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ORIGINAL PAPER

Osmotic adaptation in halotolerant yeast, *Debaryomyces nepalensis* NCYC 3413: role of osmolytes and cation transport

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Abstract *Debaryomyces nepalensis* NCYC 3413, a food spoiling yeast isolated from rotten apple, has been previously demonstrated as halotolerant yeast. In the present study, we assessed its growth, change in cell size, and measured the intracellular polyol and cations (Na^+ or K^+) accumulated during growth in the absence and presence of different concentrations of salts (NaCl and KCl). Cells could tolerate 2 M NaCl and KCl in defined medium. Scanning electron microscopic results showed linear decrease in mean cell diameter with increase in medium salinity. Cells accumulated high amounts of K^+ during growth at high concentrations of KCl. However, it accumulated low amounts of Na^+ and high amounts of K^+ when grown in the presence of NaCl. Cells grown in the absence of salt showed rapid influx of Na^+/K^+ on incubation with high salt. On incubation with 2 M KCl, cells grown at 2 M NaCl showed an immediate efflux of Na^+ and rapid uptake of K^+ and vice versa. To withstand the salt stress, osmotic adjustment of intracellular cation was accompanied by intracellular accumulation of polyol (glycerol, arabitol, and sorbitol). Based on our result, we hypothesize that there exists a balanced efflux and synthesis of osmolytes when *D. nepalensis* was exposed to hypoosmotic and hyperosmotic stress conditions, respectively. Our findings suggest that *D. nepalensis* is an Na^+ excluder yeast and it has an efficient transport system for sodium extrusion.

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Introduction

In natural habitat, microorganisms are exposed to different extreme conditions of salt, pH, and temperature. During the course of evolution, certain organisms adapted their physiology to survive and grow in such extreme environments. Organisms which grow in the presence or absence of salt in its environment are said to be halotolerant. The mechanism of halotolerance is mostly conserved in bacteria (e.g. *Brevibacterium epidermis*, *Pseudomonas mendocina*; Roberts 2005), yeast (e.g. *Schizosaccharomyces pombe*, *Zygosaccharomyces rouxii*; Kayingo et al. 2004; van Zyl et al. 1991), and algae (e.g. *Asteromonas gracilis*, *Dunaliella tertiolecta*; Ben-Amotz and Grunwald 1981; Goyal 2007). Among all halotolerant organisms, yeasts receive more attention because they play a major role in food spoilage and storage of food products. Halotolerant yeasts maintain intracellular cell homeostasis by regulating the osmotic pressure across the cell membrane. To accomplish this, they accumulate and export organic solutes (termed as compatible solutes or osmolytes) which include polyols such as glycerol, arabitol, mannitol, sorbitol, and erythritol. This helps to maintain the turgor pressure and cell volume under osmotic stress condition (Bhajekar and Kulkarni 2006).

Kayingo et al. (2001) established glycerol to be a major osmolyte produced by species of yeasts such as *Saccharomyces cerevisiae* and *Zygosaccharomyces cerevisiae* under salt stress condition. Tamas et al. (1999) showed the involvement of a channel protein, Fps1, in *S. cerevisiae*

which facilitates the accumulation and release of osmolyte under hyperosmotic and hypoosmotic conditions. *Debaryomyces hansenii*, a halotolerant food spoiling yeast, showed glycerol to be predominant osmolyte accumulated in log phase of growth whereas arabitol was predominant in stationary phase (Adler and Gustafsson 1980). The intracellular concentration of polyol in *D. hansenii* was shown to be markedly enhanced in the presence of salt and it increased linearly with increase in salinity (Larsson et al. 1990).

Another strategy used by most halophilic and halotolerant organisms to overcome salt stress condition involves regulation of intracellular concentration of cations (Na^+/K^+) abundant in nature. Regulation of cation transport under salt stress condition has been widely studied in some halotolerant yeasts such as *D. hansenii* (Almagro et al. 2001) and *Z. rouxii* (Watanabe et al. 2005). Prista et al. (1997) showed *D. hansenii* to accumulate high amount of Na^+ under salt stress condition without hindering its physiology. In addition, several groups have also reported the involvement of transporters such as Na^+-ATPases (Almagro et al. 2001), Na^+/H^+-antiporters, and H^+-ATPase (Watanabe et al. 2005) in halotolerance. They mediate Na^+/K^+ efflux and influx so as to regulate intracellular accumulation of these ions in the cytoplasm under salt stress condition.

