



CHAOTROPIC EFFECTS OF INORGANIC & ORGANIC COMPOUNDS ON BACTERIA – INFLUENCE OF GRAM NATURE

Ananda Vardhan Hebbani*, Mridula Tripathi, Veeresh, Mythreyi S Jamadagni, Chaitra M and
Snehalatha Nadigar

Department of Biotechnology, New Horizon College of Engineering, Bangalore

ABSTRACT

Inorganic and organic chaotropic agents were analyzed for their effects on pathogenic bacteria, namely *Staphylococcus aureus* ATCC 25923 a Gram positive species and *Escherichia coli* ATCC 25922 a Gram negative species, with an objective to understand these effects on the variable membrane configurations and permeability properties that exists among their cell walls and membranes respectively. The studies revealed the extent of bacterial lysis that amplified with rise in concentrations of chaotropic agents, in a time dependent mode. Furthermore findings proved that, the inorganic chaotropic agents had shown a higher efficacy against Gram positive species while the organic chaotropic agents demonstrated a higher efficacy against Gram negative species. The findings revealed, specific type of chaotropic agents affects specific Gram nature of bacteria. This paves way for dimensional understanding of the susceptibility of biological membranes towards specific chaotropic agents.

Keywords: Chaotropic agents, ammonium sulfate, urea, Gram positive, Gram negative bacteria, lysis assay

INTRODUCTION

In our preceding review (Ananda Vardhan and John Barnabas, 2012) we discussed the significance of chaotropic agents and their already vast applications, their prospect capability to be used as tools in biomolecular research. Chaotropic agents are the chemical entities with an ability to disrupt and denature macromolecules by increasing their solubility in aqueous medium (Ananda Vardhan and John Barnabas, 2012). By virtue of their minimal polarity, chaotropic agents can easily break hydrogen bonds between water molecules suppressing water structure formation and can thereby interfere with the stabilizing inter-molecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces and hydrophobic effects leading to the unfolding of any complex biomolecule (Mishra, 2011). Chaotropic agents can be either inorganic salts or organic polar solvents or any organic compound; exhibiting varied chemical structures and complexities which generally are known to alter the thermodynamics of water present in the medium, thus favoring the solubilization of hydrophobic substances (Hatefi Y., W. G. Hanstein., 1969; Samuel *et al.*, 1981), and as a result has been a major aid in the investigations of any cell with its membrane as a reliable target.

Chaotropic agents have been widely used in the previous applications as of detergents and in the cosmetic industry and proposed to have their disruptive actions especially against bacteria (Anton Middelberg, 1995). Since the process of cell division has been proven to be sensitive in bacteria (Spratt, B.G *et al.*, 1980; InGram and Thurston, 1976; Slater and Schaecter, 1974), many chemicals were reported to affect bacterial morphology and interfere with cell division (Loveless *et al.*, 1954). Therapeutics like penicillin and other β – lactam antibiotics were reported to be acting by inhibiting steps in cell wall biosynthesis (Blumberg and Stromlnger, 1974; Ghuysen, 1977; Grula and Grula, 1962); similarly, changes in phospholipid composition or fatty acid composition (Johnson and Grula, 1980; Cronan and Vagelos, 1972; Naota Oku and MacDonald, 1983), starvation for certain metal ions (Kennell and Kotoulas, 1967), and addition of ethyl alcohol, diazouracil (Previc and Richardson, 1969), or a variety of hydrophobic organic reagents were all effectively chaotropic against bacteria. Many of these treatments and agents were reported to affect cell division by altering the membrane association of proteins which function in peptidoglycan synthesis. Since these proteins are located in the periplasmic regions of the cell where they are partially protected from the changes in external environment and their positioning involves ionic interactions, hydrophobic associations, and hydrogen bonding; any chemical compound which can alter these non covalent interactions can surely have a chaotropic effect on the bacterial cell membrane and alter its growth kinetics (InGram, 1981). Similarly, *B.subtilis* cells treated with various surfactants (used for decontamination and sanitation at hospitals, food industries and homes) which resulted in their lysis was proposed to be due to the deregulation of autolytic enzymes (Daniel Rigomier *et al.*, 1980). Because of their constituent sodium and potassium ions, surfactants were thought to be stimulating the whole cell autolysis (Tetsuaki *et al.*,1990; Lekha Patel *et al.*,1975). Since many of the enzymes for the synthesis of phospholipids, cell wall, and outer membrane components are associated with cytoplasmic membrane, a perturbation of the membrane structure by any chemical entity was understood to cause a defect in morphology and the division process itself (Victor and Aaron, 1973; Hawrot and Kennedy, 1975; Rogus, 1979).

