Efficacy Enhancement of Hydrophobic Antibiotics Employing Rhamnolipid as Biosurfactant

Abdurrahim A. Elouzi[1], Abdurrauf M. Gusbi[1], Ali M. Elgerbi[2]

[1]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Tripoli University, Tripoli, P.O. Box 13645 Libya.
[2]Department of Food Technology, Faculty of Engineering and Technology, Sebha University, Brack Ashati, P. O. Box 68 Libya.
abdurahim68@hotmail.com

Abstract: Antibiotic resistance has become a global public-health problem, thus it is imperative that new antibiotics continue to be developed. Major problems are being experienced in human medicine from antibiotic resistant bacteria. Moreover, no new chemical class of antibiotics has been introduced into medicine in the past two decades. The aim of the current study presents experimental results that evaluate the capability of biosurfactant rhamnolipid on enhancing the efficacy of hydrophobic antibiotics. Serial dilutions of azithromycin and clarithromycin were prepared. A bacterial suspension (approximately $5X10^5$ CFU) from an overnight culture in MSM was inoculated into 20ml sterile test tube each containing a serial 10-folds dilution of the test antibiotic(s) in broth with or without $200mgL^{-1}$ rhamnolipid. The tubes were incubated for 24 h with vigorous shaking at 37°C. Antimicrobial activity in multiple antibiotic-resistant Gram-negative bacteria pathogens and Gram-positive bacteria were assessed using optical density technique. The results clearly demonstrated that the presence of rhamnolipid significantly improved the efficiency of both antibiotics. We hypothesized that the addition of rhamnolipid at low concentration, causes release of LPS which results in an increase in cell surface hydrophobicity. This allows increased association of cells with hydrophobic antibiotics resulting in increased cytotoxicity rates.

[Abdurrahim A. Elouzi, Abdurrauf M. Gusbi, Ali M. Elgerbi. **Efficacy Enhancement of Hydrophobic Antibiotics Employing Rhamnolipid as Biosurfactant.** *J Am Sci* 2014;10(1):93-98]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 17

Keywords: Biosurfactants, rhamnolipid, azithromycin, clarithromycin, hydrophobic antibiotics.

1. Introduction:

Hydrophobic antibiotics have received substantial interest in part because of their broad spectrum of activity. Due to rising rates of drug resistance, the need for novel antibiotics is crucial, nevertheless many cationic peptide antibiotics suffer from high dose-limiting toxicity (1,2). Surprisingly activity of such cationic peptide antibiotics on Gram-negative bacteria is markedly reduced and that is possibly either due to mutation of chromosomal genes or acquisition of resistance genes on chromosomal pieces of DNA or due to a decrease in the penetration of the antibiotic through the outer membrane (3,4,12). The distinction between these categories of resistance is imperative, because it permits the search for antibiotics to which the development of specific mechanisms of resistance are rare but to which the bacterial membrane is impermeable (5,6,7). With the emergence of numerous strains of multidrug-resistant bacteria has come a renewed attention in new antimicrobial agents. A number of studies have reported that the presence of intact phospholipids causes diffusion route to have low activity, on the other hand the hydrophilic polysaccharide chains of lipopolysaccharides (LP) prevent access of hydrophobic antibiotics to the lipid bilayer (8,9,12). Furthermore, bacteria with reduced LP and increased phospholipid in the outer layer should be more susceptible to hydrophobic antibiotics (9,12). Studies have reported that one potential solution to the problems of both resistance and toxicity is targeting outer membrane of multidrug-resistant bacteria. It is possible to modify the outer membrane of gram-negative bacteria either by, mutation (8,10,11) or by the addition of membrane active agents, such as EDTA (13-15) and rhamnolipid (16-18). This results in overall changes in cell surface properties as well as in increased permeability and hydrophilicity of the bacterial membrane. LP mutants which have lost the O antigen component have increased affinity for hydrophobic probes (10,11,19). Interestingly it is well-known that EDTA can cause partial release of LPS (13,14,15), resulting in cells that are more susceptible to the action of such active but non-penetrating hydrophobic antibiotics (20, 21). However, there is a little information in the literature about the effect of rhamnolipid (biosurfactants) on multidrug-resistant bacteria.

