INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

Volume: 2: Issue-4: Oct - Dec -2011 IJABPT *ISSN 0976-4550*

ISOLATION, CHARACTERIZATION AND GROWTH KINETICS OF CHAOTROPE INDUCED WATER STRESS TOLERANT BACTERIA FROM SOILS OF THAR DESERT

Shikha Khandelwal*[1] and Ramavtar Sharma[2]

[1]Amity Institute of Biotechnology, Amity University Haryana, Manesar, Gurgaon-122413, Haryana, India

[2]Plant Biotechnology Centre, Rajasthan Agricultural University, Bikaner-334 006, Rajasthan, India

ABSTRACT : Chaotropic compounds that freely traverse biological membranes and do not affect turgor are powerful mediators of water stress. The present study was initiated to isolate, identify and characterize the Chaotrope tolerant bacteria from soil of Thar Desert. Bacteria were isolated from soil samples, collected from the surface of sand dunes, suspended in water contained 2.5 x 106 bacteria g-1 of soil while alcohol suspended soil had 4.4 x 104 bacteria g-1. The eleven bacterial isolates studied, for the tolerance was found tolerant to 10 % of alcohol, 14 % NaCl and 26 % glycerol and 9 out of 11 isolates were found to be gram positive. More than 50 % of isolates belonged to *Bacillus* species while *Cornybacterium, Acinobacter, Aeromonas* and *Staphylococcus* were also present. Growth kinetic studies revealed that under highly stressed conditions of alcohol and temperature, the generation time was reduced as compared to generation time in plain medium. A rise in incubation temperature and concentration of alcohol added in growth medium resulted in reduction in bacterial population. All Chaotropic solute tolerant bacteria were also tolerant to water stress induced by Halotropes and Kosmotrophes. This study suggests that non saline soil /sand dunes may be a common source for the isolation of bacteria tolerant to Chaotropic, Halotropic and Kosmotropic solutes up to a greater extent, and might help to resolve the general strategy adopted by microbes to thwart desiccation induced by various types of solutes.

Key Words: Thar Desert, Soil bacteria, Chaotrope, Kosmotrope, Glycerol, Alcohol

INTRODUCTION

Soils are considered as the main reservoirs of microorganisms, since bacteria, algae, protozoans, yeasts, molds, and microscopic worms are routinely found in this environment and are continuously submitted to environmental and anthropogenic perturbations which can lead to serious modifications of microbial taxonomic and functional diversity (Bååth *et al*., 1998). Consequently, the capacity of adaptation, in terms of resistance and resilience, of indigenous microbial community to environmental stresses directly influences the maintenance of soil functions and therefore the sustainability of soil quality (Griffiths *et al*., 2005). Soils typically contain 10^9 to 10^{10} microorganisms per gram (dry weight), which may represent more than a million bacterial species (Gans, J., M. Wolinsky, and J. Dunbar. 2005). Soil microorganisms are commonly subjected to extremely low water potentials resulting from low matric water potential (cm) in dry soils and/or low solute water potential (cs) in saline soils (Kieft et al., 1987; Harris, 1981).Low solute water potential might also develop in drying soils left with negligible water adhered around soil particles in the form of a thin film that probably holds the microbes also.

Consequently, Studies of water availability in biological systems have traditionally focused on osmotic stress, which influences cell turgor, which in turn influences fundamental processes such as transport, cell extension and growth, membrane integrity and regulation of cytosol concentration. However, other factors, such as chaotropic solutes (Ethanol) that weaken electrostatic interactions in biological macromolecules, can influence water availability without having a major impact on cell turgor (Shah *et al*., 1998; Takahashi *et al*., 2000), are powerful mediators of water stress. Indeed, it may be that many so called toxic compounds have negligible specific modes of action on specific cellular targets, but rather are agents of water stress with general adverse effects on cellular macromolecules (Hallsworth, 1998). The question is whether the soil microbes commonly exposed to low water potential develop macromolecule stabilizing mechanism and illustrate tolerance to Chaotropic solutes like alcohol.

