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ROOT ASSOCIATED MODERATELY HALOPHILIC AND ALKALIPHILIC BACTERIA FROM *CONVOLVULUS ARVENSIS*

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Abstract: Seven isolates from the histoplane (Cs-1, Cs-2, Cs-3, Cs-4) and rhizoplane (RCs-1, RCs-2, RCs-3) of *Convolvulus arvensis*, growing in saline area were obtained. They were gram-negative motile rods (except Cs-2 which was gram negative motile cocci) and could tolerate 2.0-3.0M NaCl in the medium. Optimal salt concentration for their growth was 0.5M (Cs-2, Cs-3, RCs-1, RCs-3), 1.0M (Cs-4, RCs-3) and 1.5M (Cs-1), hence all of them were moderately halophilic bacteria. Strains Cs-4 and RCs-2 shared characters with genus *Halomonas*, while others belonged to group gram-negative facultative anaerobic rods (except Cs-2). Tolerance level to salts such as $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl varies for different strains. Depending upon the strain temperature for maximum growth of these bacteria was 28°C (Cs-4, RCs-2), 32°C (RCs-1, RCs-3), 37°C (Cs-2, Cs-3) or 42°C (Cs-1). They exhibited wide pH range with optimal pH 8 (except for Cs-2 where it was 9) in the presence of 1M NaCl, thus belonging to moderately alkaliphilic bacteria. However their pH optima varied from 5-10 in the absence of NaCl. These strajns confer resistance to kanamycin in the presence of 1M NaCl but not in its absence. Whereas resistance behaviour to ampicilline was different for different strains. They also exhibited multiple resistance to different metallic salts. Apart from Cs-2, other strains harbor plasmid/s.

Key words: Histoplane, rhizoplane, moderately alkaliphilic, salt-tolerant bacteria.

INTRODUCTION

Among the abiotic stresses that limit crop productivity, salinity ranks as the most detrimental. Organisms specially bacteria which can resist saline stress are being intensively investigated but the molecular basis of microbial resistance to salinity are not fully understood. Two major approaches by which microorganisms respond to raised external salinity are the accumulation of intracellular compatible solutes (osmoprotectants) and modification of membrane composition and function (Russell, 1989; Csonka, 1989; Wohlfarth *et al.*, 1990; Severin *et al.*, 1992; Russell, 1992). Two distinct classes of osmoprotectants exist, those which act as genuine osmolytes such as glycine betaine, proline or glutamate and those which act as chemical mediators *e.g.* ectoine (Talibart *et al.*, 1994). It is imperative to comprehend the mechanism/s utilize by bacteria to withstand the osmotic and ionic stresses imposed by high salinity. Bacterial population from the natural habitat provide a rich source for the understanding of osmoregulation. Bacteria growing in saline habitat fall in two categories; halotolerant, which do not require NaCl for growth but can grow under saline conditions and halophiles, which must have NaCl for growth. Halophiles can be classified into three groups on the basis of their response to NaCl. Slightly halophilic bacteria, yield optimum growth at 0.2-0.5M NaCl, moderately halophilic bacteria grow

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best in 0.5-2.5M NaCl and extremely halophilic bacteria prefer 2.5-5.2M NaCl for their optimal growth (Kushner, 1978). In this connection we are isolating and characterizing the salt tolerant/moderately halophilic bacteria from different sources *viz.*, soil, plant's histoplane, rhizoplane and rhizosphere (Sherwani and Hasnain, 1990a,b; Hasnain and Taskeen, 1989; Yasmin and Hasnain, 1993a,b). Here we are describing the isolation and characterization of moderately halophilic bacteria from the rhizoplane and histoplane of *Convolvulus arvensis*.

MATERIALS AND METHODS

Isolates were obtained from the histoplane and rhizoplane of *Convolvulus arvensis*, growing in the saline patches around Lahore, by the method of Yasmin and Hasnain (1993a). Only those isolates which could bear 1M or above concentrations of NaCl in the growth medium were selected. Purified isolates were characterized following Gerhardt *et al.* (1994). Some biochemical tests were also accomplished by using Q.T.S-20 and CO strips (DESTO Laboratories, Karachi, Pakistan).