The concept of chaotropic effects caused by chemical entities on bacteria and by large any biological membrane has been envisioned by various groups of scientists in many novel angles and slowly the use of chaotropic agents for unraveling the fundamentals of biological systems started becoming a new investigative tool in biomolecule research (Ananda Vardhan and John Barnabas, 2012). Chaotropic effects on microorganisms have been studied to understand the adaptive changes posed by the microorganisms to survive under the stressed condition of exposure to different chaotropic agents (Hallsworth *et al*, 2003; Hallsworth JE *et al* 2007). Attempts were even conducted in trying to look at the effect of chaotropic agents on lipid, protein and carbohydrate metabolisms (Daniel Rigomier *et al.*, 1980, Jennifer *et al.*, 2010; Nunn and Tropp; 1972). Certain chaotropic agents have even been used as potential agents for enhancing bioremediation of dangerous chemical carcinogenic compounds from nature by enhancing their uptake into plants - phytoremediation (Makris *et al*, 2007; Douglas & Mark, 1983).

In the present scenario we briefly present their effects on the nature of the Gram type of bacteria. This has been a subject of concern since specificity (on the Gram type) of the chaotropic agents is not really established. This study would enable to establish chaotropic agents as a tool for biomolecular research.

MATERIALS AND METHODS

- 1. Bacterial strains and growth conditions:** ATCC strains of Gram positive bacteria *Staphylococcus aureus* – ATCC 25923 and Gram negative bacteria *Escherichia coli* – ATCC 25922 procured from Department of Microbiology, Sagar Hospitals, Kumarswamy Layout, Bangalore-78, India; were used for the present study. Sub culturing of the cultures were done onto nutrient agar slants and maintained aerobically at 37⁰c in an incubator.
- 2. Preparation of log phase inoculums:** To 5ml of nutrient broth dispensed into a sterilized 25ml conical flask, a loopful of organism was added and incubated overnight on an orbital shaker maintained at 125rpm to obtain a pre- inoculum. Subsequently, 5ml of the pre-inoculum was added into 100 ml of fresh, sterile nutrient broth and continued to incubate until an OD value of 0.5-0.7 was achieved.
- 3. Preparation of chaotropic nutrient broth:** The chaotropic agents used for the analysis were chosen from two different chemical categories with varied properties, molecular sizes and degrees of

chaotropicity i.e., inorganic salt – ammonium sulphate and an organic polar compound – urea. The chaotropic agents were prepared in a range of concentrations covering 0.05M, 0.1M, 0.2M and 0.4M. Care was taken to add the chaotropic agents at double the concentrations to nullify the effect of normal anti-chaotropic agents present in nutrient broth; i.e., for the preparation of chaotropic nutrient broth with a net 0.05M concentration of a chaotropic agent, amount equivalent to 0.1M concentration of the chaotropic agent is added. The same strategy is used for the preparation of both the chaotropic nutrient broths with slight modifications for urea.

