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Salt stress Tolerance of Methylotrophic Bacteria *Methylophilus* sp. and *Methylobacterium* sp. Isolated from Coal Mine Spoils

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Abstract

Two methylotrophic strains of Bina coalmine spoil BNV7b and BRV25 were identified based on physiological traits and 16S rDNA sequence as *Methylophilus* and *Methylobacterium* species. The strains exhibited similar carbon utilization but differed in N utilization and their response to the metabolic inhibitors. *Methylophilus* sp. was less tolerant to salt stress and it viability declined to one tenth within 4 h of incubation in 2M NaCl due to membrane damage and leakage of the intracellular electrolytes as evident from malondiaaldehyde (MDA) assay. In 200 mM NaCl, they exhibited increased superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activity while in 500 mM NaCl, enzyme activities declined in *Methylophilus* sp. and increased in *Methylobacterium* sp. Among exogenously applied osmoprotectants proline was most efficient; however, polyols (mannitol, sorbitol and glycerol) also supported growth under lethal NaCl concentration.

Key words: *Methylophilus* sp., *Methylobacterium* sp., salt stress tolerance

Introduction

Approximately 397 m.h.a. (million hectares) unproductive saline soils are required to be reclaimed at the global level (FAO, 2006). In saline soil, agricultural productivity is affected due to excessive ions concentration which interferes with plant water uptake leading to physiological and metabolic dysfunctions, Available plant nutrients also get adversely affected since salinity influences microbe mediated soil C and N mineralization processes as excessive salt inhibits microbial growth mainly due to ion toxicity (Pathak and Rao, 1998). Under osmotic stress, reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) are generated in microbes causing oxidative damage of their various cellular components and biomolecules (Halliwell and Gutteridge, 1986). To cope with ROS caused injury, enzyme super oxide dismutase (SOD) gets induced and catalyzes dismutation of O_2 to H_2O_2 which is further scavenged by catalase (CAT), ascorbate peroxidase (APX) and other enzymes (Gómez *et al.*, 2004). The other adaptive processes developed by microbes to cope with salinity stress include accumulation of protective chemical substances such as polyamines, polysaccharides, amino acids and its derivatives, other compatible solutes including proline, serine, glutamate, betaine, ectoine and inorganic cations (K^+) that protect bacterial cells from salt stress (Robert *et al.*, 2000; Shamseldin *et al.*, 2006).

Coal mine spoils are characterized by low nutrients and high toxic heavy metals load along with the salinity. Such spoils harbor salt tolerant Gram-positive (*Bacillus*, *Arthrobacter* and *Corynebacterium*) and Gram-negative (*Pseudomonas*, *Alcaligenes*, *Ralstonia*, and *Burkholderia)* bacterial genera as the dominant microflora (Ellis *et al.*, 2003). Recently, stress tolerant methylotrophic bacterial genera have been reported from such desiccated environment (Giri *et al.*, 2012) that survive by utilizing trace amounts of nutrients and a wide variety of organic compounds for their carbon energy source (Pasamba *et al.*, 2007). These methylotrophs play an indispensable role in the ecosystem by active participation in various biogeochemical cycles.

The methylotrophic bacterial genera *Methylobacterium* sp. and *Methylophilus* sp. had earlier been employed in metal tolerance, plant growth promotion, plant disease resistance, and soil fertility enhancement

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(Madhaiyan *et al.*, 2001; Sy *et al.*, 2001; Ivanova *et al.*, 2007). These genera have also been used in industrial production of amino acids, polyhydroxy alkanoates, carotenoids and in bioremediation (Yellore, 1998; Fournier *et al.*, 2005). However, these genera have not been studied in relation to their physiological and biochemical response to salt stress and till now. The knowledge of their salt stress responses could be applied in innovative designing of gene expression systems to augment the microbe mediated soil bioremediation, industrial application as well as in saline soil reclamation. In the present study the two bacterial isolates of coal mine spoil BNV-7b (*Methylophilus* sp.*)* and BRV-25 (*Methylobacterium* sp.) were tested for their physiological and biochemical responses to lethal salt concentrations.

Experimental

Materials and Methods

Study site and soil analyses. Soil samples were collected from a two year old coal mine dumping site of Bina in the Sonbhadra district of Uttar Pradesh (24° 11' N, 82° 38' E), India Soil texture, bulk density and water holding capacity along with the other physiochemical properties like pH, ammonium-N, nitrate-N, organic C and total N were described earlier (Giri *et al.*, 2012). The soil cations (Na^+, K^+ and Ca^{+2}) were extracted in ammonium acetate solution by repeated leaching (Simard, 1993), and their concentrations were determined by Atomic Absorption Spectrophotometer (Perkin-Elmer-167, USA).