Resistance profile of these strains for NaCl (0.5-3.0M) and some other salts involve in salinity such as $NaHCO_3$ (0.1-0.5M), Na_2SO_4 (0.1-1.0M), $MgSO_4$, KNO_3 and KCl (0.1-2.0M) were studied. Resistances to $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl were examined both in the presence and absence of 1M NaCl. Following Yasmin and Hasnain (1993b), bacterial growth curves, temperature (25°, 28°, 32°, 37°, 42°C) and pH ranges (5, 6, 7, 8, 9, 10, 11) of these isolates were accomplished both in the presence and absence of NaCl. Resistance pattern of these isolates to antibiotics kanamycin (Km, 10-50 $\mu g\ ml^{-1}$), ampicillin (Ap, 100-500 $\mu g\ ml^{-1}$), tetracycline (Tc, 5-25 $\mu g\ ml^{-1}$), streptomycin (Sm, 100-500 $\mu g\ ml^{-1}$), chloramphenicol (Cm, 5 $\mu g\ ml^{-1}$) as well as metallic salts of Ni ($NiSO_4$), Sn ($SnCl_2$), Ba ($BaCl_2$), Pb [$Pb(NO_3)_2$], Cr ($CrCl_3$), Mn ($MnSO_4$), Zn ($ZnSO_4$), Fe (Fe_2SO_4), Mo (Na_2MoO_4), Co ($CoCl_2$) and Cu ($CuSO_4$) were also determined. Media (L-agar or L-broth) was supplemented with the respective antibiotics and metallic salts and after inoculation/ streaking and incubation at 37°C for 24 hours, growth was checked or OD was monitored at 600 nm, as the case may be.

Bacteria were screened for the presence of plasmid/s by the gel electrophoresis of total cell lysate (Thomas, 1984).

RESULTS

Isolates were obtained from the histoplane (Cs-1, Cs-2, Cs-3, Cs-4) and rhizoplane (RCs-1, RCs-2, RCs-3) of *Convolvulus arvensis* growing in the saline area around Lahore. All the isolates, both from rhizoplane and histoplane of the plant, had circular, entire (except Cs-3) and convex colonies with different shades of yellow except for Cs-1 and Cs-2, where colonies were dirty white and white, respectively. Isolates Cs-4 and RCs-2 were strictly aerobic while rest of them were facultative anaerobes. All isolates were gram-negative motile rods except Cs-2 which was found to be gram-negative motile cocci. Isolates Cs-4, and RCs-2 could be affiliated with genus *Halomonas*, while

Cs-1, Cs-3, RCs-1, RCs-3 were identified as the members of group facultatively anaerobic gram-negative rods (Holt *et al.*, 1994). Affinities of Cs-2 (gram-negative facultative anaerobic cocci) remained uncertain. Excluding Cs-1 and RCs-1 for few tests, they showed similar biochemical attributes (Table 1).

Table 1: Biochemical characterization of isolates from *Convolvulus arvensis*.

Name of Test	Strains						
	Cs-1	Cs-2	Cs-3	Cs-4	RCs-1	RCs-2	RCs-3
Gram staining	-	-	-	-	-	-	-
Oxidation fermentation	+	+	+	-	+	-	+
Cytochrome oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Denitrification	-	-	-	-	-	-	-
Voges Proskaur	-	+	-	-	-	-	-
Methyl red	-	+	-	-	-	-	-
Phenylalanine deaminase	+	+	+	+	+	+	+
ONPG	+	-	-	-	+	-	-
Sodium citrate	-	-	-	-	-	-	-
Sodium malonate	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-
Tryptophane deaminase	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-
Acetoin	+	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+	+
Acid from glucose	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+
Acid from maltose	-	-	-	-	-	-	-
Acid from sucrose	-	-	-	-	-	-	-
Acid from manitol	-	-	-	-	-	-	-
Acid from arabinose	+	+	+	+	+	+	+
Acid from rhamnose	-	-	-	-	-	-	-
Acid from sorbitol	-	-	-	-	-	-	-
Acid from inositol	-	-	-	-	-	-	-
Starch hydrolysis	+	-	-	-	-	-	-
Pigment production	-	-	-	-	-	-	-