- a. **Ammonium sulphate:** To 100ml of normal nutrient broth, respective amounts of ammonium sulphate (calculated at double the concentration) was added and swirled gently in order to ensure the complete dissolution of the salt. Later the pH was adjusted to 6.8 to 7.9 and aliquoted (9ml each) into five different boiling test tubes for subsequent analysis at 0, 30, 60, 90 and 120 minutes and autoclaved. An aliquot of this chaotropic broth was used as a blank.
 - b. **Urea:** Since urea is thermo-labile, care was taken to avoid the autoclaving of broth with urea; instead a 10M stock solution of urea was prepared using sterile water and appropriate volumes of it were directly added to the sterile nutrient broth under aseptic conditions i.e., to prepare chaotropic nutrient broth with a net 0.05M concentration of urea; from the 100ml of normal sterile nutrient broth, 1ml was pipetted out and replaced with 1ml of 10M urea solution (calculated at double the concentration). The same procedure was followed for the other concentrations of urea as well. Later the pH in each case was adjusted to 6.8 to 7.9 and 9ml of it was aliquoted into five boiling test tubes for further analysis at 0, 30, 60, 90 and 120 minutes. An aliquot of this chaotropic broth was used as a blank.
4. **Lysis assay:** Method employed by L.O.InGram with slight modifications was used for the assay (InGram L. O., 1981 and 1982). Accordingly, 1ml of log phase inoculum was added to 9ml of chaotropic nutrient broth taken in the first test tube (0th minute), briskly swirled and immediately an aliquot was taken to measure its OD at 660nm against respective blanks; to obtain the growth patterns of a bacteria at 0th minute. The same procedure was followed for the other test tubes after their respective times intervals of incubation (30, 60, 90,120 minutes). In duplicates, 1 ml aliquots of the resuspended inoculum at every time interval were used for measuring the colony forming units (CFU) on nutrient agar plates using pour plate method.

Care was taken to avoid shaking of the tubes after the inoculation i.e. only the supernatant was used for measuring OD. All the tests were conducted in duplicates and a set of test tubes with normal nutrient broth devoid of chaotropic agents is run for both the organisms in order to generate a normal growth curve (NGC).

RESULTS AND DISCUSSION

The susceptibility of *S.aureus* to the increasing concentrations of ammonium sulfate and urea are represented in figure 1 and 3 respectively. Figures 2 and 4 represent the susceptibility of *E.coli* to the increasing concentrations of ammonium sulfate and urea respectively.

It is evident from all the figures that the extent of lysis of both the bacteria enhanced considerably with the increase in concentration of chaotropic agent in a time dependent manner, which is seen as a shift of curves from their respective normal growth curves.

Inorganic chaotropic agent (ammonium sulfate) appears to be better effective in lysing the Gram positive bacteria (figure 1) than Gram negative (figure 2) whereas organic chaotropic agent (urea) appeared to be better effective against Gram negative (figure 4) than Gram positive bacteria (figure 3).