Rhamnolipids are anionic biosurfactants produced by the bacterium *Pseudomonas species* (22). They consist of hydrophilic and hydrophobic domains, which tend to partition preferentially at the interface between fluids of different degrees of

polarity and hydrogen bonding (23, 24). Our previous published work has concluded that the isolated bacteria from the soil of local site were found to have the ability of producing the biosurfactant (rhamnolipid) (Figure 1) in the form of biological molecules (25). Our recent studies investigated the feasibility of using the rhamnolipid to remove or reduce heavy metals from contaminated water (26). Its ability to wash out the hydrocarbon contaminated soil was also evaluated by our team (27).

In this study we have used rhamnolipid (biosurfactant) at low concentrations to improve the action of hydrophobic antibiotics. We hypothesized that rhamnolipid would effectively improve the solubility of hydrophobic antibiotics and increase the permeability of the outer membranes of gram negative bacteria and render these microorganisms susceptible to many hydrophobic antibiotics that allow antibiotics work at low concentration and presumably a higher therapeutic ratio.

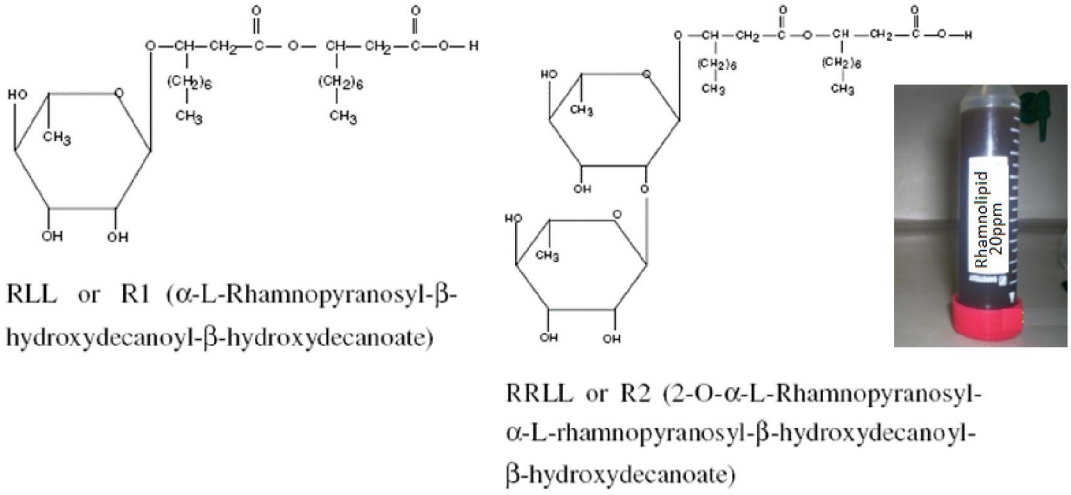

Figure 1: Structure of Rhamnolipid.

2. Material and Methods

2.1. Production of rhamnolipid (Bio-surfactants):

Rhamnolipid produced by *Pseudomonas aeruginosa* was purified as mentioned in our previous published work (25). The quantification of rhamnolipids expressed in rhamnose (g/L) was measured in the culture medium using the phenol sulfuric acid method as described previously (25). The purified rhamnolipid was diluted at concentration of 20mg/L and pH was adjusted to 7.2 using NaOH solution.

2.2. Microorganism

The strain *Pseudomonas aeruginosa* NBIMCC33347 (National Bank of Industrial Microorganisms and Cell Cultures) and *Staphylococcus aureus* (BCRC 10780) were used during this study. Bacteria were grown on agar slants (nutrient agar; Difco Laboratories, Detroit, Mich.) and kept at 4°C for up to 3 weeks.