+ = Positive reaction; - = Negative reaction

Apart from RCs-1, they could bear upto 2.5M NaCl in the medium (Fig.1). These strains showed their best growth in the salt medium between 0.5-1.5M NaCl, hence belong to moderately halophilic bacteria. As regards the resistance profile against other salts, pertaining to saline habitat, Cs-1 could not tolerate even low level of $NaHCO_3$ (0.1M) in the medium. Whereas other strains showed variable behaviour to this salt. Cs-2, Cs-3 and RCs-1 could grow at low level of this salt in the absence of NaCl but in the presence of NaCl these strains showed quite sensitive behaviour (Fig.2a, 2b). Cs-4 could grow, both in the presence and absence of NaCl, relatively in wide range of $NaHCO_3$. Whereas RCs-2 and RCs-3 showed better growth response in this salt in the presence of NaCl (Fig.2b). Cs-2, Cs-3, Cs-4, RCs-1, RCs-2 and RCs-3 showed comparatively better growth response in the presence of $NaHCO_3$ in NaCl free media reflecting the halophilic nature of these isolates. These strains also showed relatively better growth response (Fig.2a, 2b) in the presence of Na_2SO_4. All strains could tolerate Na_2SO_4 in NaCl supplemented as well as NaCl free medium. Generally these strains gave comparatively better growth yield at low concentrations of Na_2SO_4 in the presence of NaCl, but at higher concentrations in the absence of 1M NaCl (Fig.2a, 2b). They could also tolerate $MgSO_4$, KNO_3 and KCl from 0.1-2.0M both in the presence and absence of 1M NaCl. In general growth of these strains was better in these salt media when supplemented with NaCl. But some strains such as Cs-2, RCs-1, RCs-2 (in case of KCl), Cs-1, Cs-2, RCs-1 (in case of KNO_3) and Cs-2, RCs-2, RCs-3 (in case of $MgSO_4$) yield better growth at some concentrations in the absence of NaCl.

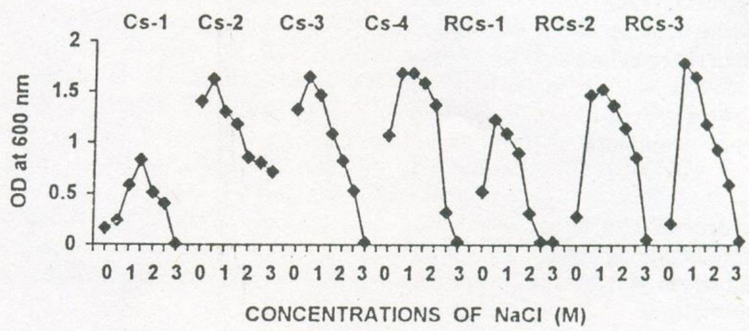


Fig. 1: **Growth responses of moderately halophilic bacterial strains, obtained from *Convolvulus arvensis*, in the presence of different concentrations of NaCl after 24 hours of growth at 37°C.**

Growth pattern of these strains revealed that they require 2 to 6 hour of lag period and stationary phase could be achieved between 18-24 hours of incubation at 37°C (Fig.3). These isolates could grow under wide range of temperatures (25°-42°C) (Fig.4) with different optima. The optimum temperature for the growth of Cs-1 was 42°C, whereas Cs-2 and Cs-3 gave best growth at 37°C. Not much difference in the bacterial growth yield of Cs-4, RCs-1, RCs-2 and RCs-3 was observed over the range of 28°-37°C, hence optimal temperature for these strains ranged from 28°-37°C. Excluding Cs-2 and Cs-3, all strains could grow over wide pH range both in the presence and absence

Table 2: **Resistance of isolates against 100 $\mu g\ ml^{-1}$ salts of heavy metal in the presence and absence of 1M NaCl.**

Metallic salts/NaCl conc.		Strains						
		Cs-1	Cs-2	Cs-3	Cs-4	RCs-1	RCs-2	RCs-3
Nickle sulphate	0M	++	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Stannous chloride	0M	++	++	w+	++	-	-	-
	1M	++	++	++	++	+	+	-
Barium chloride	0M	-	++	++	-	-	++	++
	1M	++	++	++	++	++	++	++
Lead nitrate	0M	+	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Chromium oxide	0M	+	+	++	+	++	-	++
	1M	-	+	++	+	++	-	++
Manganese sulphate	0M	++	++	++	++	++	-	++
	1M	++	++	++	++	++	++	++
Zinc sulphate	0M	-	++	++	-	++	-	-
	1M	-	-	++	-	++	++	++
Ferrous sulphate	0M	++	++	++	w+	++	++	++
	1M	++	++	++	++	++	++	++
Sodium molybdate	0M	++	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Cobalt chloride	0M	++	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Copper sulphate	0M	++	++	++	-	++	++	++
	1M	++	++	++	++	++	++	++

of 1M NaCl. Cs-2 and Cs-3 had a bit narrow pH range in the NaCl supplemented medium. In the presence of 1M NaCl optimal pH for all strains was 8 (except for Cs-2 where it was 9), whereas in the absence of NaCl it was 5 (Cs-1), 6 (RCs-3), 7 (Cs-3), 8 (Cs-4, RCs 2), 9 (Cs-2) and 10 (RCs-1) for different strains (Fig.5).