Bacterial isolation and identification. From the above mentioned soil, putative methylotrophic bacteria were isolated by plating serially diluted soil suspension (10^{-1} to 10^{-6}) on nitrate mineral salt (NMS) agar containing per litre Na_2HPO_4 0.036 g, KH_2PO_4 0.014 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, $CaCl_2 \cdot 2H_2O$ 0.20 g, $FeSO_4 \cdot 7H_2O$ 0.005 g, and 1 ml of trace elements stock (Na_4EDTA 0.00 gl^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.007 gl^{-1}, $MnCl_2 \cdot 4H_2O$ 0.030 gl^{-1}, H_3BO_3 0.03 gl^{-1}, $CoCl_2 \cdot 6H_2O$ 0.20 gl^{-1}, $CuCl_2 \cdot 2H_2O$ 0.001 gl^{-1}) supplemented with 0.5% methanol under standard microbiological conditions (Hanson, 1998). The bacterial isolates were maintained in bacteriological incubator (30°C) on NMS agar slant and broth. Exponentially growing bacterial culture (0.1 ml) was inoculated in culture tubes containing 9.9 ml NMS medium with a specific salt concentration and growth was measured in terms of optical density(OD_{600}) at 72 h intervals for 12 days using spectrophotometer (Systronics-117, India). The effect of methanol (0.001 to 10%) as the C source was checked at the same inoculation period.

Bacterial isolates were taxonomically characterized by amplifying, sequencing and analyzing the 16S r RNA as described earlier (Giri et al., 2012). A phylogenetic tree was constructed using MEGA 4.1 program with the neighbor-joining (N-J) method based on a matrix of distances.

Effect of NaCl on growth. Log phase methylotrophic strains (A_{600} = 0.5 to 0.6) were inoculated into 50 ml of fresh NMS medium containing NaCl (100, 200 and 500 mM) and specific growth rate (μ day^{-1}) was calculated on the basis of (OD_{600}) at various time intervals following Myers and Kratz (1955) using the formula

$$\mu = \{2.303(\log N_2 - \log N_1)\}/T_2 - T_1,$$

Where N_1 and N_2 are the $OD_{600,}$ at time T_1 and T_2, respectively.

Estimation of Na^+. Intracellular Na^+ was estimated in cell biomass grown for 4 h in 200 mM and 500 mM NaCl following Li and El-Mallakh (2004). The time course of Na^+ uptake was studied only in culture medium with 200 mM NaCl. Samples were withdrawn at regular time intervals and cell biomasses were harvested. Cell pellets were washed three times with isotonic $MgCl_2$ solution (100 mM) and lysed overnight with 30% HNO_3. Na^+ was measured by using flame atomic absorption spectrophotometer (Perkin-Elmer-167, USA) with lamp current (8 mA), wavelength (589.0 nm), and slit width (0.4 nm). All the samples were prepared in Milli 'Q' water and various NaCl concentrations (0.1, 0.5, and 1 μM) were used for preparation of standard curve.

Assay of antioxidative enzymes. To study the salt induced antioxidative enzymatic activity in the bacteria, cell homogenate was extracted in 50 mM phosphate buffer (pH 7.0), containing 1 mM EDTA, Triton X-100 (0.05%), 2% (w/v) polyvinyl pyrrolidone (PVP) and 1 mM ascorbic acid. The SOD activity was measured as inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm in a 3 ml reaction mixture followed by illumination under a fluorescent lamp for 15 min (Beauchamp and Fridovich, 1971). An illuminated blank without cell free extract, gave the maximum reduction of NBT on the basis of OD_{560}. Further, an unilluminated blank without cell free extract was used as the reference. SOD activity was expressed as U μg $protein^{-1}$.

Catalase (CAT) activity was measured following the method described by Clairbone (1985). For its estimation, decrease in H_2O_2 absorbance was measured by scanning the 2 ml assay mixture at 240 nM for one minute. The extinction coefficient for catalase was 0.039 mM cm^{-1} and the activity was calculated as mol min^{-1} g $protein^{-1}$.

APX activity was measured as the decrease in absorbance of H_2O_2 at 290 nM as described by Asada (1984). The 1.2 ml reaction mixture was scanned for one minute in spectrophotometer. The extinction coef-

ficient was 2.8 mM cm^{-1} for APX and the activity was presented as μmol min^{-1} μg $protein^{-1}$.

Peroxide assay. Peroxide content was measured following Sagisaka (1976). In brief, bacterial cell were harvested, suspended in cell lysis buffer and sonicated for 15 minutes followed by addition of TCA (20%). Resulting suspension was centrifuged and supernatant (1.6 ml) was added into reaction mixture (0.4 ml of 50% TCA, 0.4 ml of 10 mM ferrous ammonium sulfate and 0.2 ml of 2.5 M potassium thiocyanate), centrifuged and the OD_{480} of supernatant was compared with standard curve for measuring the concentration of H_2O_2.