Previously, we isolated *Debaryomyces nepalensis*, a food spoiling yeast isolated from rotten apple (Gummadi and Kumar 2006a). This strain could utilize pectin as the sole source of carbon and produce pectinases (Gummadi and Kumar 2006b). The strain could grow well in the absence of salt and in the presence of high salt concentration. It was reported to tolerate 2 M NaCl and 3 M KCl and growth was enhanced in the presence of 0.5 M NaCl and 1 M KCl. *D. nepalensis* could also grow in medium containing LiCl up to 1 M, which was reported to be toxic to similar food spoilage halotolerant yeast, *D. hansenii*, at all concentration (Gummadi et al. 2007). This strain could also grow in a wide range of pH (3–11) and temperature (8–42°C) (Gummadi and Kumar 2008; Kumar et al. 2008).

The objective of the present study is to understand the mechanism of osmoadaptation of the halotolerant yeast, *D. nepalensis*. We investigate salt tolerance of *D. nepalensis* on defined medium. Osmotic adjustments of sodium and potassium in this halotolerant yeast have been determined at different external salinities in an attempt to understand the physiological basis of halotolerance. It is known from a previous study on *D. hansenii* that osmolytes are released during hypoosmotic shock in proportional to shock intensity. Based on kinetics of osmolyte released, they suggested the involvement of a channel protein (Kayingo et al. 2001). There are no further reports on the osmolyte transport across the halotolerant yeast cells under hypoosmotic and hyperosmotic conditions. These facts prompted us to gain further insight into the characteristics of halotolerant yeast using *D. nepalensis* upon hypoosmotic and hyperosmotic shock. Different osmolytes accumulated by this strain at various salinities and after exposure to hypoosmotic and hyperosmotic stress have been studied. The results obtained in this study revealed the physiological basis underlying the halotolerance of *D. nepalensis*.

Materials and methods

Strain source and maintenance

Debaryomyces nepalensis, non-pathogenic ascomycetous yeast strain used in this study, has been previously isolated in our laboratory from rotten apple and the strain was deposited at National Collection of Yeast Culture (NCYC), Norwich, UK, with accession number 3413. It was maintained and propagated on solid YEPP plates which contained (all values are in g/l) yeast extract (10), peptone (20), pectin (5), and agar (20) incubated at 30°C.

Media and growth condition

For all experiments, cells were first grown on YEPP agar plates incubated at 30°C for 24 h and one loopful of the organism was transferred from the plate to 50 ml YEPD broth in a 250 ml conical flask. YEPD medium contained (all values are in g/l) yeast extract (10), peptone (20), and glucose (20). After 12 h of incubation at 30°C and 180 rpm, 3% (v/v) inoculum was aseptically transferred to 250 ml Erlenmeyer flasks containing 50 ml of YEPD/defined media. The salinity of the medium was adjusted with various concentration of NaCl and KCl. Chemically defined minimal medium contained (all values are in g/l): glucose (10), $(NH_4)_2SO_4$ (3), $MgSO_4$ (0.1), K_2HPO_4 (3), and Na_2HPO_4 (6). Cells were harvested at exponential and stationary phase of growth and analyzed for intracellular osmolytes and cell viability.

Measurement of growth and cell viability

Cell growth was measured by monitoring the optical density of the medium at absorbance 600 nm (BioRad Smart Spectrometer 3000, BioRad Laboratories, CA). The biomass concentration was calculated from the absorbance values using predetermined correlation factor. OD_{600} corresponds to 0.34 g cell dry weight/l (Gummadi and Kumar 2008).