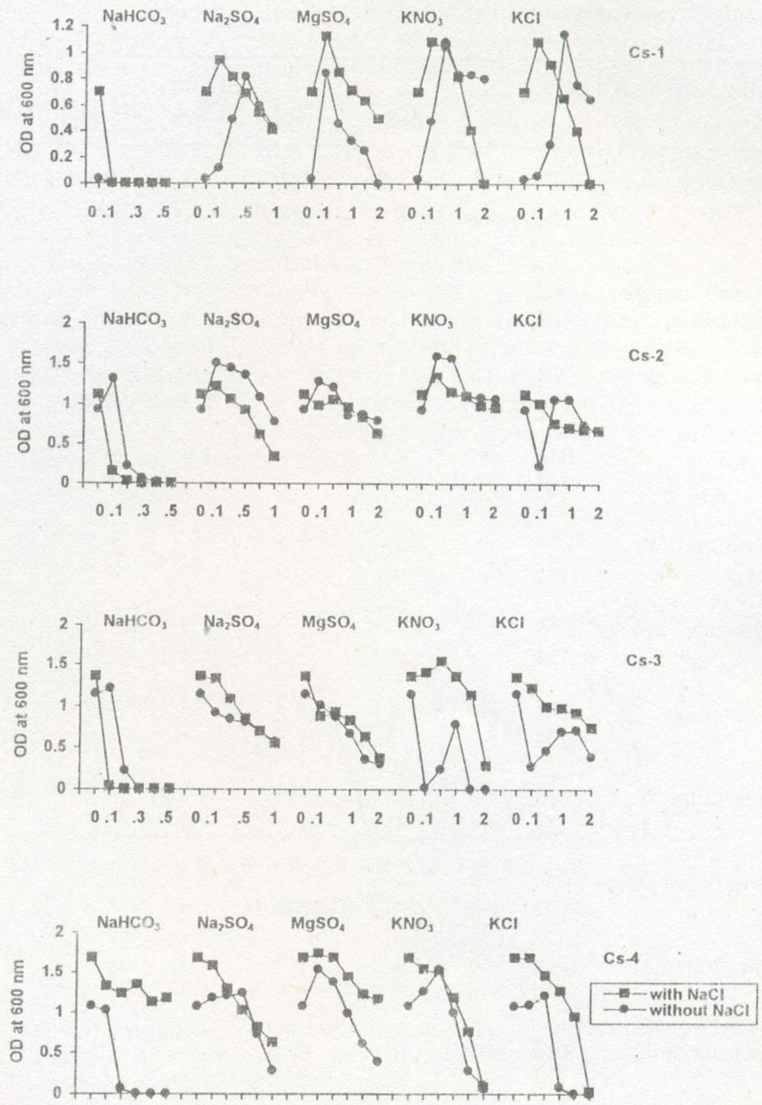


Fig. 2a: Effect of $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl (both in the presence and absence of 1M NaCl) on the growth of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*, at 37°C after 24 hours incubation.