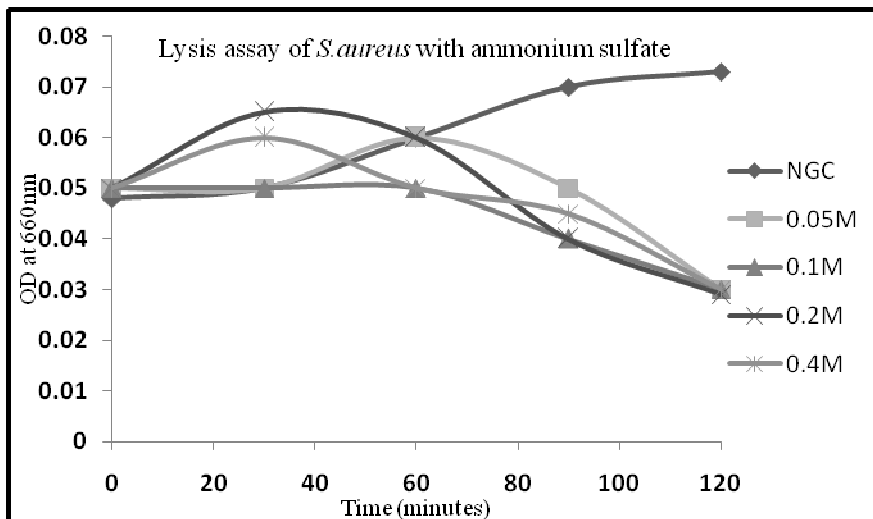


Figure 1: Chaotropic effects of inorganic salt on Gram positive bacteria

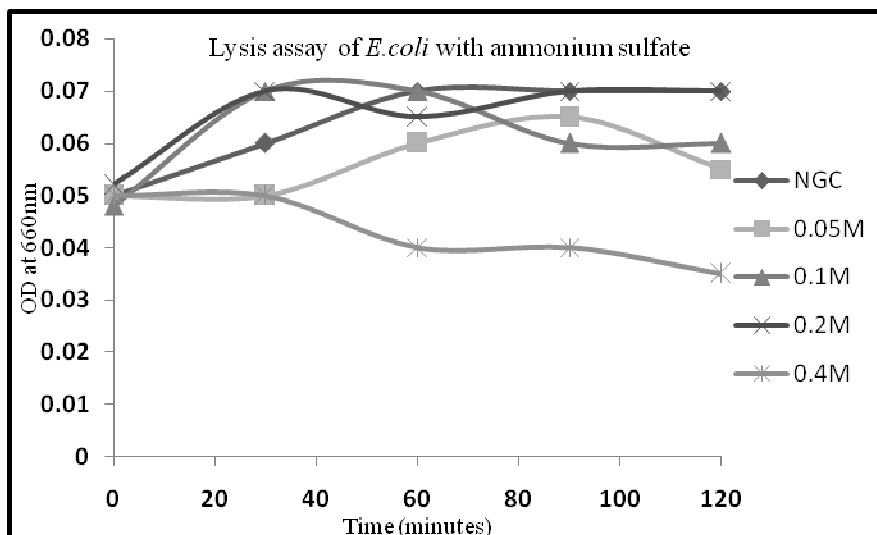


Figure 2: Chaotropic effects of inorganic salt on Gram negative bacteria

It is evident from figure 1 that the effective lysis of *S.aureus* is concentration independent and all the concentrations of ammonium sulfate (0.05M, 0.1M, 0.2M and 0.4M) are equally effective in making a considerable impact on the growth kinetics of Gram positive bacteria. In comparison, figure 2 shows that the chaotropic effect caused by ammonium sulfate on Gram negative bacteria is purely concentration dependant and maximum lysis of *E.coli* occurred at 0.4M concentration of ammonium sulfate compared to the other concentrations, which have a minimal chaotropic efficacy on the bacteria.

Interestingly, a similar trend in reverse is observed in the case of organic chaotropic agent (i.e., urea) used against the two bacterial strains. Figure 3 shows the chaotropic effect caused by urea on *S.aureus*, which is concentration dependant with a maximum lysis happening at 0.4M concentration of urea compared to the other concentrations. Whereas figure 4 clearly depicts that the chaotropic effect caused by urea on *E.coli* is concentration independent and all the concentrations possess a considerable chaotropic effect on the bacteria.