2.3. Preparation of culture medium and growth conditions

The bacteria cells were grown in a mineral salts medium (MSM) supplemented with $CaCl_2$ (2mM), casein hydrolysate (Fluka, 0.5% w/v), maltose (0.5% w/v), pH 7.2 (28). Inoculum was prepared by transferring the cells from agar slants to 2ml of MSM medium (pH 7.2) in 20ml flasks and cultivated for overnight at 37°C with agitation at 200 rpm. The experimental culture of 20ml was inoculated with 1%(v/v) inoculum and incubated in 250ml flasks until late exponential phase (20hrs) Growth conditions were the same as those used for preparing the inoculum. The growth was monitored by measuring the absorbance at 570nm.

2.4. Preparation of antibiotics

Azithromycin was provided by Pfizer Inc. (New York, N.Y.) and Clarithromycin was provided by Abbott Japan Co. (Tokyo, Japan) were used in this study. Stock solutions of both antibiotics were prepared (1mg mL^{-1}) in sterile water and serial 10-fold dilution of studied antibiotics were made in MSM as shown in table 1 and table 2.

2.5. Measurement of antimicrobial activity

Antimicrobial activity of azithromycin and clarithromycin in multiple antibiotic-resistant Gram-negative bacterial pathogens and Gram-positive bacteria was measured in the presence and absence of rhamnolipid. The technique is based on measuring

the optical density of bacteria suspension at wave length 470 nm. Optical density, measured in a spectrophotometer can be used as a measure of the concentration of bacteria in a suspension. Greater scatter indicates that more bacteria is present (29, 30). In brief, the assay is carried out as following, cells were grown overnight at 37°C in MSM and were diluted in the same medium as described previously. 1ml of a bacterial suspension (approximately $5X10^5$ CFU) from an overnight culture in MSM was inoculated into 20ml sterile test tube each containing a serial 10-fold dilution of the test antibiotic(s) in broth with or without rhamnolipid (Tables 1 and 2). The tubes were incubated for 24 hrs with vigorous shaking at 37°C. 500µl of bacteria suspension containing test antibiotic(s) with or without rhamnolipid were placed in disposable polystyrene cuvette using sterile technique, thereafter the absorbance was measured at 470 nm. Values were expressed as a percentage of the control to which no antibiotics were added (negative control) which produced 100% viability. MSM was used as a negative control (blank) and produced 0% viability. The percentage of microbial growth inhibition for each concentration was measured by the following formula: Inhibition % = (A control – A test) / A control x 100

Experiments were performed in triplicate, and the results are expressed as means ± standard deviations.

Table1: Preparation of serial dilutions of Azithromycin and Clarithromycin in MSM without rhamnolipid [Biosurfactants, (20mg L^{-1})], followed by the addition of 1ml of bacteria to give a final inoculum of 5×10^5 CFU/ml. Stock antibiotics = 1mg mL^{-1}.

Volume of Antibiotic solution (mL)	Volume of MSM (mL)	Volume of Rhamnolipid Solution (mL)	Volume of Bacteria Suspension (mL)	Final Antibiotic concentration (mg L^{-1})
0	9	0	1	(-ve) Control
1	8	0	1	100
2	7	0	1	200
3	6	0	1	300
4	5	0	1	400
5	4	0	1	500

Table2: Preparation of serial dilutions of Azithromycin and Clarithromycin in MSM with rhamnolipid [Biosurfactants, (20mg L^{-1})], followed by the addition of 1ml of bacteria to give a final inoculum of 5×10^5 CFU/ml. Stock antibiotics = 1mg mL^{-1}.