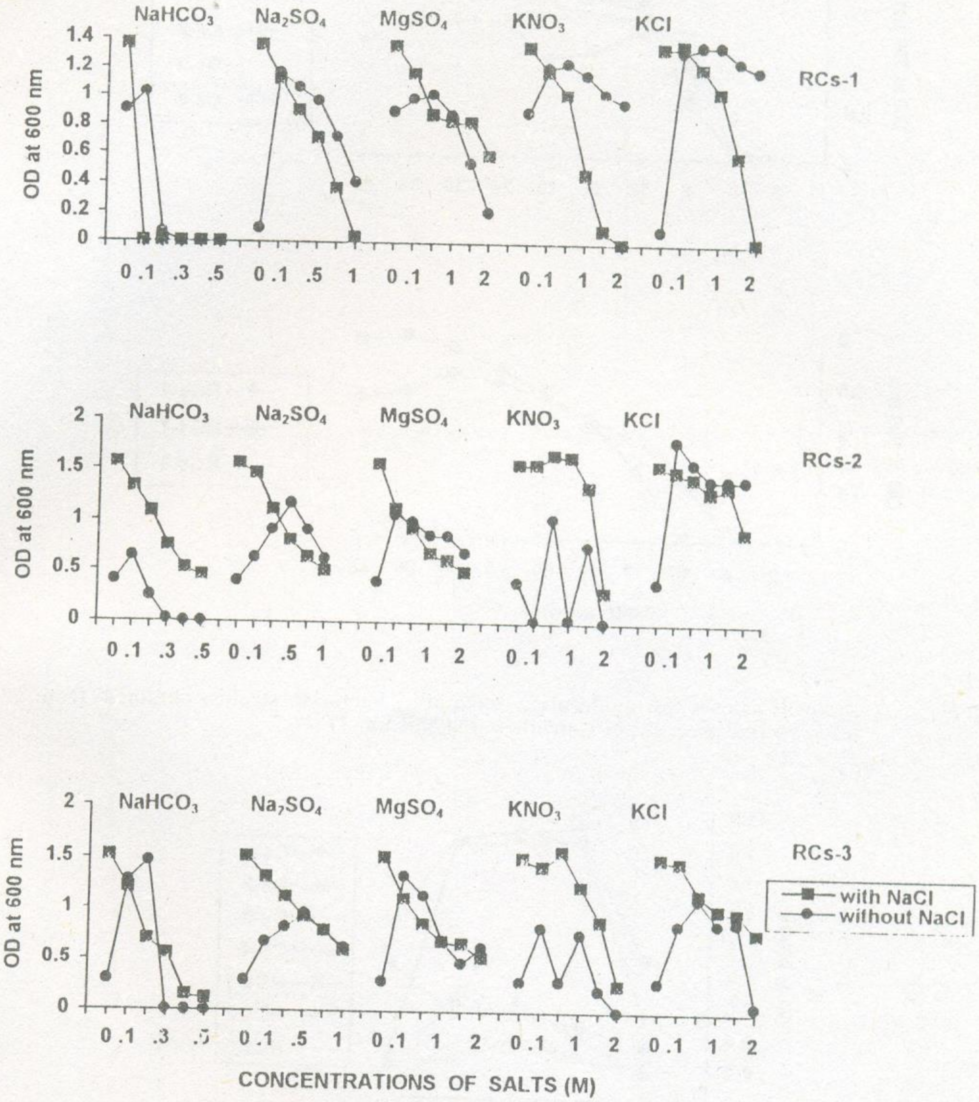
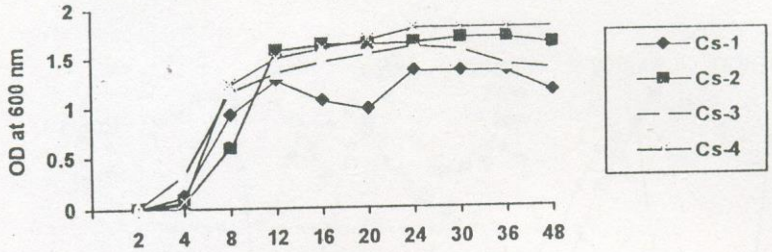


Fig. 2b: Effect of $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl (both in the presence and absence of 1M NaCl) on the growth of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*, at 37°C after 24 hours incubation.



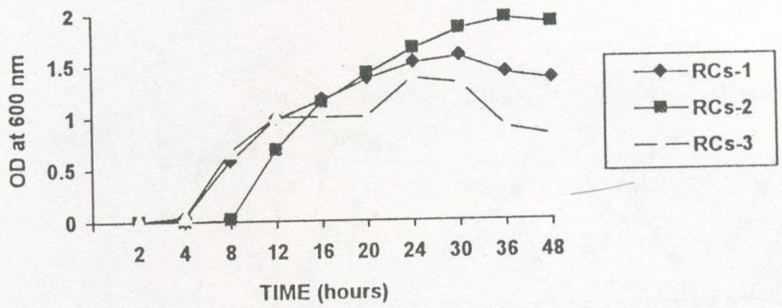


Fig. 3: Growth curves of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*, in L-broth + 1M NaCl at 37°C