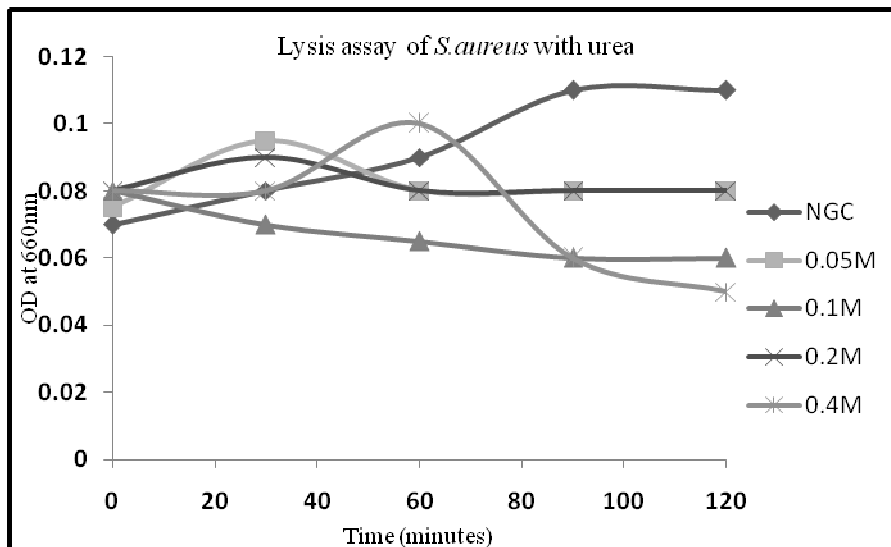


Figure 3: Chaotropic effects of organic compound on Gram positive bacteria

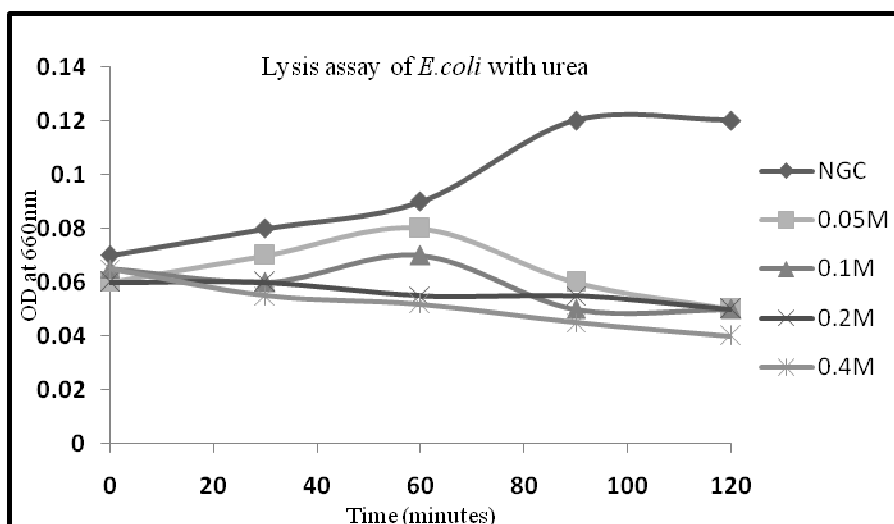


Figure 4: Chaotropic effects of organic compound on Gram negative bacteria

The data obtained with respect to the CFU's for each of the chosen bacterial species in the presence of either the inorganic or organic chaotropic agents showed a similar trend of concentration dependent decline in the viable cell populations, which is averaged to get the ranges of CFU's plotted against time intervals (Figure 5). The result obtained was observed to be very similar with that of lysis assay, with Gram positive species appearing more susceptible for inorganic chaotropic agent and Gram negative species more susceptible for organic chaotropic agents.

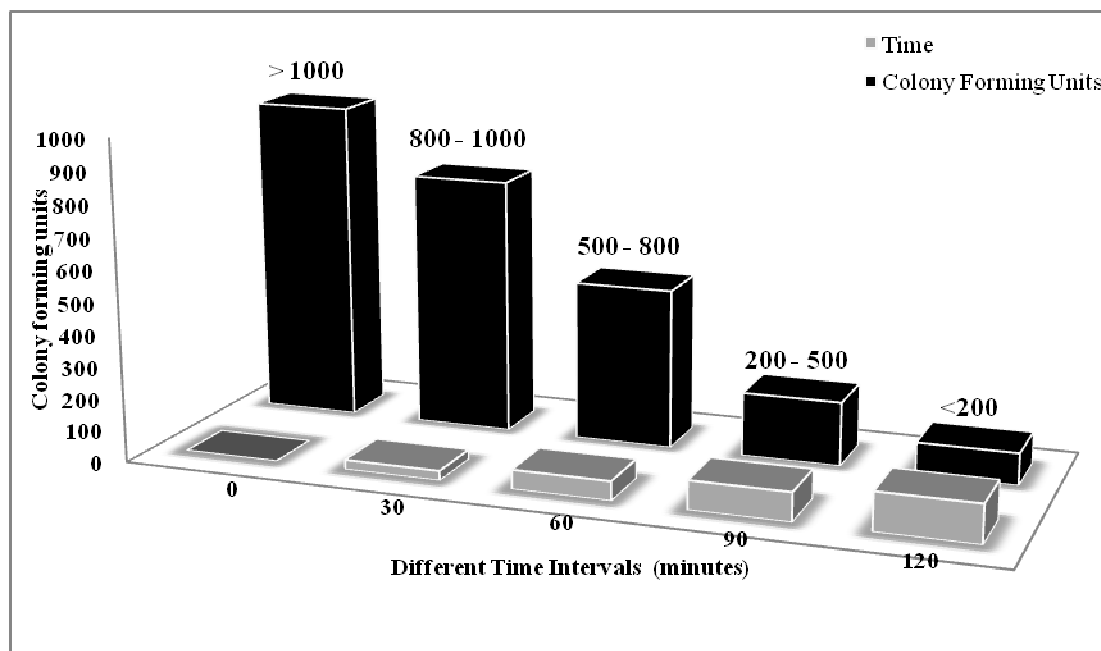


Figure 5: A general trend of colony forming units (reported in ranges) for Gram positive and Gram negative bacteria.