Lipid peroxidation. Oxidative damage of lipid was estimated indirectly by measuring the total content of 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalent of MDA (malondialdehyde) as described by Cakmak and Horst (1991). In brief, bacterial culture (1.0 ml) was mixed with 1.0 ml of 20% (w/v) trichloroacetic acid (TCA) centrifuged(10,000 × g, for 10 min at 4°C) and supernatant was suspended in 0.5 ml of Phosphate buffer saline followed by the addition of TBA (1.0 ml of 0.67%) and incubated at 90°C (20 min). The reaction stopped in ice-bath and OD_{532} was compared using culture medium as the blank. MDA concentration was calculated based on a standard curve of 1, 1, 2, 3-tetramethoxypropane.

Electrolyte leakage. Electrolyte leakage was measured as described by Lutts *et al.* (1996). Cell pellets of bacteria grown in 200 and 500 mM NaCl were washed thrice with deionized water to remove the surface-bound electrolyte, suspended in 10 ml of Milli 'Q' water and incubated on shaker (120 × g, 30°C, 24 h). Electric conductivity of the suspension solution was determined before (EC_1) and after (EC_2) steam sterilization at 120°C for 20 minutes using conductivity meter (Systronics, India). Electrolyte leakage is defined as:

$$\text{Electrolyte leakage (\%)} = (EC_1 / EC_2) \times 100.$$

Organic solute leakage. Carbohydrate content was determined by Yemm and Willis (1954). Alcoholic extract of cell pellet (0.1 ml) mixed with 3 ml freshly prepared anthrone reagent (150 mg anthrone and 100 ml 72% H_2SO_4), placed in a boiling water bath for 10 min reaction was terminated on ice, and OD_{625} was compared with D-Glucose standard curve for concentration measurement of carbohydrate. Total amino acid content was estimated by Moore and Stein (1948) and total protein content by Bradford (1976). To estimate carotenoids content, a known volume of bacterial culture was centrifuged and the pellet was incubated overnight in 5ml acetone (80%v/v) at 4°C, followed by centrifugation and OD_{480} of supernatant to calculate specific absorption coefficient (Myers and Kratz, 1955). Total RNA content was estimated following the modified orcinol method (Kamali and Manhouri, 1969).

Tetrazolium assay for cell viability. Cell viability against elevated NaCl concentration was tested by TTC assay (Maness *et al.*, 1999) with some modifications. The exponentially growing cells were harvested by centrifugation (10,000 × g, 10 min, 4°C) and pellet were suspended in 5 ml phosphate buffer (pH 7.0) containing 0.12% TTC and 1.2% sodium succinate. The pellet in phosphate buffer without TTC and sodium succinate served as a control. The reaction mixture was incubated in the dark (37°C, 4 h). The red formazan formed was extracted in 1ml acetone and absorbance was measured at 490 nm.

Effect of osmoprotectants. Bacterial cells were inoculated in various flasks containing 50 ml NMS medium having 500 mM NaCl to study the effect of different osmoprotectants (glycine, betaine, sucrose, proline, mannitol, glycerol and sorbitol) and each osmoprotectant was added at its final concentration of 100 mM. Osmoprotective potential of osmolyte was expressed in terms of specific growth rate of the bacterial (**something missing**).

Chemicals. All the chemicals were of reagent grade from Sigma-Aldrich (USA) or Merck India Ltd. They were filter sterilized through Millipore filter when required (Millipore, India) before use (data not shown).

Results

Soil properties. The coal mine spoil at the study site was sandy loam, with low water holding capacity, soil organic C and t otal N. The soil sample had high levels of exchangeable Na^+ 0.43 mgg^{-1}, K^+ 0.16 and Ca^{++} 43.1mgg^{-1}.

Bacterial identification. The selected bacterial strains were pink coloured, round shaped and convex elevated colonies and differed only in size. The colonies of strain BNV7b were large (< 1 mm) compared to BRV25 (0.5–1 mm) on NMS agar medium. The selected strains were similar in their response to urease, catalase, oxidase, indole and H_2S production. The details of the phenotypic and biochemical characteristics of these strains have been described elsewhere (Giri *et al.*, 2012). The two strains differed with respect to their organic substrate utilization as *Methylophilus* BNV7b did not utilize formate and *Methylobacterium* BRV25 did not utilize citrate as C source. The former strain did not utilize ammonium acetate, aspartate, cysteine and glutamate as the N substrate and later strain was unable to utilize alanine, aspartate, glutamate, glycine, isoleucine, lysine, proline and sodium nitrate as N source. The growth rate of the two strains was fast in KNO_3 and arginine supplemented NMS medium. Increase in methanol concentration from 0.1% to 0.5% resulted in enhanced the growth; however, a further increase was