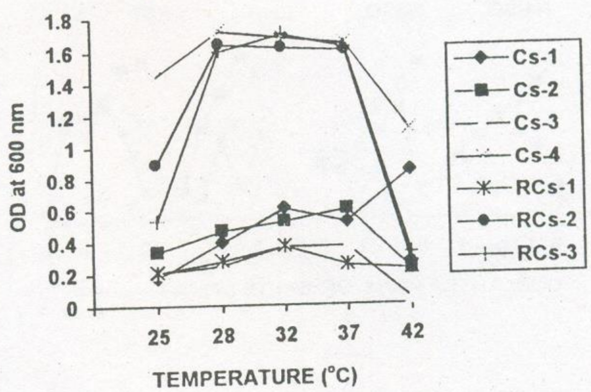


Fig. 4: Effect of different temperatures on the growth of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*.

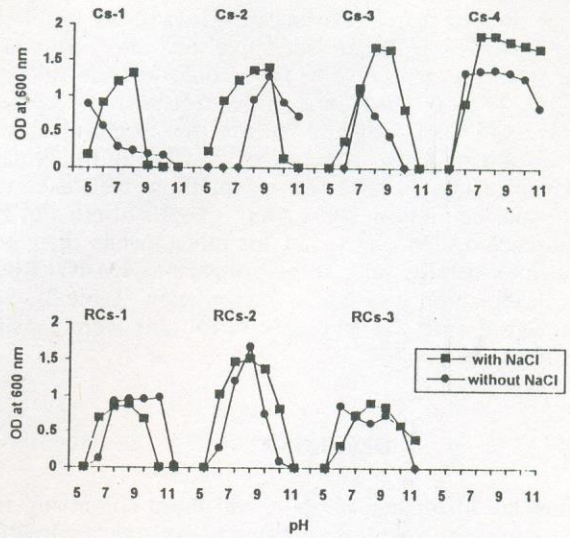


Fig. 5: Effect of pH (in simple as well as 1M NaCl supplemented L-broth) on the growth of moderately halophilic bacterial strains, obtained from *Convolvulus arvensis*, at 37°C after 24 hours of growth.

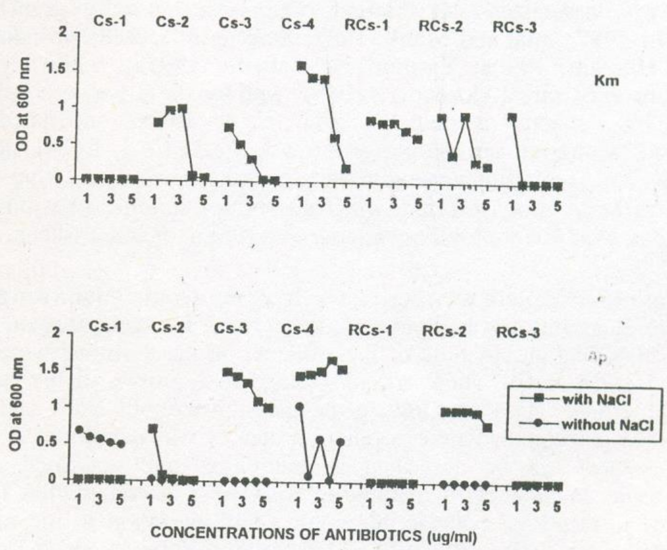


Fig. 6: Resistance of moderately halophilic bacterial strains, obtained from *Convolvulus arvensis*, against different concentrations of kanamycin at 37°C after 24 hours of growth.

Excluding Cs-1 all isolates shared resistance to Km (10-50 $\mu g\ ml^{-1}$) in the presence of 1M NaCl, but were sensitive to Km in NaCl free medium. For ampicillin resistance behaviour of different strains varied (Fig.6). Cs-1 could tolerate Ap (100-500 $\mu g\ ml^{-1}$) in the absence of salt, Cs-2, Cs-3 and RCs-2 confer resistance to Ap in the presence of 1M NaCl, while Cs-4 could resist Ap both in the presence and absence of salt, but resistance was better exhibited in the presence of NaCl (Fig.6). None of them could resist to Tc, Sm and Cm. These isolates showed multiple resistance to salts of heavy metals (Table 2). All strains could bear 100 $\mu g\ ml^{-1}$ of salts of Ni, Pb, Fe, Mo, Co both in the presence and absence of 1M NaCl, but for other metals their behaviour varied, they conferred resistance to metallic salts in the presence of 1M NaCl but were sensitive to some metallic salts in the absence of NaCl or vice versa (Table 2). Some strains *i.e.*, Cs-1, Cs-4 (for Zn), RCs-2 (for Cr) and RCs-3 (for Sn) were sensitive both in the presence and absence of NaCl.