In all the experiments performed, cells harvested from culture collected at exponential and stationary phase of growth and exposed to (hypo- and hyper-) osmotic stress condition were tested for viability. Harvested cells were

washed and serially diluted (10^6–10^8 factor) with isoosmotic solution and 50 µl of diluted sample was plated in triplicate on YEPD plates containing same concentration of salt as in the culture medium. The plates were incubated at 30°C till the formation of visible colonies. The viability was expressed in terms of mean values of colony-forming units, CFU/ml. The survival percentage was determined by dividing the CFU/ml of isolate under hypoosmotic and hyperosmotic stress to CFU/ml of the strain when grown in the isoosmotic condition.

Preparation of samples for scanning electron microscope (SEM) analysis

Debaryomyces nepalensis cells grown in YEPD medium containing different concentration of NaCl (0–2.5 M), KCl (0–2.5 M) and LiCl (0–1.0 M) were harvested at exponential phase of growth. Sample preparation for SEM analysis was done by modification of the method of Andreishcheva et al. (1999). For fixing, the cells were resuspended in glutaraldehyde solution (3%, v/v) prepared in 100 mM phosphate buffer solution, pH 7.3 and incubated for more than 4 h at 4°C. Thereafter, cells were washed twice with phosphate buffer (100 mM, pH 7.3) followed by rinsing with double-distilled water. Cells were dehydrated using ethanol gradient at 25, 50, 75, and 95% with 10-min incubation at each step. Cell suspension was left overnight in desiccator. Cells were then coated with gold particles, deposited on silicon plate, and analyzed with SEM (Hitachi, S-3400N).

Preparation of cell extract

Osmolyte extraction was done using a modification of the method of Bligh and Dyer (1959). Cell sample (30 mg dry weight) was resuspended in 0.5 ml of Bligh and Dyer solution (methanol:chloroform:water, 10:5:4, v/v) and vigorously shaken at 30°C for 30 min. Thereafter, 130 µl each of chloroform and double-distilled water were added. The suspensions were again vigorously shaken for 30 min at 30°C followed by centrifugation at 4,000×*g* (Eppendorf 5810 R) for 8 min. The upper aqueous phase was separated out using Pasteur pipette, which was used for intracellular osmolyte and intracellular ion concentration measurements.

Measurement of intracellular Na^+ and K^+ content

Debaryomyces nepalensis cells grown in YEPD/defined medium containing different concentrations of Na^+ (NaCl) and K^+ (KCl) were harvested at mid-log and stationary phase of its growth by centrifugation at 8,200×*g* (Eppendorf 5810 R) for 5 min at 4°C. Cell pellets were resuspended and washed three times with isoosmotic solution of sorbitol. Cell-free extract was prepared using a modification of the method of Bligh and Dyer as described above. Intracellular Na^+ and K^+ content were measured in the cell extract using ion chromatography (Dionex LC 20 ion chromatograph, Dionex, CA). Methane sulfonic acid (3 mM) was used as mobile phase at a flow rate of 1 ml/min. Detection was done using electrochemical detector and cation exchange column (Ion pac SCS 1, Dionex, CA) operated at 30°C.

Na^+ and K^+ influx experiments

Cells were grown in complex YEPD medium in the absence of salt, harvested at mid-log phase by centrifugation, and washed thrice with isoosmotic solution of sorbitol. 30 mg of cells was resuspended in 20 ml of sodium phosphate or potassium phosphate buffer (20 mM, pH 7) containing different concentrations of NaCl and KCl ranging between 0 and 2 M. Samples were taken periodically, washed thrice with isoosmotic solution of sorbitol, and treated for determination of intracellular Na^+ and K^+ content.

Cation (Na^+/K^+) exchange experiments

Exponential phase cells grown in YEPD medium containing 2 M NaCl were incubated in medium containing 2 M KCl and vice versa. Samples were taken every 5 min, washed, and treated for determination of intracellular Na^+ and K^+ content as described above.