Volume of Antibiotic solution (mL)	Volume of MSM (mL)	Volume of Rhamnolipid Solution (mL)	Volume of Bacteria Suspension (mL)	Final Antibiotic concentration (mg L^{-1})
0	8	1	1	Control
1	7	1	1	100
2	6	1	1	200
3	5	1	1	300
4	4	1	1	400
5	3	1	1	500

3. Data Statistical analysis

Statistical analysis was carried out on the data using analysis of variance followed by one-way analysis of variance (ANOVA) in trends of possible clinical importance and post tests carried out using Fisher's pair wise comparisons with the statistical package Minitab TM13 windows. *P* value of less than 0.05 was considered to represent a statistically significant difference. Data are reported as means ± standard deviations.

4. Result and Discussion

Macrolide antibiotics have been used in the treatment of infections caused by clinically important Gram-positive cocci, such as *Streptococcus* spp. and *Staphylococcus* spp., and Gram-negative bacteria such as *Pseudomonas aeruginosa* (1). The antibacterial activity of macrolides is known to result from their ability to inhibit protein synthesis by binding to the transpeptidation site of the larger ribosomal subunit (7).

The measurement of optical density of bacteria suspension reflected the viability of microorganisms in each treated sample. The results in figure 2 showed the bactericidal activity of serial dilution of azithromycin and clarithromycin on *p. aeruginosa* in the presence and absence of rhamnolipid. From figure 2, it can be seen that

azithromycin and clarithromycin were effectively decreased the viability of *p. aeruginosa*. The highest antibacterial activity level of both antibiotics was seen at concentration of 400mgml^{-1} and this was nearly 2-fold higher than 100mgml^{-1} and 1.5-fold higher than 200mgml^{-1} concentration. On the other hand, antimicrobial activity of azithromycin and clarithromycin in the presence of rhamnolipid against gram- negative bacteria was statistically significantly (p <0.05) higher than the efficacy of azithromycin and clarithromycin in the absence of rhamnolipid. Comparing with the control (MSM only),the efficiency of azithromycin and clarithromycin in the presence of rhamnolipid at the 400mgml^{-1} was significantly high, which was 3-fold higher than the control. Furthermore, the effect of azithromycin in the presence of rhamnolipid was greatly enhanced than that observed with clarithromycin.