DISCUSSION

Bacteria inhabiting the histoplane of roots and those colonizing in the rhizoplane encounter diverse environmental conditions. Different ecological conditions might affect bacterial population, their growth behaviour, biochemical and genetic attributes. Hence seven bacterial isolates were obtained from the histoplane (Cs-1, Cs-2, Cs-3, Cs-4) and rhizoplane (RCs-1, RCs-2, RCs-3), of *Convolvulus arvensis* from saline locality and were studied. Isolates belonging to two sources were slightly different from one another. All of them were gram-negative rods (except Cs-2 from histoplane). Many workers have reported that root associated salt tolerant bacteria are usually gram-negative rods (Reinhold *et al.*, 1987; Bilal and Malik, 1987; Zafer *et al.*, 1988; Hasnain *et al.*, 1989; Sherwani and Hasnain, 1990a; Yasmin and Hasnain, 1993a), while cocci and gram-positive rods are very rare (Alexender, 1985). Mainly these bacteria belonged to two main groups *i.e.*, strictly aerobic bacteria (Cs-4, RCs-2, affiliated with genus *Halomonas*) and facultative aerobic bacteria (Cs-1, Cs-2, Cs-3, RCs-1, RCs-3). Gram-negative aerobic and facultative anaerobic bacteria are also reported from the rhizoplane and histoplane of *Desmostachya bipinnata, Heleochloa schnoites* (Yasmin and Hasnain, 1993a) and *Leptochloa fusca, Atriplex rhogodoides* (Hasnain and Taskeen, 1989).

In juxtapose to halotolerant bacteria, which do not require NaCl for growth but can grow under saline conditions, halophiles require NaCl for their growth. To determine the halophilic/ halotolerant attribute of the isolates, bacterial strains were grown in the presence of 0.5-3.0M NaCl. These strains yielded best growth in the range 0.5-1.5M NaCl. Bacterial growth yield was low in the medium without NaCl and was declined above 1.0M NaCl (except for where population density was maximum at 1.5M). Hence the bacterial isolates can be described as halophiles and classified as moderately halophilic bacteria. In moderately halophilic eubacteria haloadaptation is required via accumulation of intracellular compataible solutes and alteration in the membrane lipid composition (Csonka, 1989; Russell, 1989). Aerobic moderate halophiles usually cumulate amino acids (*e.g.*, glutamate and proline) and related compounds such as glycine betaine as their major compatible solutes, when grown on complex media (Imhoff, 1986; Wohlfarth *et al.*, 1990; Severin *et al.*, 1992). On contrary, when cultures are grown on defined media the major compataible solutes are commonly the tetra-hydropyrimidines, ectoine and hydroxy ectoine, which are made by *de novo* biosynthesis process (Bernard *et al.*, 1993). Talibert *et al.* (1994) reported that ectoine is not involved in reiteration of osmotic balance of cells, rather its role is in triggering the synthesis of endogenous osmolytes. The most commonly observed salinity-dependent change in lipid composition of this group of eubacteria is an increase in the proportion of anionic phospholipids (Russell, 1992; Kuchta and Russell, 1994). At higher concentration of NaCl (2.0-3.0M NaCl), growth rate of the halophilic bacteria decreased and cell yield was reduced to 90%. Drastic decrease in cell population at higher osmolarity is also reported by Barnard *et al.* (1993). They attributed abrupt reduction in cell density to decreased ectoine content in the cells. *de novo* ectoine synthesis depends on the external osmolarity and from 1.0-2.0M NaCl the ectoine content decreased, which caused decrease in self-osmoprotection of bacteria.