In response to chaotropic solutes (e.g., LiCl, urea, and ethanol), microbial cells also up-regulate proteins involved in protein stabilization, lipid metabolism and membrane structure, protein synthesis, and energy metabolism (Hallsworth *et al.*, 2003a). Ethanol and other chaotropes readily traverse lipid bilayers (Takahashi *et al*., 2000) but nevertheless reduce water activity and decrease electrostatic interactions in biological macromolecules (Hribar *et al.,* 2002; Wiggins, 2001). It has also been found that compatible solutes protect against ethanol, which is consistent with the hypothesis that ethanol's primary effects on microbial metabolism are through perturbation of water-macromolecule relations (Hallsworth *et al*., 2003b).

The goal of this work was to determine whether bacteria present in the sand dunes of Thar Desert that are naturally subjected to desiccation and salts concentrating around soil particles during soil drying, are tolerant to chaotropic solutes. For this study, we isolated the microbes from soil sample collected from the sand dunes of Thar Desert and identified and characterized the bacterial isolates on the basis of microbiological and biochemical techniques. The effects of varying temperatures and different stress solutes were analyzed and their growth kinetics was studied. However, characterization of the small fraction of bacterial isolates that has been investigated provides only a glimpse of their potential physiological capacity and influence on soil ecosystems.

MATERIALS AND METHODS

Collection of soil samples

The soil samples were collected from sand dunes of Bikaner situated in the Western region of Rajasthan. Samples were taken from uncultivated fallow barren lands during the period of June (hot season). Soil samples from soil surface (0 - 5 cm) were taken in sterilized polyethylene bags using sterilized spatula and stored at 4 ºC until examination.

Isolation and identification of Chaotrope tolerant bacteria from soil sample

The soil samples were passed through a sieve (1.7 mm mesh) to remove large pieces of debris and vegetation. The soil samples were diluted to 1:100 ratios i.e. 1 gm of soil was dispersed in 100 ml of sterile distilled water and absolute alcohol and kept for 1 h. Then 100 µL from each dilution was spread on nutrient agar medium containing different percentage of alcohol (0 % - 5 %) and incubated at 37ºC, 45°C and 50 °C for over night. The developed colonies were counted in plates and the average number of colonies was determined.

The number of total bacteria (CFU) per gram dry weight soil was determined. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar. The bacterial isolates were kept on nutrient agar at 4 °C and re-cultured every 4 weeks. The bacterial isolates were identified on the basis of classification schemes published in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

For the identification of bacterial isolates, Gram's staining was performed by commercially available chemicals to detect the Gram's reaction of the bacterium (Harold J Benson,). Endospore staining was also performed to detect the presence of spores and the position of spore whether terminal or center (Harold J Benson,). The motility of the bacteria was studied by hanging drop technique (Harold J Benson,). Biochemical tests such as Oxidation – Fermentation test, catalase test, oxidase test, sugar-fermentation test were carried out using the respective Hi-media reagents.

Determination of Tolerance to various solute concentrations

The bacterial isolates were evaluated for their capability to tolerate osmotic potential generated by various solutes in combination with various temperatures. The solutes were directly added to nutrient agar medium in required concentration. The isolates were tested for their tolerance on high concentration of alcohol (up to 10 %), NaCl (up to 15 %) and glycerol (up to 30 %) at an interval of 5 % on two temperatures viz. 45 °C and 50 °C. Further finer details of tolerance to various solutes were investigated with an increment of 1 %. The measurements consisted of visual observations of the colonies that appeared in the plates after 24 hrs of incubation.

Determination of Tolerance to Higher Temperatures

Temperature tolerance was studied in the absence of any solute stress using culture media onto which a 24 hrs old culture was streaked and incubated at different temperatures such as 37 °C, 45 °C, 55 °C, 60 °C and 65 °C and observed for growth after 24 hrs. The plates were examined for differential growth density. The maximum growth temperature of the isolates was thus determined.

Determination of growth kinetics of Chaotrope tolerant isolates

Overnight cultures of bacteria were inoculated in a flask containing 100 mL medium to obtain 0.1 O.D. at 600nm. The flasks were incubated in a incubator at 37 °C (150 rpm) and O.D. was recorded periodically until the growth reached the stationary phase. The most tolerant bacterial isolate was used for this purpose and growth pattern was studied at various concentrations of alcohol (0 %, 4 %, and 8 %) in combination with different temperatures (40 °C, 50° C, 60 °C). A growth curve was also constructed for 37 °C in the absence of osmotic stress in order to find out normal growth pattern of the culture.