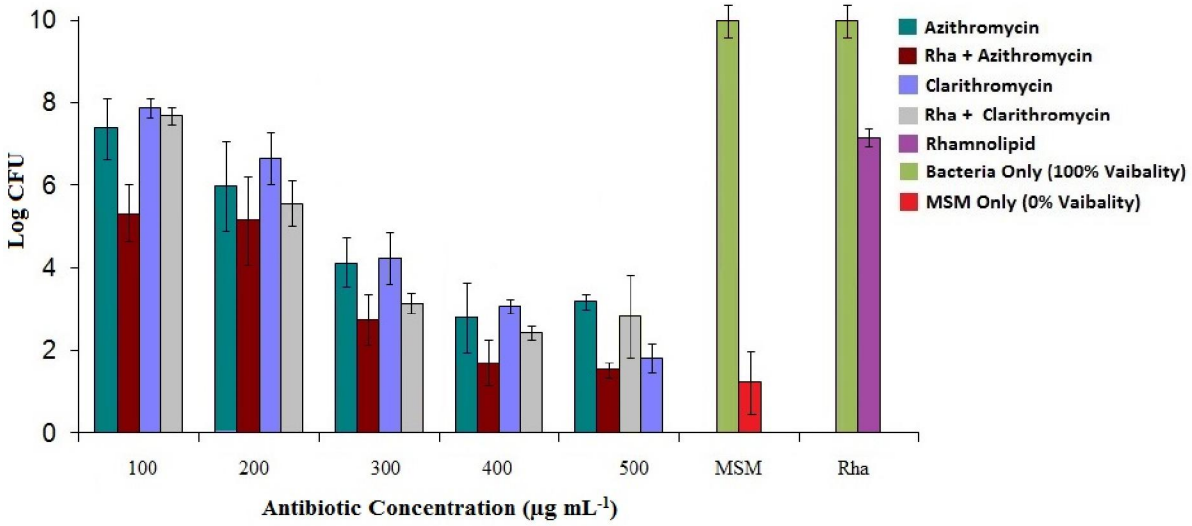

Figure 2: Influence of rhamnolipid addition on susceptibility of *P. aeruginosa* to azithromycin and clarithromycin. Bacterial suspension (approximately 5X10^5 CFU) was inoculated into 20ml sterile test tubes each containing a serial 10-fold dilution of the test antibiotic(s) in broth with or without rhamnolipid (20 mgL^{-1}). After coincubation for 24 h at 37°C, viable-cell numbers were determined. Experiments were performed in triplicate, and the results are expressed as mean ± standard deviation.

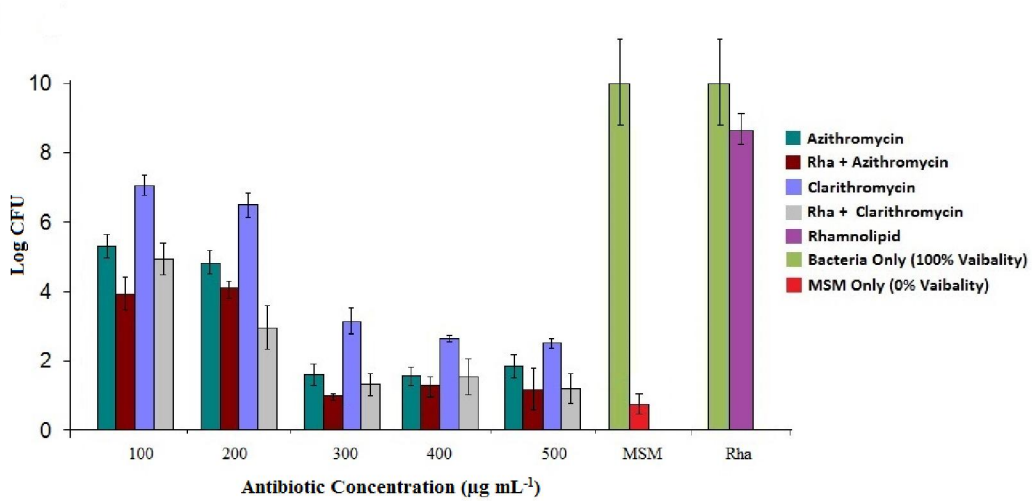

Figure 3: Influence of rhamnolipid addition on susceptibility of *Staph. aureus* to azithromycin and clarithromycin. Bacterial suspension (approximately 5X10^5 CFU) was inoculated into 20ml sterile test tubes each containing a serial 10-fold dilution of the test antibiotic(s) in broth with or without rhamnolipid (20 mgL^{-1}). After coincubation for 24 h at 37°C, viable-cell numbers were determined. Experiments were performed in triplicate, and the results are expressed as mean ± standard deviation.