Measurement of intracellular osmolytes

Cell extract prepared as above were analyzed using high-performance liquid chromatography (Jasco LC 2000 plus HPLC, JASCO, UK) for common intracellular osmolytes (trehalose and polyhydroxy alcohols, e.g. glycerol, arabitol, mannitol, sorbitol, erythritol) accumulated by yeasts under saline stress condition (Kayingo et al. 2001; Kogej et al. 2007; Larsson et al. 1990). HPLC analysis was performed with Zorbax carbohydrate column (4.6 × 250 mm, 5.0 µm, Agilent Technologies, USA) and operated at 35°C. Acetonitrile (HPLC grade, 70%, v/v) was used as mobile phase at a flow rate of 1 ml/min. Detection was done using refractive index detector. Intracellular glycerol concentration was also quantified spectrophotometrically at 540 nm (Jasco V 660 UV-VIS Spectrophotometer, JASCO, UK) using glycerol kinase enzyme-based commercial assay kit (Free Glycerol Reagent, Sigma-Aldrich, USA). Samples were diluted using double-distilled water.

Osmolytes in salt-adapted cells

Yeast cells were grown in defined media containing various concentrations of NaCl and KCl and harvested at the mid-exponential, early stationary, and late stationary phase of growth. Cells harvested were used for measurement of intracellular polyol and viability test.

Hypoosmotic shock experiments

Cells of *D. nepalensis* were grown to exponential and stationary phase in defined medium containing 1 M NaCl, harvested, and washed. Subsequently, cells were resuspended in medium of same salinity (control) and in medium containing 0.2, 0.5, and 0.8 M NaCl. Cell suspension was then incubated at 30°C and 180 rpm for 1 h. Similarly, cells grown in the presence of 2 M NaCl were harvested and exposed to hypoosmotic shock by incubating the cells in medium containing 0.4, 1, and 1.6 M NaCl. Similar experiments were carried out using KCl in medium. The samples for viability test and determination of polyols were taken after 1 h and treated as described above.

Hyperosmotic shock experiment

To study the effect of hyperosmotic shock on *D. nepalensis*, cells were grown on defined medium supplemented with 0.5 M NaCl. At exponential and stationary phase of growth, cells were harvested and washed. Thereafter, cells were resuspended in minimal media containing 2 M NaCl and incubated at 30°C, and 180 rpm. The samples for viability test and determination of polyols were taken at 15 min, 1 h and 3 h after the shock. Similar experiment was performed with medium containing KCl.

Results

Growth of *D. nepalensis* NCYC 3413 at different concentrations of NaCl and KCl

Debaryomyces nepalensis NCYC 3413 has been known for its remarkable ability to grow on YEPD medium containing 2 and 3 M of NaCl and KCl, respectively (Gummadi et al. 2007). In this study, we have determined the growth of *D. nepalensis* on minimal defined medium containing glucose (10 g/l) as the sole source of carbon. Specific growth rate was not affected by the presence of salt in the medium. However, the presence of 2 M NaCl and 2 M KCl did significantly increase lag phase of growth and reduction in maximum biomass produce per unit time (Table 1). Growth of *D. nepalensis* was relatively lowered in the presence of NaCl as compared to that of KCl. Cells grown on medium with salinities amended with 0.5 M KCl produced maximum biomass (Table 1). As compared to the growth on YEPD (Gummadi et al. 2007), *D. nepalensis* grown on minimal medium showed poor growth and considerably less biomass was produced. Although, *D. nepalensis* could tolerate a maximum of 2 M NaCl and 2 M KCl during growth on defined medium, better growth was observed on medium with no salt or with 0.5 M KCl.

Changes in cell morphology (size) in response to saline stress

Cells of *D. nepalensis* were grown on YEPD medium containing different concentrations of NaCl, KCl, and LiCl and change in cell size was determined using SEM. The mean cell diameter decreased with increase in salt concentration when measured using SEM (Fig. 1). The decrease in cell size was linear to the decrease in salt concentration with R^2 (0.8–0.99). The decrease in cell size

Table 1 Specific growth rate, maximum cell dry weight produced per unit time and lag time of growth for yeast *Debaryomyces nepalensis* NCYC 3413 grown at different salinities of NaCl and KCl in defined medium