RESULTS AND DISCUSSION

The total bacterial count (2.5 x 10^6 CFU/gm soil) was comparatively less in desert soils as compared to other soils reported (Echigo et al., 2005). However, a considerable proportion of it consisted of temperature tolerant microbes. About 5.2 % bacteria could tolerate 45 °C of temperature and developed visible colonies on nutrient agar medium. Further rise in temperature to 50 °C also maintained a considerable fraction of bacterial population (4.4 %) that amounts to $9.0X10^3$ bacteria g^{-1} of soil.

Exposed to extremely hot and dry conditions coupled with sudden soaking wet with scanty precipitation and rapid drying desert soils are expected to harbor microbes tolerant to desiccation and equipped to adapt rapid change in moisture regime. Ethanol a chaotropic solute having fast penetrating ability through cell membrane likely to induce rapid desiccation effect in the cell. Chaotropic compounds however, do not affect turgor but reduce water activity, perturb macromolecule–water interactions and thereby destabilize cellular macromolecules that eventually inhibit growth of bacteria. Since choatropic solute, ethanol is one of the most commonly used powerful mediators of water stress in bacterial isolates it was considered for the present study.

There was approximately 50 times reduction in bacterial population i.e. 4.4 x 10^4 CFU/gm soil, when soil suspension was made in alcohol and plated on nutrient agar medium at 37 °C and 45 °C. However it makes a considerable proportion 1.76 % bacterial population that sustain the absolute alcohol for 1 h and this percentage though dropped to 0.36 % when it was incubated at 50 °C. Thus, the results indicated that the total bacterial count in water suspended soil was higher than that in alcohol suspended soil and the number of bacteria decreased with increasing temperatures. Similarly, the bacterial population decreased with increasing concentration of alcohol and temperature in nutrient agar medium. The reduction in population of colonies ranged from 30 to 50 times on nutrient agar medium supplemented with 2 % and 5 % alcohol respectively with varying temperature.

When both the stresses viz. temperature and alcohol were applied simultaneously, the population of colonies further decreased. At the highest temperature (50 °C) and alcohol concentration (5 %) used for isolation of bacteria in the present investigation did not yield any bacterial colony with the soil suspension in alcohol. It was supposed that Bacteria only sporulated bacteria survived the one hour suspension in alcohol and all of them formed spores at 50°C in the presence of 5% alcohol. In order to avoid sporulating bacteria for further study we

isolated temperature and alcohol tolerant bacteria growing at 50 °C with 5 % alcohol from water suspended soil. On these conditions we do not expect bacterial colonies developed by sporulating bacteria. All these bacteria were represented in surface soils only (Table 1).

Table 1: Number of colony forming units per gram of soil when soil is dissolved in water and alcohol.

S. No.	Alcohol concentration in nutrient agar plates	Soil + water			Soil + alcohol		
		37 °C	45°C	50°C	37 °C	45°C	50°C
1.	0% alcohol conc.	$2.5X10^6$	$1.3X10^5$	$1.1X10^5$	$4.4X10^4$	$4.3X10^4$	$9.0X10^3$
2.	2% alcohol conc.	$9.3X10^4$	$7.9X10^4$	$6.8X10^4$	$3.0X10^4$	$2.6X10^4$	$5.0X10^3$
3.	3% alcohol conc.	$6.7X10^4$	$5.0X10^4$	$4.5X10^4$	$2.5X10^4$	$2.0X10^4$	$3.0X10^3$
4.	4% alcohol conc.	$4.2X10^4$	$3.8X10^4$	$3.4X10^4$	$1.6X10^4$	$1.1X10^4$	$1.0X10^3$
5.	5% alcohol conc.	$3.9X10^4$	$1.5X10^4$	$1.4X10^4$	$9.0X10^3$	$7.0X10^3$	0.0