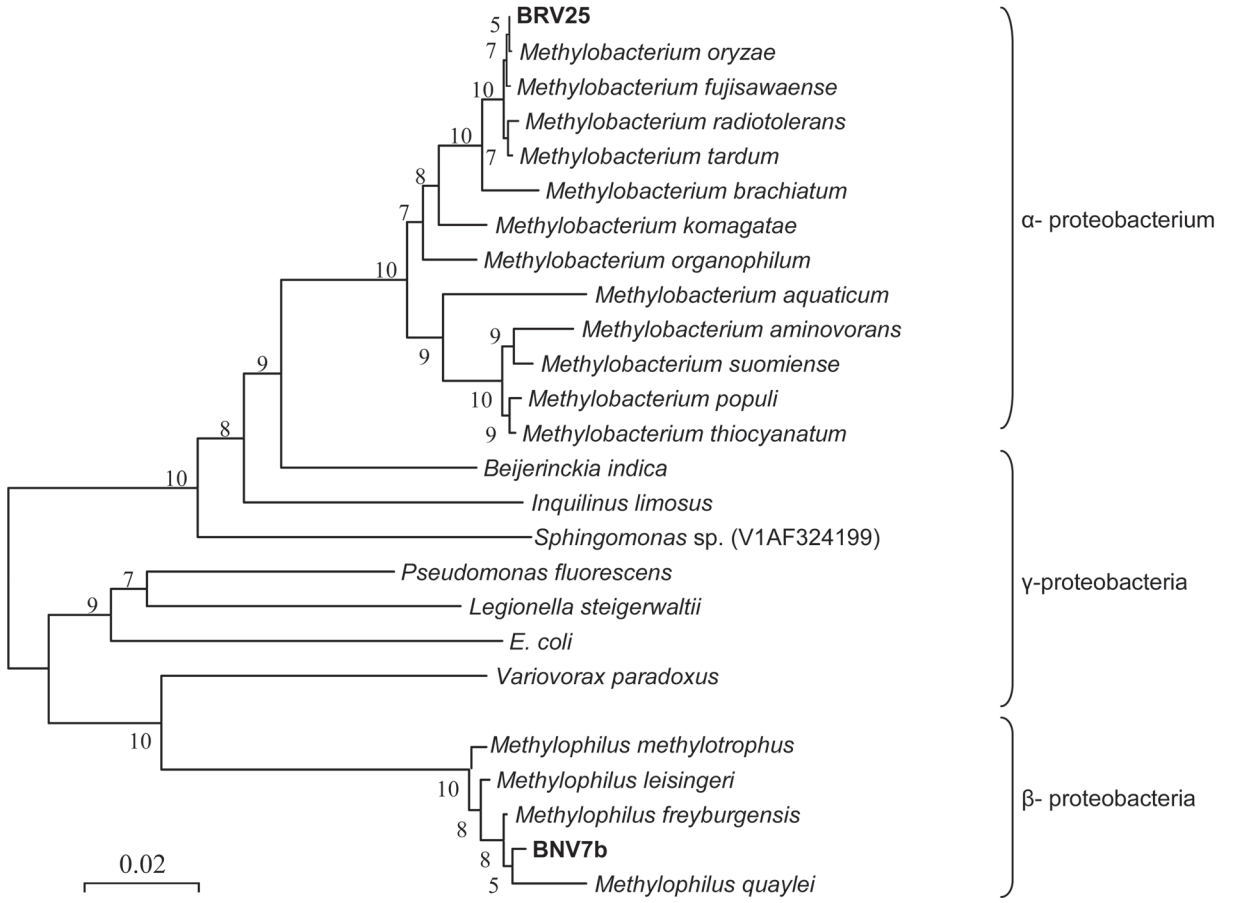

Fig. 1. Neighbor-joining phylogenetic tree showing the relationship between 16S rRNA gene sequences of methylotrophic strains isolated from coalmine spoil and their closest relative sequences retrieved from Gene bank database.

inhibitory. Both the strains tolerated SDS (2%), but only *Methylobacterium* tolerated crystal violet (0.01%) and NaN_3 (0.02%).

Based on 16S r RNA gene sequence, strains BNV7b revealed 98% similarity with *Methylophilus freyburgensis* and *Methylophilus. quaylei* whereas BRV25 formed a monophyletic clade having 99% similarity with *Methylobacter oryzae* which exhibited close relationship with *Methylobacter fuisawanse* and *Methylobacter radiotolerans*. Further, on their morphology, growth characteristics and biochemical tests revealed that strains BNV7b and BRV25 were the member of genera *Methylophilus* and *Methylobacterium*, respectively (Fig. 1).

Effect of NaCl on bacterial growth. In *Methylobacterium* sp. specific growth rate declined nearly 17% in 100 mM NaCl whereas this decline was as high as 75% in 500 mM NaCl. In case of *Methylophilus* sp. there was no apparent change in specific growth in 100 mM NaCl, but a decrease was nearly 16% and 86% in 200 mM and 500 mM NaCl, respectively (Fig. 2).

