Biochim. Biophys. Acta.* 1256, 1995**,** 52-56.
7. Takahashi M, Morita T, Fukuoka T, Imura T, Kitamoto D, Glycolipid biosurfactants, mannosylerythritol lipids, show antioxidant and protective effects against H(2)O(2)-induced oxidative stress in cultured human skin fibroblasts. *J. Oleo Sci.* 61, 2012**,** 457- 464.
8. Parihar A, Parigar MS, Milner S, Bhat S, Oxidative stress and anti-oxidative mobilization in burn injury. Burns, 34, 2008, 6-17.
9. Patil JR, Chopade BA, Studies on bioemulsifier production by *Acinetobacter* strains isolated from human skin, J. Appl. Microbiol, 91,2001,290-298.
10. Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giulietti AM. 1996. Isolation and selection biosurfactant-producing bacteria. *World J. Microbiol. Biotechnol.* **12**: 82-84.
11. Kiran GS, Hema TA, Gandhimathi R, et al. 2009. Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. *Colloides Surf. B: Biointerfaces*, 73: 250-256.
12. Ellaiah P, Prabhakar T, Sreekanth M, Taleb AT, Bhima P, Saisha V. 2002. Production of glycolipids containing biosurfactant by *Pseudomonas* species. *Indian J Exp. Biol.* **40**:1083-1086.
13. Zhang G, Wu Y, Qian X, Meng Q (2005) Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. *J Zhejiang Univ SCI 6B(8):725-730*
14. Seghal G, T. Thomas, J Selvina, B. Sabarathnama, A.P. Lipton (2010) Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresource Technology 101 2389–2396.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 793. 265-275, 1951
16. Dubois M, Gills KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugar and related substances. *Anal. Chem.* **28**: 350-356.
17. Manocha MS, San-Blas G, Centeno S. 1980. Lipid composition of *Paracoccidioides brasiliensis*: possible correlation with virulence of different strains. *J Gen. Microbiol.* **117**: 147–154.
18. Das P, Mukherjee S, Sen R (2008) Antimicrobial potential of a lipopeptide biosurfactant derived from a marine Bacillus circulans. J Appl Microbiol. 104:1675–1684
19. Burton. K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., 62. 315-323. 1956.
20. Schneider, WC. 1957. Determination of nucleic acids in tissues by pentose analysis, p. 680-684. In SP. Colowick and NO. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.
21. Olabinri BM, Odedire OO, Olaleye MT, Adekunle AS, Ehigie LO. Olabinri PF. *In vitro* evaluation of hydroxyl and nitric oxide radical scavenging activities of artemether. Research Journal of Biological Science. 2010; 5(1): 102-105.
22. Decker EA, Welch B. 1990. The role of ferritin as a lipid oxidation catalyst in muscle foods. *J. Agri. Food Chem.* **38**: 674.
23. Lin, S.C., Minton, M.A., Sharma, M.M. and Georgiou, G. (1994) Structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. Appl Environ Microbiol 60, 31–38.
24. Kitamoto, D., Yanagishita, H., Shinbo T., Nakane, T., Kamisawa, C. and Nakahara, T. (1993) Surface-active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by Candida antarctica. J Biotechnol 29, 91–96.
25. Singh, P. and Cameotra, S.S. (2004) Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol 22, 142–146.
26. Hingley ST, Hastie AT, Kueppers F. Effect of ciliostatic factors from *Pseudomonas aeruginosa* on rabbit respiratory cilia. Infect Immun. 1986; 51: 254-258.
27. Novo D, Perlmatter N, Hunt R, Shapiro H. Multiparameter flow cytometric analysis effect on membrane potential, membrane permeability and bacterial counts for Staphylococcus aureus and *Mycrococcus luteus*. Antimicrob Agent Chemother. 2000; 44: 827-838.
28. Vander BH. Biochemical targets for antifungal azole derivatives, hypothesis the mode of action. Curr Top Med Mycol. 1985; 1: 313-351.
29. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet. 1994, 344,721–724.

Source of support: Nil, Conflict of interest: None Declared