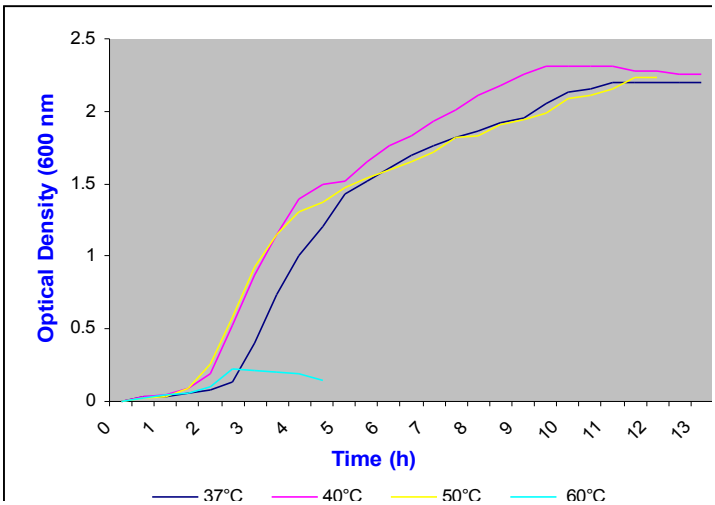

Figure: 1. Effect of temperatures on growth curve of a bacterial isolate.

All the 11 isolates were screened for their ability to grow on alcohol containing media at different concentrations ranging from 4 % to 6 %. The isolates were then purified and identified at the genus level by standard procedures described in Bergey's Manual of Systematic Bacteriology. Out of 11 isolates, 1 was found gram +ve cocci and 8 gram +ve bacilli while 2 were gram –ve bacilli. Among Gram-positive bacteria, *Bacillus (*54.54 %) was the most frequent genus isolated followed by *Cornybacterium* (18.18 %), and *Staphylococcus* (9.09 %). This showed that *Bacillus* species was in greater abundance among alcohol tolerant bacteria. One isolate each of two Gram-negative genera *Acinobacter* and *Aeromonas* were recorded.

The bacterial isolates were then evaluated for their capability to tolerate osmotic potential generated by various solutes (Kosmotrope (NaCl) and osmotrope (glycerol) in combination with two temperatures i.e. 45°C & 50°C. It was observed that bacterial isolates had a broad range of growth tolerating various concentrations of alcohol, NaCl and glycerol. Besides, simultaneous tolerance for osmotic and temperature stress was remarkable for most strains. Some of the cultures exhibited growth at as high as 10 % concentration of alcohol, 14 % of NaCl and 26 % of glycerol at 45°C. However, detrimental effect of higher temperature tested (50 °C) was evident even at lower concentrations viz. 6 %, 10 % and 18 % of alcohol, NaCl and glycerol respectively. Similarly, the reduction in growth with increasing levels of three solutes was more pronounced at 50 °C (Table 2).

The bacterial isolate S4 showed reduced growth on concentration of 10 % alcohol at 45°C whereas isolates S1, S3, S5, S6, S7, S8, S9 and S10 showed highly reduced growth, but other isolate S2 and S11 showed no growth on 10 % alcohol at this temperature. Similarly, only four bacterial isolates S1, S3, S4 and S5 showed highly reduced growth on 14 % NaCl at 45 °C while remaining isolates failed to grow. Furthermore, isolates S1, S3, S4 and S5 showed reduced growth whereas isolates S6, S7 and S9 showed highly reduced growth on 26 % glycerol. Most tolerant isolates based on the three solutes turned out to be the two-gram negative bacteria (S1 and S4).

Table 2: Effect of various solutes on the 24 h growth of Bacterial isolates at two different temperatures.