Concentration (M)	Specific growth rate, μ (h^{-1})		X_{max} (mg/l h)		Lag time of growth	
	NaCl	KCl	NaCl	KCl	NaCl	KCl
0	0.13 ± 0.02	0.13 ± 0.01	155 ± 11	155 ± 11	2	2
0.5	0.11 ± 0.01	0.14 ± 0.02	174 ± 7.4	199 ± 16	4	2
1	0.11 ± 0.01	0.13 ± 0.01	170 ± 6.0	157 ± 9.2	6	6
2	0.10 ± 0.01	0.11 ± 0.01	66.2 ± 5.2	88 ± 5.9	8	8

Specific growth rate was measured during the exponential phase of the curves. Cells were grown as described in "Materials and methods". Results are the mean of three different experiments each performed in duplicates

X_{max} corresponds to maximum cell dry weight produced during growth

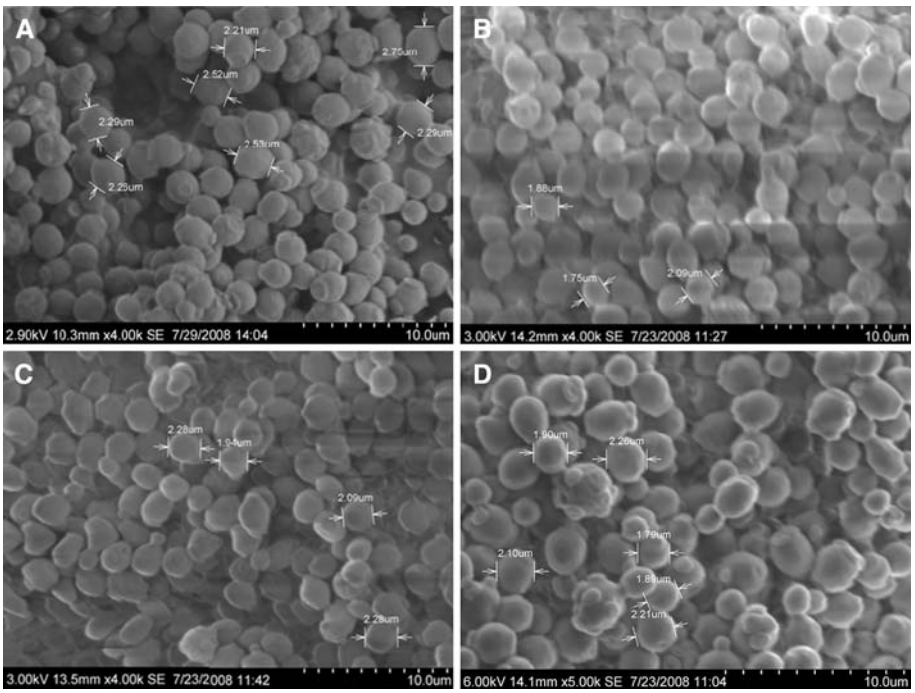

Fig. 1 Scanning electron microscopic images of *D. nepalensis* cells grown on **a** YEPD medium with no salt, **b** YEPD medium containing 2 M NaCl, **c** YEPD medium containing 2 M KCl, **d** YEPD medium containing 1 M LiCl. Cells were harvested in exponential phase of growth and viewed under electron microscope as described in "Materials and methods"

was largest for cells grown in the presence of LiCl followed by NaCl and KCl (Fig. 2).

Intracellular cation concentration in *D. nepalensis* grown in YEPD and defined medium at different concentrations of NaCl, KCl, and LiCl

Debaryomyces nepalensis was grown on YEPD and defined medium containing different concentrations of NaCl and KCl and intracellular cation contents were measured using ion chromatography. It has been found that cells grown in media in the absence and presence of NaCl accumulated both Na^+ and K^+ (Table 2). In both the cases of growth in YEPD and defined medium containing NaCl, K^+ was accumulated at relatively higher concentration as compared to that of Na^+. With the increase in NaCl concentration, the amount of intracellular Na^+ and K^+ concentrations increased for the exponential phase grown cells. However, for stationary phase cells, the intracellular Na^+ content increased with NaCl concentration and K^+ content was maintained at constant level for growth in defined medium, although for YEPD, 2 M NaCl accumulated more K^+ as compared to that of no salt condition (Table 2). The ratio of K^+/Na^+ was highest in cells grown in medium without salt and it decreased with increase in NaCl concentration. Similar pattern of intracellular K^+ and Na^+ concentration was observed for growth in either YEPD or defined medium. However, the ratio of internal K^+/Na^+ was comparatively higher for cells grown in YEPD medium with or without NaCl as compared to that of defined medium (Table 2).