Na^+ uptake. Bacterial strains were treated with 200 mM Na^+ and intracellular Na^+ estimated at 2 h interval till 6 h exhibited exponential and linear increasein *Methylophilus* and *Methylobacterium* sp., respectively (Fig. 3).

Electrolyte and organic solute leakage. In *Methylophilus*, electrolyte leakage was intense in 200 mM NaCl and marginal in 500 mM NaCl. In contrast, there was concentration-dependent electrolyte leakage in *Methylobacterium* sp. A significant decrease in the cellular solute in both the bacterial sp. was observed though decrease was more in *Methylophilus* sp. The high level

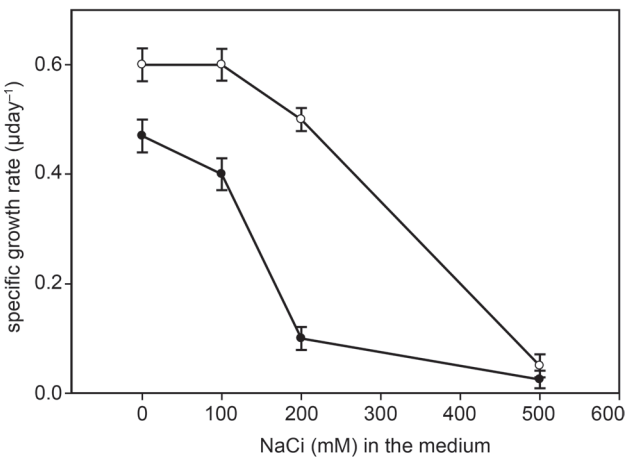

Fig. 2. Specific growth rate (μ day $^{-1}$) of *Methylophilus* sp. BNV7b (–●–) and *Methylobacterium* sp. BRV25 (–○–) with NaCl concentrations in the medium. Data are mean ± 1SE of three replicates.

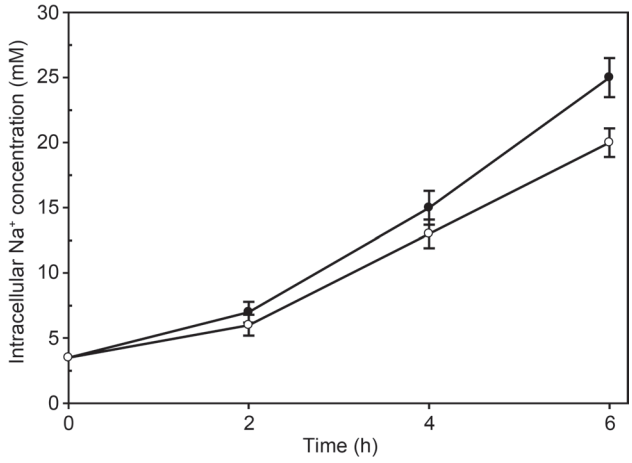

Fig. 3. Increase in intracellular Na^+ concentration content (a) and decrease in relative water content (b) of *Methylophilus* sp. BNV7b (–●–) and *Methylobacterium* sp. BRV25 (–○–) in 200 mM NaCl in the medium. Data are mean ± 1SE of three replicates.

Antioxidative enzymes. The CAT activity of *Methylobacterium* sp. and *Methylophilus* sp. was almost same in control where as exhibited slight activation and suppression in the respective strains at 200 mM salt, but its significant reverse result was observed in 500 mM salt (Fig. 4a)

APX activity was less than half in *Methylobacterium* sp. compared to *Methylophilus* sp. in control. APX activity was nearly equal to control in 200 Mm and increased three times in 500 mM NaCl in *Methylobacterium* sp. whereas in *Methylobacterium* sp. APX activity increased 1.4 times in 200 mM and decreased to one third in 500 mM salt compared to the control. (Fig. 4b)

In *Methylobacterium* SOD activity increased linearly up to 500 mM NaCl where as in *Methylophilus* sp. activity increased in 200 mM and slightly decreased in 500 mM compared to the control (Fig. 4c).