CONCLUSION

The present work is an attempt to look at the chaotropic effects of basic chemical compounds on the biological membranes. Both the chosen chaotropic agents are routinely used in laboratory experiments and this work signifies a new angle of applying them in membrane analysis.

It is already known that the cross-linking of peptidoglycan can be inhibited by ethanol which can ultimately result in cellular lysis (InGram and Vreeland, 1980). As the periplasmic space of a cell is surrounded by an outer membrane, which contains transmembrane pores which are freely accessible for small molecules, such as ethanol and inorganic ions; the differential susceptibilities of Gram positive and Gram negative bacteria for inorganic and organic chaotropic agents, as observed in the present work might be possible, which can be better understood if seen at the molecular level; wherein the probable inhibiting role that these agents possess on the enzymatic machinery of cell membranes, involved in the cross linking can be better understood. As Gram positive and Gram negative species significantly differ with respect to their membrane compositions and permeability properties, the present work opens up a new scope for further looking at such differential chaotropic effects caused by a vast array of organic and inorganic compounds that are part of our daily life.

REFERENCES

1. Ananda Vardhan Hebbani and John Barnabas 2012. Chaotropic agents: a new investigative tool in biomolecule research. International Journal Current Life Sciences - Vol.2, Issue, 9, 42-44.
2. Anton P. J. Middelberg., 1995. Process-scale disruption of microorganisms. Biotechnology Advances, Vol. 13, No. 3, pp. 491-551.
3. Blumberg, P. AL, and J. L Stromlnger. 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev. 38:291-335.
4. Cronan, J. E., Jr., and P. R. Vagelos. 1972. Metabolism and function of membrane phospholipids of Escherichia-coli. Biochim. Biophys. Acta 265:25-60.

5. Daniel Rigomier, Jean-Pierre Bohin and Bernard Lubochinsky, 1980. Effects of Ethanol and Methanol on Lipid Metabolism in *Bacillus subtilis*. *Journal of General Microbiology*, 121, 139-149.
6. Douglas A. Wait and Mark D. Sobsey., 1983. Method For Recovery Of Enteric Viruses From Estuarine Sediments With Chaotropic Agents Applied And Environmental Microbiology, Vol. 46, No. 2, P. 379-385.
7. Ghuysen, J. IL 1977. The concept of the penicillin target from 1965 until today. *J. Gen. Microbiol.* 101:13-33.
8. Grula, E. A., and M. ML Grula 1962. Cell division in a species of *Erwinia*. HI. Reversal of inhibition of cell division caused by D-amino acids, penicillin, and ultraviolet light. *J. Bacteriol.* 83:981-988.
9. Hallsworth JE, Yakimov MM, Golyshin PN, Gillion JL, D'Auria G, de Lima Alves F, La Cono V, Genovese M, McKew BA, Hayes SL, Harris G, Giuliano L, Timmis KN, McGenity TJ, 2007. Limits of life in MgCl₂-containing environments: chaotropicity defines the window. *Environ Microbiol.* 9(3):801-13.
10. Hallsworth, John E, Sabina Heim and Kenneth N. Timmis., 2003. Chaotropic solutes cause water stress in *Pseudomonas putida*. *Environmental Microbiology*, 1-11.
11. Hatefi Y., W. G. Hanstein., 1969. Solubilization of Particulate Proteins And Nonelectrolytes By Chaotropic Agents. *Proc. N.A.S. Biochemistry*, Vol. 62, 1129-1136.
12. Hawrot, E., and E. P. Kennedy. 1975. Biogenesis of membrane lipids: mutants of *Escherichia coli* with temperature-sensitive phosphatidylserine decarboxylase. *Proc. Natl. Acad. Sci. U.S.A.* 72:1112-1116.
13. InGram L. O., N. S. Vreeland., 1980. Differential Effects of Ethanol and Hexanol on the *Escherichia coli* Cell Envelope. *Journal of Bacteriology*, Vol. 144, No. 2. p. 481-488.
14. InGram L. O., 1982. Regulation of Fatty Acid Composition in *Escherichia coli*: A Proposed Common Mechanism for Changes Induced by Ethanol, Chaotropic Agents, and a Reduction of Growth Temperature. *Journal of Bacteriology*, Vol. 149, No. 1 p. 166-172.
15. InGram L. O., 1981. Mechanism Of Lysis Of *Escherichia coli* By Ethanol And Other Chaotropic Agents. *Journal Of Bacteriology*, 146, No. 1, P. 331-336.
16. InGram, L O., and E. L Thurston. 1976. Potassium requirement for cell division in *Anacystis nidulans*. *J. Bacteriol.* 125:369-371.
17. Jennifer L. Proc, Michael A. Kuzyk, Darryl B. Hardie, Juncong Yang, Derek S. Smith, Angela M. Jackson, Carol E. Parker, and Christoph H. Borchers., 2010. A Quantitative Study of the Effects of Chaotropic Agents, Surfactants, and Solvents on the Digestion Efficiency of Human Plasma Proteins by Trypsin. *J Proteome Res.* ; 9(10): 5422–5437.
18. Johnson, J. H., and E. A. Grula. 1980. Cell membrane phospholipids and their constituent fatty acids in dividing and non dividing cells of *Micrococcus lysodeikticus*. *Can. J. Microbiol.* 26:658-66.
19. Kennell, D., and A. Kotoulas. 1967. Magnesium starvation of *Aerobacter aerogenes*. IV. Cytochemical changes. *J. Bacteriol.* 93:367-378.
20. Lekha Patel, Shimon Schuldiner and H. R. Kaback., 1975. Reversible effects of chaotropic agents on the proton permeability of *Escherichia coli* membrane vesicles. *Proc. Nat. Acad. Sci.* Vol. 72, No. 9, pp. 3387-3391.
21. Loveless, L E., E. Spoerl, and T. H. Weiman. 1954. A survey of effects of chemicals on division and growth of *Escherichia coli*. *J. Bacteriol.* 68:637-644.
22. Makris KC, Shakya KM, Datta R, Sarkar D, Pachanoor D., 2007. Chemically catalyzed uptake of 2,4,6-trinitrotoluene by *Vetiveria zizanioides*. *Environ Pollut.* ;148(1):101-6.