In saline environment salts other than NaCl are also present and different cations and anions such as Ca^{++}, Mg^{++}, K^{+}, SO_4^{-}, HCO_3^{-}, NO_3^{-} are also important. Hence bacteria were also exposed to the medium containing $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl. Their individual effects as well as combined effects with 1M NaCl revealed that $NaHCO_3^{-}$ was the most toxic salt and potassium salts were least toxic (Fig.2a, 2b), but the resistance pattern of individual isolates differed. Some isolates were more sensitive to single salt as compared to when that salt was present with 1M NaCl, whereas others showed the opposite behaviour. This may be due to the involvement of more than one osmoregulatory mechanisms (Yasmin and Hasnain, 1993b). Requirement of Na^{+} for halophilic microorganisms is indispensable, but it may be replaced with Mg^{++} or Ca^{++}, although Na^{+} with either Mg^{++} and Ca^{++} is necessary for the most rapid growth (Sheikh, 1993), but Bernard *et al.* (1993) observed that replacement of NaCl with other ionic salts did not change the growth pattern of bacteria. Among the sodium salts Cl^{-} was preferred by all strains and with HCO_3^{-} growth was relatively inhibited. Na^{+} and Cl^{-} might be helping in keeping the osmotic balance of the cell as compared with other cations and anions. We have also observed similar responses of Pseudomonad strains to different salts (Yasmin and Hasnain, 1993b). In mesophilic halophilic bacteria Na^{+} is needed for a number of complex and crucial cellular function such as permease systems involved in uptake of exogenous substrates (Fein and Macleod, 1975), preservation of solutes within the cells (Wong *et al.*, 1969) and the perpetuation of cell wall integrity (Forsberg *et al.*, 1970). In mesophilic halophic Na^{+} has also been found to stimulate the activities of amylase (Onishi, 1972) and cytochrome oxidase (Kushwaha *et al.*, 1977). Whether Na^{+} have any of these functions in the strains described here has to be investigated. All strains could grow in the presence of high level of salts of K and Mg in the absence of NaCl. Hence the requirement for Na^{+} could be satisfied by K^{+} or Mg^{++}, indicating that requirement for Na^{+} is an osmotic function. Shieh (1993) reported a halophilic thermophilic bacterium in which requirement for Na^{+} could be replaced by Mg^{++} or Ca^{++}.

All the isolates reported here were mesophilic in nature with different optima (Fig.4). Generally halophilic and halo-tolerant bacteria are mesophilic in nature (Sheih, 1993; Yasmin and Hasnain, 1993a, b). They have a wide pH-range and might be placed in alkaliphilic bacteria, as they could grow well at pH 10/11, with optimum pH 8 or 9 in the presence of NaCl (Fig.5). Growth of most isolates was better in the pH medium supplemented with NaCl as compared to the medium when NaCl was absent. An increase in Na^+ concentration allowed the cells to grow at alkaline pHout (Nakamura *et al.*, 1992). When pH of medium was checked at the end of experiment, a rise in the pH of the medium initially adjusted to pH 6-8 and a decrease in the pH of highly alkaline media was recorded. Previously moderately halophilic gram-negative rods which prefer alkaline pH have been described (Del-Moral *et al.*, 1988; Yasmin and Hasnain, 1993a, b). Ni *et al.* (1994) described a moderately halophilic and alkaliphilic methanogen, grew over a wide pH range from 6.8-9.0. Cells suspended in medium with a pH above 8.2 reversed their transmembrane pH gradient by making their cytosol more acidic then the medium.

Antibiotic profile of the mesophilic halophilic strains revealed that they confer resistance to ampicillin and kanamycin, but resistance pattern for these antibiotics was different for different strains (Fig.6). They also showed multiple resistances to a number of heavy metal salts (Table 2). Some strains could resist some metals only in the presence of 1M NaCl or vice versa. Cd toxicity was reported to be decreased (Onishi *et al.*, 1984), while Zn toxicity increased (Babich and Stozky, 1978) with the increase in NaCl level, but our observation for Zn was of two types, Cs-2 (from histolane) was resistant to Zn in the absence of 1M NaCl, while RCs-2, RCs-3 (both from rhizoplane) confer resistance to Zn in the presence of 1M NaCl. Hence bacterial isolates from different sources behaved differently. Many workers reported moderately halophilic/ salt tolerant bacteria having multiple resistances to antibiotics and heavy metals (Nieto *et al.*, 1989; Del-Moral *et al.*, 1988; Hasnain and Yasmin, 1991; Yasmin and Hasnain, 1993a, b). In many cases these resistances were plasmid encoded, in present case isolates harbor plasmid, whether these resistances are plasmid borne is still to be determined.

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