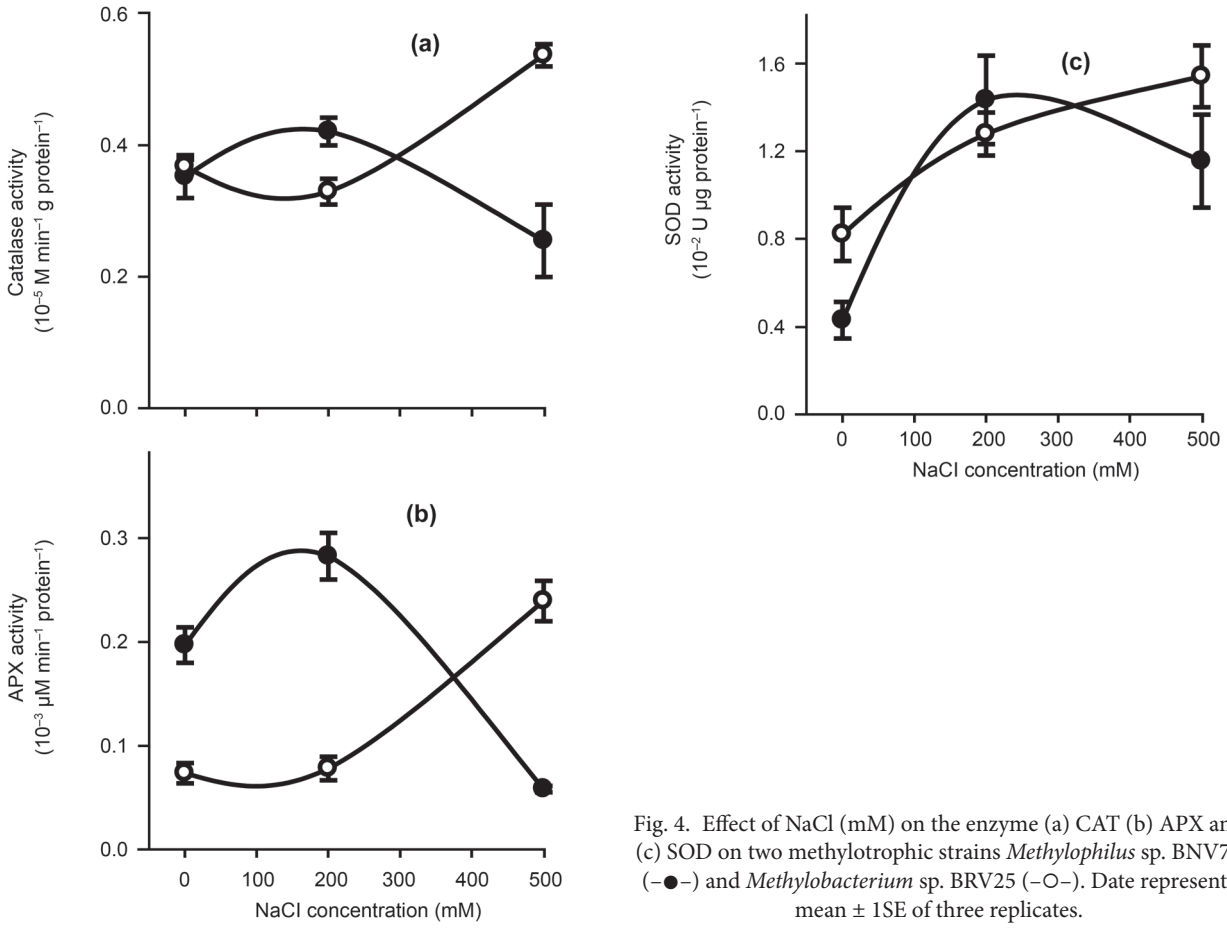

Fig. 4. Effect of NaCl (mM) on the enzyme (a) CAT (b) APX and (c) SOD on two methylotrophic strains *Methylophilus* sp. BNV7b (–●–) and *Methylobacterium* sp. BRV25 (–○–). Date represents mean ± 1SE of three replicates.

of leakage was noticed for amino acids, carbohydrate and carotenoids, while low leakage of RNA was noticed (data not shown).

Peroxide and malondialdehyde production. In 200 mM NaCl, peroxide and malondialdehyde (MDA) production were also significantly higher in *Methylophilus* sp. as compared to *Methylobacterium* sp.

Cell viability. Cell viability remained nearly equal in 1.5 M NaCl and TTC reduction was recorded even in 2 M NaCl in both the strains (Fig. 5.)

Effect of osmoprotectants. Among the various osmoprotectants used proline was the most effective and betaine was the least effective in maintaining the cell viability in selected methylotrophs. Exogenous

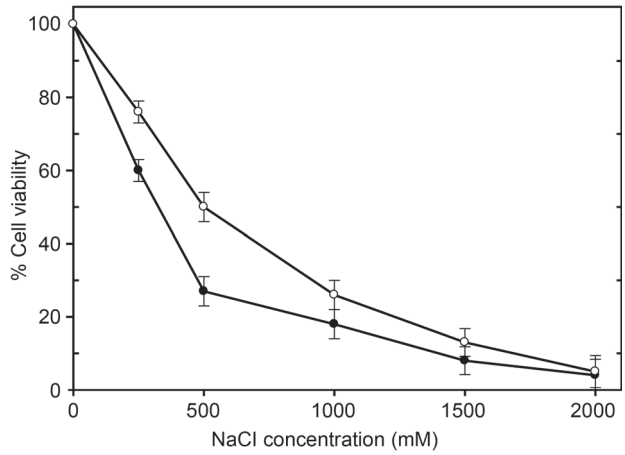

Fig. 5. Decrease in cellular viability (%) of *Methylophilus* sp. BNV7b (–●–) and *Methylobacterium* sp. BRV25 (–○–) with increase in NaCl concentrations in the medium. Data are mean ±1 SE of three replicates.