Further proof of the hypothesis that rhamnolipid is crucial to improve the efficiency of azithromycin and clarithromycin was found on *staph. aureus* (Fig. 3). The result of figure 3 showed that azithromycin and clarithromycin were also effectively decreased the viability of *staph. aureus*. In the presence of rhamnolipid, the highest efficacy level of azithromycin was seen at concentration 300µgmL^{-1} and this was approximately 2-folds higher than the effect of 300µgmL^{-1} azithromycin without rhamnolipid and 4-folds higher than the effect of 200µgmL^{-1} azithromycin alone. On other hand, no significant results were observed between the effect of azithromycin at concentration of 300µgmL^{-1}, 400µgmL^{-1} and 500µgmL^{-1} and this is suggested that the minimum effect concentration (MIC) of azithromycin with rhamnolipid was at concentration of 300µgmL^{-1}**.** Similarly, a statistically significant difference of p <0.05 was also observed between the clarithromycin in the presence of rhamnolipid and the efficiency of clarithromycin in the absence of rhamnolipid (Fig 3). Moreover, the antibacterial effect of clarithromycin at 300µgmL^{-1} was statistically indistinguishable from the antibacterial effect of 400µgmL^{-1} and 500µgmL^{-1} clarithromycin. For the 100µgmL^{-1} and 200µgmL^{-1} of clarithromycin with rhamnolipid (Fig 3), the results also showed clearly reducing in the bacteria viability but were less than that observed in the presence of 200µgmL^{-1} rhamnolipid. furthermore, the efficacy of azithromycin with or without rhamnolipid on *staph. aureus* was higher than that observed within clarithromycin in the presence or absence of rhamnolipid respectively (Fig 3). In a mouse model of skin infection with *S. pyogenes,* azithromycin proved to be more effective than clarithromycin or roxithromycin while erythromycin had no effect (30). These results were an agreement with our results (Figs 2 and 3).

It's clearly that presence of rhamnolipid improved the efficiency of both antibiotics. We hypothesized that the addition of rhamnolipid at low concentration, causes release of LPS which results in an increase in cell surface hydrophobicity. This allows increased association of cells with hydrophobic antibiotics resulting in increased cytotoxicity rates. Makin *et al.* concluded that the quantity and type of LPS found on the cell surface of bacteria has a profound effect on the nature of interactions between the cell and its environment. Study by Herman *et al.* (31) demonstrated that the addition of rhamnolipid at concentrations less than the critical micelle concentration (CMC) induced formation of multi-cellular aggregates, implying that the cells forming these aggregates are hydrophobic in nature. Other study by Zhang and Miller (32) showed that rhamnolipid not only increased apparent hydrocarbon solubility but also modified the cell surface, resulting in increased hydrophobicity.

5.Conclusion:

Due to increased bacterial drug resistance in the past two decades, there is an imperative need for development of new classes of antibiotics. The outcome of present study concluded that low concentration of rhamnolipid biosurfactant enhanced the antimicrobial activity of azithromycin and clarithromycin. Employing rhamnolipid biosurfactant showed promising approach to overcome the problems associated with the rapid emergence of multi-drug-resistant pathogens. Further investigations are proposed to support the present study for possibility of the application of this compound for human therapeutics.

References

1. Findlay B., Zhanel G. G., Schweizer F.(2010): Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold. Antimicrob. Agents Chemother. 54:4049–4058.
2. Guani-Guerra E., Santos-Mendoza T., Lugo-Reyes S. O., Teran L. M.. 2010. Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin. Immunol. 135:1–11.
3. Nikaido H., Varra M.1985 Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1–32
4. Friedrich G., Tammy B., Karl-heinz S.(2006): The Genera Staphylococcus and Macrococcus. Prokaryotes 4:5–75.
5. Vaara, M. 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. 56:395-411.
6. Poole K. 2001. Multi drug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J Mol Microbiol Biotechnol.3:255–64.
7. Denyer SP, MaillardJ-Y. (2002): Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. J Appl Microbiol. 92:35S–45S.
8. Y. Ni, R.R. Chen. Accelerating whole-cell biocatalysis by reducing outer membrane permeability barrier, Biotechnol Bioeng, 87 (2004), pp. 804–811.
9. H. Nikaido, Molecular basis of bacterial outer membrane permeability(2003): Microbiol Mol Biol Rev, 67 pp. 593–656.
10. Leive L. (1965) Release of lipopolysaccharide by EDTA treatment of *E. coli.* Biochem. Biophys. Res. Commun. 21:290–296.