S. No.	Bacterial isolates	Temp.	Alcohol conc. (in %)				NaCl conc. (in %)						Glycerol conc. (in%)				
			6%	7%	8%	9%	10%	10%	11%	12%	13%	14%	18%	20%	22%	24%	26%
1.	S1	45°C	++++	++++	+++	+++	+	+++	+++	+++	+++	+	++++	++++	+++	++	++
		50°C	+++	+++	++	+	-	++	++	++	+	-	+++	++	+	-	-
2.	S2	45°C	++++	++++	++	+	-	+++	+++	+	+	-	++++	++++	+	+	-
		50°C	+++	+++	+	-	-	++	++	+	-	-	+++	++	-	-	-
3.	S3	45°C	++++	++++	+++	++	+	+++	+++	+++	+++	+	++++	++++	++	++	++
		50°C	+++	++++	++	+	-	++	++	++	++	-	+++	+++	+	-	-
4.	S4	45°C	++++	+++	+++	+++	++	+++	+++	+++	+++	++	++++	++++	++	+++	++
		50°C	+++	+++	++	+	-	++	++	++	++	-	+++	+++	+	-	-
5.	S5	45°C	++++	++++	+++	+++	+	+++	+++	+++	+++	+	++++	++++	+++	+++	++
		50°C	+++	+++	++	+	-	++	++	++	+	-	+++	++	++	+	-
6.	S6	45°C	++++	+++	++	+	+	+++	+++	+++	++	-	++++	++++	++	+	+
		50°C	+++	+++	+++	-	-	++	++	++	+	-	+++	++	+	-	-
7.	S7	45°C	++++	++++	++	++	+	+++	+++	+++	++	-	++++	++++	++	+	+
		50°C	+++	+++	+++	+	-	++	++	++	+	-	+++	++	+	-	-
8.	S8	45°C	++++	++++	++	++	+	+++	+++	+++	++	-	++++	++++	++	+	-
		50°C	+++	+++	+	+	-	++	++	++	-	-	+++	++	+	-	-
9.	S9	45°C	++++	++++	+++	+++	+	+++	+++	+++	+++	-	++++	++++	+++	+++	+
		50°C	+++	+++	++	+	-	++	++	++	+	-	+++	++	+	+	-
10.	S10	45°C	++++	++++	+++	++	+	+++	++	++	-	-	++++	++++	++	-	-
		50°C	+++	+++	++	+	-	++	+	-	-	-	+++	++	+	-	-
11.	S11	45°C	++++	+++	-	-	-	+++	++	-	-	-	++++	++++	++	-	-
		50°C	++	+	-	-	-	++	+	-	-	-	+++	++	+	-	-

++++ = Very Good growth, +++ = Good growth, ++ = Reduced growth
+ = highly reduced growth, = No growth

All the isolates were also tested for their capability to stand higher temperatures ranging 37°C to 65 °C in the absence of stressful solute concentrations. It can be seen that most bacterial isolates showed good growth up to 50 °C with gradual reduction on subsequent increase in temperature. Four isolates viz., S1, S2, S3 and S4 offered resistance up to 65 °C temperature. However, none of the isolates could stand such a high temperature in the presence of stressful concentrations of any of the solutes tested (Table 3).

Table 3: Studies on temperature tolerance profile on the 24 hrs growth of the Bacterial isolates.

S.No.	Bacterial isolates	Temperature						
		37°C	45°C	50°C	55°C	60°C	63°C	65°C
1.	DH5α	++++	+++	+	-	-	-	-
2.	S1	++++	++++	++++	+++	+++	++	+
3.	S2	++++	++++	++++	+++	+++	++	+
4.	S3	++++	++++	++++	+++	+++	++	+
5.	S4	++++	++++	++++	+++	+++	++	+
6.	S5	++++	++++	++++	+++	+++	+	-
7.	S6	++++	++++	++++	++	++	+	-
8.	S7	++++	++++	+++	-	-	-	-
9.	S8	++++	++++	+++	-	-	-	-
10.	S9	++++	++++	+++	++	+	-	-
11.	S10	++++	++++	+++	++	+	-	-
12.	S11	++++	+++	++	-	-	-	-

++++ refers to very good growth +++ refers to good growth
++ refers to reduced growth + refers to highly reduced growth - refers to no growth

Figure: 2. Growth Curve of isolate S4 at different temperatures with different concentrations of alcohol.

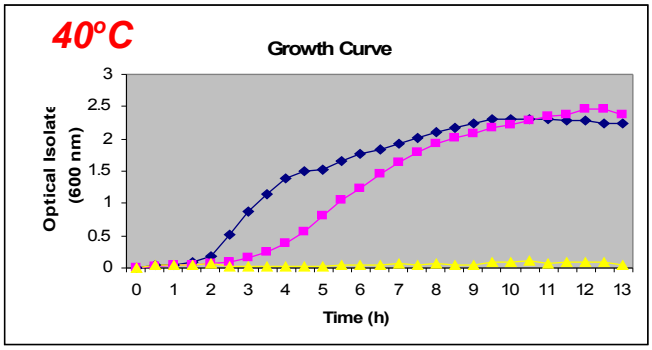

Fig. 2(a). Growth pattern of Bacterial isolate at 40°C temperature at 0, 4 and 8% alcohol concentration in nutrient broth.