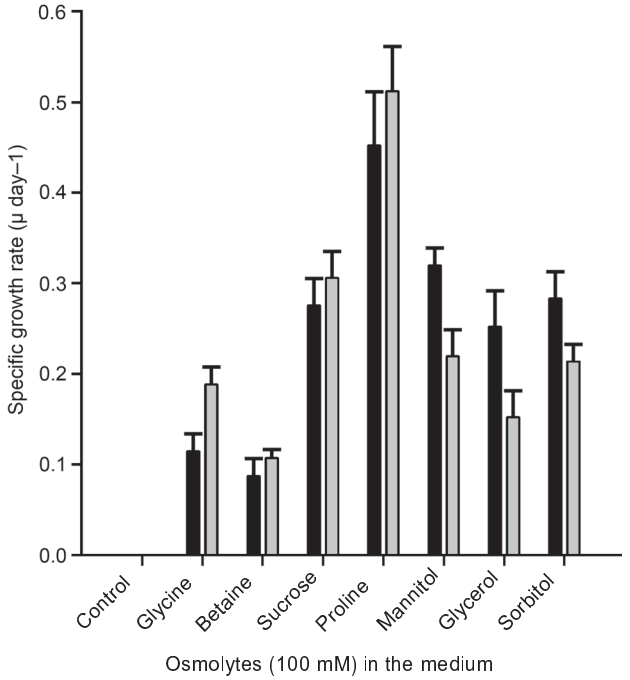

Fig. 6. Specific growth rate of methylotrophs, *Methylophilus* sp. BNV7b (Black bars) and *Methylobacterium* sp. BRV25 (light shaded bars), incubated with 100 mM concentration of various osmoprotectants and 0.5 M NaCl. Data represented as mean ±1SE of three replicates.

betaine, glycine and proline were more effective in *Methylobacterium* sp., while glycerol, mannitol and sorbitol were effective in *Methylophilus* sp. (Fig. 6).

Discussion

The two selected methylotrophs, *Methylophilus* sp. and *Methylobacterium* sp. utilized a number of organic C compounds as the carbon source, differed with respect to inorganic and organic N utilization. *Methylophilus* utilized the combined inorganic N sources and amino acids whereas *Methylobacterium* relied mostly on inorganic N. These bacteria exhibited similarity in their lysis tolerance and differed in their response to chemical inhibitors. *Methylobacterium* sp. tolerated respiration inhibiting compounds azide and cell division inhibiting crystal violet (Walker *et al.*, 1971), while *Methylophilus* was sensitive to both of them.

We observed up to 20% and 40% decreased relative water content in *Methylobacterium* and *Methylophilous* sp. respectively when grown in 200 mM NaCl. Ronsch and coworkers (2003) grew *Corynebacterium glutamic* to study the influence of osmotic stress, when the cells grew in constant osmotic stress, the maximal cytoplasmic volume decreased significantly with increase in external osmolalities. Further, under severe osmotic stresses maximum cytoplasmic volume were lower and required long time period to attain the maximal cytoplasmic volume of non stressed cells. The critical threshold of NaCl was 400 mM and concentration above it inactivated most of the enzymes due to perturbation of hydrophobic-electrostatic interaction necessary for protein integrity (Wyn Jones and Pollared, 1983). The decreased anti-oxidative enzyme activity of *Methylophilous* sp. in 500 mM NaCl can be explained in the same terms. Again, enzyme inactivation under salt stress depends upon bacterial capabilities of ions transport across membrane. *Methylobacterium* sp. was more efficient in managing influx and efflux of Na^+; the apparent increased Na^+ accumulation in *Methylophilus* sp. was due to its failed regulation of Na^+. When the methylotrophic bacteria were treated with 200 mM NaCl, such sudden hyperosmotic shift in the laboratory condition obviously did not resemble the conditions in their natural growth habitat. In suddenly altered salt in the environment the microbes immediately started to accumulate different ions from the environment but in long term prevailing salt stress they accumulated osmoprotectants available in the environment or synthesized them de novo inside the cell.

The methylotrophic bacteria *Methylobacter* sp. and *Methylophilous* sp. differed in their preferred antioxidant for growth revival under deleterious salt. The intracellular level of solutes was increased to regulate the cellular osmotic balance, but in extreme extracellular salt led to damage the cell wall resulting in drastic leakage of solutes (Korber *et al.*, 1996). Electrolyte and organic solute leakage were more prominent in 500 mM NaCl due to membrane instability and lipid peroxidation in the methylotrophs (Table I).