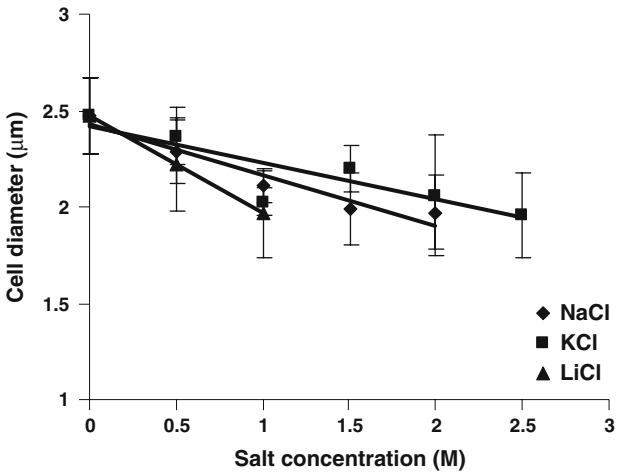

Fig. 2 Effect of salt concentration on size of *D. nepalensis* grown on YEPD medium containing different concentration of NaCl, KCl, and LiCl. Cells were harvested at exponential phase of growth and cell diameter was measured with SEM. The values shown are average and standard deviation of 20 measurements

During growth on defined medium containing KCl, exponential phase cells of *D. nepalensis* accumulated comparatively high concentration of K^+, with the increasing KCl concentration. In case of stationary phase cells, intracellular K^+ content was maintained at constant level with concentration higher than that of no salt condition (Table 2). However, as compared to defined medium, cells grown in YEPD medium containing KCl accumulated higher level of K^+ at exponential and stationary phase of growth which increased with increase in KCl content of the

Table 2 Changes in intracellular concentration of Na^+ and K^+ (μmol/g cell dry weight) of cells grown in YEPD and defined medium in the presence or absence of different concentrations of NaCl or KCl

Medium	Salt (M)	NaCl				KCl			
		Mid-log		Stationary		Mid-log		Stationary	
		Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+
Defined medium	0	34.4 ± 2.8	116 ± 10	37.5 ± 3.0	146 ± 5.2	34.4 ± 2.8	116 ± 10	37.5 ± 3.0	146 ± 5.2
	1	62.8 ± 10.3	171 ± 23.8	53.3 ± 0.7	147 ± 6.8	0	201 ± 15	0	238 ± 15
	2	123.4 ± 8.8	201 ± 4.4	135 ± 11	145 ± 7.4	0	274 ± 21	0	239 ± 9.3
YEPD	0	43.5 ± 6.4	231 ± 13	36.2 ± 3.4	162 ± 10.6	43.5 ± 6.4	272 ± 11	36.2 ± 3.4	162 ± 10.6
	1	60.6 ± 3.3	237 ± 10	61.8 ± 3	158 ± 10.6	0	307 ± 6	0	259 ± 11
	2	134 ± 11	272 ± 11	75.3 ± 7.8	182 ± 6	0	398 ± 53	0	338 ± 6.4