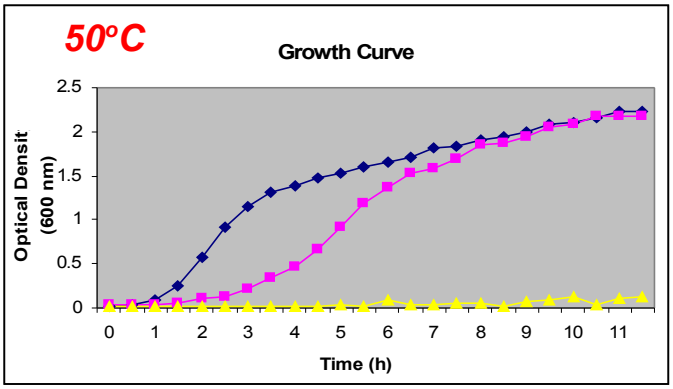

Fig. 2(b). Growth pattern of Bacterial isolate at 50°C temperature at 0, 4 and 8% alcohol concentration in nutrient broth.

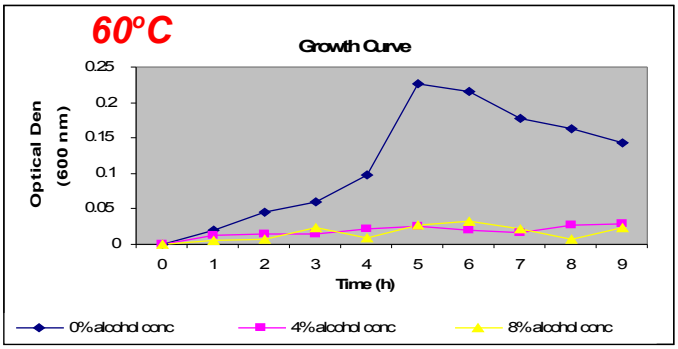

Fig. 2(c). Growth pattern of Bacterial isolate at 60°C temperature at 0, 4 and 8% alcohol concentration in nutrient broth.

The most chaotropic resistant isolate viz. S4 *Aeromonas* sp. was monitored for growth kinetics studies in nutrient broth containing different concentrations of alcohol (0 %, 4 %, 8 %) in combination with different temperatures (40 °C, 50 °C, 60 °C). Figure 1 showed the kinetics of the isolates under different temperature conditions (in plain nutrient broth), whereas Figure 2 (a, b and c) show the growth curve of resistant S4 *Aeromonas* sp., at 40°C, 50°C, 60°C temperature at 0, 4 and 8 % alcohol concentration in nutrient broth. The growth curve constructed for various temperatures revealed that the isolate grew faster at 40 ºC and 50 ºC as compared to 37 ºC. The lag phase lasted for 3 hrs at 37 ºC as against 2 hrs at higher temperatures (40 ºC and 50 ºC). However, by the end of log phase, the growth at 50 ºC approximated the growth at 37 ºC, keeping high at 40 ºC. However, stationary phase was reached after 11 hrs at all the 3 temperatures (Figure 1). The even higher temperature i.e. 60 ºC was detrimental to growth where bacteria maintained a very subdued growth.

Nutrient broth is a stress free; complete medium containing most of the preformed growth requirements which are directly available to the cells (Madigan *et al.,* 1997). The generation time of the resistant isolates in nutrient broth containing (0 %, 4 %, and 8 %) at various temperatures (40 ºC, 50 ºC, 60 ºC) showed a reduction because of the alcohol stress. The 4 % level of alcohol delayed lag phase for 4 h as against one and half hours on control medium devoid of alcohol, at 40 ºC and 50 ºC whereas 8 % of alcohol in nutrient broth was found to be enough to almost stop growth as represented by straight line nearing base. Moreover, the growth at 4 % lagged behind the control during log phase also at 40ºC and 50ºC (Figure 2). However, the cell concentration approximated to control while reaching to stationary phase. Both the concentrations of alcohol i.e. 4 % and 8 % were found enough to check the growth of bacterial isolate at 60ºC whereas under control (without supplemental alcohol) conditions, lag phase though got delayed, abrupt increase in cellular growth was observed in log phase with subsequent sudden drop in cell mass. These observations with reference to generation time under stress of alcohol in nutrient medium are in agreement with the extended generation time of soil bacteria grown in lab conditions with phenol stress (Ajaz *et al.,* 2004).