All microorganisms have their own genetic capabilities to regulate various enzymes under stress condition. *Methylobacterium* sp. and *Methylophilous* sp. variably expressed three anti-oxidative enzymes SOD, CAT and

Table I
Influence of NaCl concentration on leakage of electrolyte and various cellular organic solutes

	Methylophilous sp.			*Methylobacterium* sp.		
NaCl (mM)	0	200	500	0	200	500
Electrolyte leakage (%)	1.5 ± 0.1	2.8 ± 0.1	3.3 ± 0.1	0.8 ± 0.1	1.6 ± 0.2	2.2 ± 0.2
Cellular water (%)	98	65	54	97	88	72
Carbohydrate released (%)	8.2 ± 1.1	12.1 ± 1.2	32.3 ± 1.4	5.1 ± 0.9	6.2 ± 1.0	15.1 ± 1.1
Protein released (%)	0.1 ± 0.01	5 ± 0.3	10.5 ± 0.6	0.08 ± .01	3 ± 0.1	5.9 ± 0.6
Amino acid released (%)	4.1 ± 0.1	10.2 ± 0.4	38.3 ± 0.5	3.1 ± 0.1	8.1 ± 0.5	32.0 ± 0.5
Carotanoids released (%)	3.3 ± 0.3	10.3 ± 0.8	38 ± 0.5	5.2 ± 0.4	8 .3 ± 0.4	2.3 ± 0.2
RNA released (%)	0.33 ± 0.10	1.34 ± 0.20	2.54 ± 0.30	0.25 ± 0.10	0.63± 0.22	1.71 ±0.22

APX in the absence of salt, and in 200 mM and 500 mM NaCl. *Methlophilus* sp. the activity of two antioxidative enzymes APX and CAT was increased in 200 mM, and the activity of both enzymes decreased below the control in 500 mM. Only SOD activity remained higher compared to the control in 500 mM. Salt stress induced ROS acts as the signal for programmed cell death in prokaryotic cells (Ning *et al.*, 2002). Oxdative stress induced membrane damage and excessive solute leakage are the primary factors responsible for decreased viability. Plasmolysis caused by salt stress may also result in cell death (Korber *et al.*, 1996). In extreme salt concentration most cells die but a very small proportion of cells has the ability to survive. This cell viability decreased by 95% in *Bacillus subtilis* facing 500 mM NaCl (Ikeuchi *et al.*, 2003).

The compatible solutes have two important functions, first they increase the internal osmolality leading to redirection of water flux to after osmotic upshift and restore growth. The second function of compatible solute is to stabilize the native state of the globular cellular proteins. Exogenous proline and betaine mitigated detrimental effects of Na^+ (Okuma *et al.*, 2000; Harinasut *et al.*, 1988). Proline stimulates cell growth by improving metabolism, reduced oxidation of the lipid membranes and increased the antioxidative enzymes activities (Hoque *et al.*, 2007). Under conditions of low and medium osmolality, the addition of betain did not accelerate growth nor changed the time at which maximum growth rate occurred. But under severe osmotic stress, the addition of betaine shortened the time required to reach the maximum growth (Ronsch *et al.*, 2003). The methylotrophic genera selected in present when supplemented with various compatible solutes at 100 mM concentration in the growth medium containing 500 mM NaCl, specific growth rate was very lowest in betaine, highest in proline and intermediate for glycine and various polyols. This was due to the fact that proline acts as predominant compatible solute independent of constant higher osmolality (Ronsch *et al.*, 2003) or sudden upshift (Guillouet and Engasser, 1996). However, proline accumulation is affected by the presence of other compatible solutes such as betaine. The intracellular proline was as high as 815 mM and 300 mM in the cytoplasm in the absence and presence of betaine, respectively. *Pseudomonas putida* produced mannitol in response to osmotic stress (Kets *et al.*, 1996), though polyols are not a preferred compatible solute in bacteria. The presence of polyols like mannitol, glycerol and sorbitol in the extracellular environment was beneficial for methylotrophs in stress and provided better protection.

Microbial adaptation in a degraded habitat is a long term process and requires perhaps a decade (Wichern *et al.*, 2006). In the heterogeneous habitat of coal mines spoils with multiple stress conditions like low water, high salt and nutrient limiting conditions, salinity tolerant pink pigmented methylotrophic bacteria *Methylophilous* sp. and *Methylobacterium* sp. attained high population size (10^4 to 10^6) in a time interval of four to 12 years (Giri *et al.*, 2012), and are supposed to be adapted to such an environment. The *Methylobacterium* sp. in coal mine spoils could be of phyllospheric origin (Madhaiyan *et al.*, 2001), adapted to stressed coal mine spoils.

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