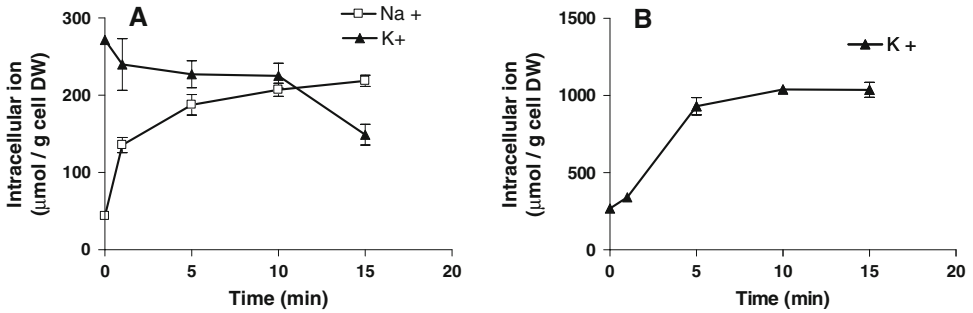

Fig. 3 Changes in intracellular concentration of Na^+ and K^+ of cells grown in the absence of salt and subjected to hyperosmotic stress. *D. nepalensis* was grown to exponential phase in the absence of salt in YEPD and incubated with either of **a** 2 M NaCl or **b** 2 M KCl, respectively. Samples were taken every 5 min, washed, and treated for determination of intracellular Na^+ and K^+ content by ion exchange chromatography as described in "Materials and methods". The data shown are the mean and standard deviation of three replicates

medium. Interestingly, no accumulation of intracellular Na^+ was observed for cells grown in the presence of KCl.

D. nepalensis cells grown on YEPD medium containing 0.5 M LiCl was found to accumulate 358 ± 49 and 323 ± 21 μmol/g cell DW of Li^+ in mid-log and stationary phase of growth. At 1 M LiCl, the amount of biomass was low such that determination of intracellular Li^+ was below determinable levels. Similarly, the growth was very low in the defined medium. However, Li^+ has been shown to act as analog of Na^+ for many transport systems (Haro et al. 1991). Therefore, further studies were restricted on studying the transport of Na^+ and K^+ in cells grown under different stress condition of NaCl and KCl.

Intracellular sodium and potassium ions in the cells of *D. nepalensis* after hyperosmotic shock

Cells of *D. nepalensis* grown in YEPD medium without salt were harvested at exponential phase of growth and subjected to hyperosmotic shock by incubation in the presence of high salt concentration (2 M NaCl/KCl). Incubation with 2 M NaCl for 5 min caused an immediate increase in Na^+ content of the cell from 48 to 187 μmol/g cell dry weight (Fig. 3a). On further incubation for 15 min, Na^+ content reached a steady state and increased up to 220 μmol/g cell dry weight, which is approximately twice the Na^+ content measured in cells grown in YEPD amended with 2 M NaCl. K^+ content of the cell dropped from 272 to 240 μmol/g cell dry weight within 5 min of incubation, which further declined to 150 μmol/g cell dry weight on further incubation for 15 min.

Immediate increase in intracellular content of K^+ was observed during hyperosmotic shock when cells were incubated with 2 M KCl. Within 5 min of incubation, intracellular K^+ increased from 270 to 931 μmol/g cell dry weight and a steady state was reached after 10 min, and accumulated K^+ approximately four times the K^+ content measured in cells grown in YEPD medium containing 2 M KCl (Fig. 3b).

Intracellular Na^+ and K^+ in the cells of *D. nepalensis* grown in the presence of 2 M KCl and incubated with 2 M NaCl and vice versa

Cells of *D. nepalensis* grown in YEPD medium with 2 M NaCl were harvested at exponential phase of growth and

resuspended in medium containing 2 M KCl. During growth in the presence of 2 M NaCl, cells accumulated approximately 130 µmol/g cell dry weight of Na^+ and 230 µmol/g cell dry weight of K^+. However, within 1 min of incubation with 2 M KCl, an immediate efflux of Na^+ accumulated by the cell was observed which was accompanied by uptake of K^+ (Fig. 4a). Thereafter, cells reached a steady state and no significant exchange of cation (Na^+ and K^+) was observed.

Similarly, exponential phase cells grown in medium containing 2 M KCl showed an immediate decline in intracellular K^+ content from 400 to 170 µmol/g cell dry weight on incubation for 1 min with medium containing 2 M NaCl. This was compensated by immediate increase of intracellular Na^+ from 0 to 132 µmol/g cell dry weight (Fig. 4b). On further incubation, cells reached a steady state.