11. Nikaido H. (1976) Outer membrane of *Salmonella typhimurium*: transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta 433:118–132.
12. Li, C., Budge, L. P., Driscoll, C. D., Willardson, B. M., Allman, G. W. & Savage, P. B. (1999). Incremental conversion of outer-membrane permeabilizers into potent antibiotics for gram-negative bacteria. Journal of the American Chemical Society 121, 931–40.
13. Rogers S. W., Gilleland H. E. Jr., Eagon R. G. (1969) Characterization of a protein-lipopolysaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. Can. J. Microbiol. 15:743–748.
14. Nikaido H., Varra M. (1985) Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1–32.
15. S.P. Denyer, J.-Y. Maillard. (2002) Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. J Appl Microbiol, 92 pp. 35S–45S
16. Y. Zhang, R.M. Miller. (1994). Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl Environ Microbiol, 60: 2101–2106
17. Singh P, Cameotra SS. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol 2004;22:142–6.
18. R.A. Al-Tahhan, T.R. Sandrin, A.A. Bodour, R.M. Maier. (2000) Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates. Appl Environ Microbiol, 66:3262–3268.
19. Poxton I. R. (1993) Prokaryote envelope diversity. J. Appl. Bacteriol. 74 (Suppl.) S1–S11.
20. Leive L. (1965) Release of lipopolysaccharide by EDTA treatment of E. coli. Biochem. Biophys. Res. Commun. 21:290–296
21. Leive L. (1974) The barrier function of the Gram-negative envelope. Ann. N. Y. Acad. Sci. 235:109–127.
22. P. Singh, S.S. Cameotra. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol, 22 (2004), pp. 142–146
23. Siegmund Lang (2002): Biological amphiphiles (microbial biosurfactants). Current Opinion in Colloid & Interface Science, 7(1-2): 12-20.
24. Sung-Chyr Lin, Yi-Chuan Chen and Yu-Ming Lin. (1998): General approach for the development of high-performance liquid chromatography methods for biosurfactant analysis and purification, Journal of Chromatography A. 825 (2):149-159.
25. Abdurrahim A. Elouzi, Rehab Bashir, Ryma Sandouk, Bassam Elgammude. (2009): Isolation and characterization of rhamnolipid (Biosurfactant) from petroleum contaminated soil. Bulletin for Environmental Researches Ass. Uni. Bull. Environ. Res. 12 (1): 95-107.
26. Abdurrahim A. Elouzi1, Abdulrhman A. Akasha1, Ali M. Elgerbi, Mokhtar El-Baseir1, Bassam A. El Gammudi (2012): Removal of Heavy Metals Contamination By Bio-Surfactants (Rhamnolipids). Journal of Chemical and Pharmaceutical Research. 4(9):4337-4341.
27. Abdurrahim A. Elouzi, Rehab Bashir, Ryma Sandouk and Bassam Elgamode. (2012) The Effect of Biosurfactant (Rhamnolipid) on Contaninated Soil By Petroleum Hydrocarbons From Oil Spills in El-zawia City-Libya. Ass. Univ. Bull. Environ. Res. 15(1):17-30.
28. Spizizen J. (1958): Transformation of biochemically deficient strains of Bacillus subtilis by deoxiribonucleate. Proc Natl Acad Sci USA 44:1072–8.
29. Arthur L. Koch (1970): Turbidity Measurements of Bacterial Cultures in Some Available Commercial Instruments. Analytical Biochemistry 38: 252-59.
30. Pechere, J.-C. (Ed.) (1994). Comparative activity of antibiotics against group A streptococci in experimental infections. In *Acute Bacterial Pharyngitis,* pp. 68-70. Cambridge Medical Publications, Worthing.
31. Herman, D. C., Y. Zhang, and R. M. Miller. (1997): Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. Appl. Environ. Microbiol. 63:3622–3627.
32. Zhang, Y., and R. M. Miller. (1994): Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol. 60:2101–2106.
33. Makin, S. A., and T. J. Beveridge. (1996): The influence of A-band and B-band lipopolysaccharide on the surface characteristics and adhesion of *Pseudomonas aeruginosa* to surfaces. Microbiology 142:299–307.

1/15/2014