23. Naota Oku and Robert C. MacDonald., 1983. Solubilization Of Phospholipids By Chaotropic Ion Solutions. *The Journal of Biological Chemistry*, Vol. 258, No. 14, P. 8733-8738.
24. Nunn, W. D., and B. E. Tropp. 1972. Effects of phenethyl alcohol on phospholipid metabolism of *Escherichia coli*. *J. Bacteriol.* 109:162-168.
25. Previc, E., and S. Richardson. 1969. Growth-physiological changes in *Escherichia coli* and other bacteria during division inhibition by 5-diazouracil. *J. Bacteriol.* 97:416-425.
26. Rogus, H. J. 1979. Biogenesis of the cell wall in bacterial morphogenesis. *Adv. Microb. Physiol.* 19:1-62.
27. Samuel R. Farrah, Dinesh O. Shah and Lonnie O. Ingram., 1981. Effects of chaotropic and antichaotropic agents on elution of poliovirus adsorbed on membrane filters. *Proc. Natl Acad. Sci.* Vol. 78, No. 2, pp. 1229-1232.
28. Slater, M., and M. Schaecter. 1974. Control of cell division in bacteria. *Bacteriol. Rev.* 38:199-221.
29. Spratt, B. G., A. Boyd, and N. Stoker. 1980. Defective and plaque-forming lambda transducing bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the lip-dacA-rodA-pbpA-leuS region of the *Escherichia coli* chromosome. *J. Bacteriol.* 143: 569-581.
30. Tetsuaki Tsuchido, Ancharida Svarachorn, Hiroko Soga and Mitsuo Takano., 1990. Lysis And Aberrant Morphology Of *Bacillus Subtilis* Cells Caused By Surfactants And Their Relation To Autolysin Activity. *Antimicrobial Agents And Chemotherapy*, Vol. 34, No. 5 P. 781-785.
31. Victor A. Fried, Aaron Novick., 1973. Organic Solvents as Probes for the Structure and Function of the Bacterial Membrane: Effects of Ethanol on the Wild Type and an Ethanol-Resistant Mutant of *Escherichia coli* K-12. *Journal of Bacteriology*, Vol. 114, No. 1, p. 239-248.