Thus, from the present investigation it could be concluded that surface soils of sand dunes of Thar Desert are full of microbial activity and harbors desiccation tolerant bacteria along with capabilities to survive at higher temperatures.

REFERENCES

1. Bååth, E., D.R. Montserrat, Å. Frostegard, and C.D. Campbell (1998). Effect of metal-rich sludge amendments on the soil microbial community. *Appl Environ Microbiol* 64: 238– 245.
2. Kieft T.L., E. Soroker, and M.K. Firestone (1987). Microbial biomass response to a rapid increase in water potential when a dry soil is wetted. *Soil Biol. Biochem*., 19:119–126.
3. Harris, R.F. (1981). Effect of water potential on microbial growth and activity. p. 23–96. *In* J.F. Parr et al. (ed.) Water potential relations in soil microbiology. *SSSA Spec. Publ*. 9. SSSA, Madison, WI.

4. Takahashi. H., A. Matsuo, and I. Hatta (2000). Effects of chaotropic and kosmotropic solutes on the structure of lipid cubic phase: monoolein-water systems. *Mol. Cryst. Liq. Cryst.*, 347: 475–482.

5. Hallsworth, J.E. (1998). Ethanol-induced water stress in yeast. *J. Ferment. Bioeng*., 85:125–137.

6. Hallsworth, J.E., A.P. Bernard, N. Yoshiyuki, I. Masayoshi, and N.T. Kenneth (2003b). Compatible Solutes Protect against Chaotrope (Ethanol)-Induced, Nonosmotic Water Stress. *Applied and Environmental Microbiology*, Dec, p. 7032–7034 Vol. 69, No. 12.

7. Hallsworth, J.E., S. Heim, and K.N. Timmis (2003a). Chaotropic solutes cause water stress in *Pseudomonas putida*. *Environ. Microbiol*., 5: 1270-1280.

8. Hribar, B., N.T. Southall, V. Vlachy, and K.A. Dill (2002). How ions affect the structure of water. *J. Am. Chem. Soc*., 124:12302–12311.

9. Wiggins, P.M. (2001). High and low density intracellular water. *Cell. Mol. Biol.,* 47: 735–744

10. Griffiths, B.S., P.D. Hallet, H.L. Kuan, Y. Pikin, and M.N. Aitken (2005). Biological and physical resilience of soil amended with heavy metal-contaminated sludge. *Eur J Soil Sc* 56: 197–205.

11. Gans, J., M. Wolinsky, and J. Dunbar (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science*. 309(5739):1387-1390.

12. Shah, S. Li. J., B.A. Moffatt, and B.R. Glick (1998). Isolation and characterization of ACC deaminase genes from two different plant growth-promoting rhizobacteria. Can. J. Microbiol. 44:833-843

13. Krieg, N.R. and J.G. Holt (1984) Bergey's Manual of Systematic Bacteriology, Vol. 1, eds: Williams and Wilkins. Baltimore.

14. Echigo, A., M. Hino, T. Fukushima, T. Mizuki, M. Kamekura, and R. Usami (2005). Endospores of halophilic bacteria of the family *Bacillaceae* isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm). *Saline Systems, 1*, 8.

15. Madigan, M.T., J.M. Martinko, and J. Parker (1997). *Brock Biology Microrganisms*, 8th ed., Prentice Hall International Inc., New Jersey. 1-986 pp.

16. Ajaz, M., N. Noor, S.A. Rasool, and S.A. Khan (2004). Phenol resistant bacteria from soil: identification-characterization and genetical studies. *Pak. J. Bot.,* 36(2): 415-424.