Influence of salinity of the medium on the accumulation of polyols by *D. nepalensis*

We analyzed the extracts from cells of *D. nepalensis* grown on defined medium containing different concentrations of salts (NaCl and KCl) for common polyols, viz., glycerol, arabitol, mannitol, sorbitol, erythritol, and trehalose. HPLC analysis showed that the intracellular polyol pool consisted of glycerol, arabitol, and sorbitol (Fig. 5). HPLC chromatogram of polyols accumulated by the cells as osmolyte for one of the sample has been represented in comparison with that of corresponding standards in Fig. 5a. The proportion of these polyols varied with salinity of the medium and phase of growth of *D. nepalensis*. The level of arabitol responded only slightly to the medium salinity but it appeared to be regulated with respect to different growth phases. Maximum arabitol (0.6–1 mmol/g cell dry weight) was accumulated by cells in late stationary phase.

In exponential phase of growth, cells grown on medium without salt accumulated glycerol (0.26 mmol/g cell dry weight), fairly less concentration of arabitol (0.10 mmol/g cell dry weight), and trace amount of sorbitol (0.02 mmol/g cell dry weight) (Fig. 5b). Cells grown in the presence of NaCl accumulated glycerol and sorbitol as a function of salinity of the medium. However, intracellular glycerol accumulated by cells grown in medium containing KCl was not unaffected with medium salinity and it was maintained at a concentration of approximately 1.1 mmol/g cell dry weight. In addition, intracellular concentration of sorbitol increased with increase in concentration of KCl in medium. Among *D. nepalensis* grown in medium with different salinity, exponential phase cells grown at 2 M NaCl accumulated highest level of glycerol (2.3 mmol/g cell dry weight) and sorbitol (2.6 mmol/g cell dry weight) (Fig. 5b).

In stationary phase of growth, intracellular concentration of glycerol in cells grown in different salt concentration was maintained at almost same concentration as that of exponential phase (Fig. 5c). However, approximately 20% increase in sorbitol concentration was observed in all the cases. Interestingly, cells grown in 1 M NaCl and 1 M KCl accumulated approximately same level (1 mmol/g cell dry weight) of glycerol and sorbitol.

In late stationary phase of growth, polyol pool was dominated by sorbitol and intracellular concentration of glycerol was found to decline in all the cases. Glycerol and sorbitol were totally diminished in cells grown in the absence of salt. Cells grown in the presence of KCl were found to accumulate 0.2 mmol/g cell dry weight of glycerol in late stationary phase of growth. Amount of sorbitol accumulated by cells at late stationary phase of growth was highest among all the phases of growth. It was maximum (3.5 mmol/g cell dry weight) for cells grown in the presence of 2 M NaCl (Fig. 5d).

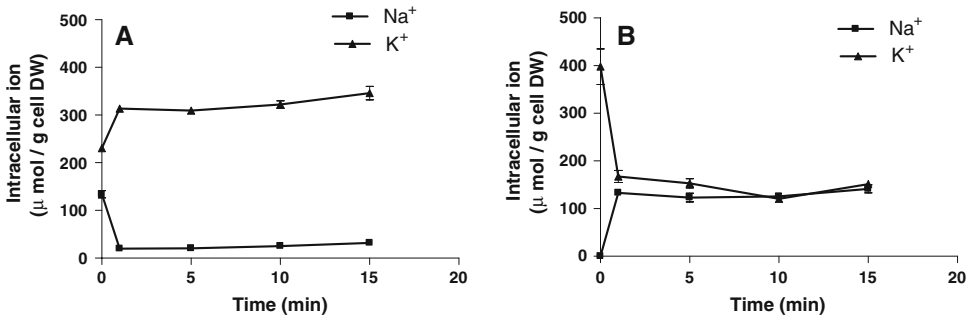

Fig. 4 Changes in intracellular concentration of Na^+ and K^+ of cells grown in YEPD medium at high salt concentration (NaCl/KCl) and incubated with medium of same osmolarity (KCl/NaCl) at exponential phase of growth. **a** Cells grown in the presence of 2 M NaCl were incubated with 2 M KCl. **b** Cells grown in the presence of 2 M KCl were incubated with 2 M NaCl. Samples were taken every 5 min, and treated for determination of intracellular Na^+ and K^+ content by ion chromatography as described in "Materials and methods". Each experiment was performed thee times and data shown are the mean and standard deviation of three replicates

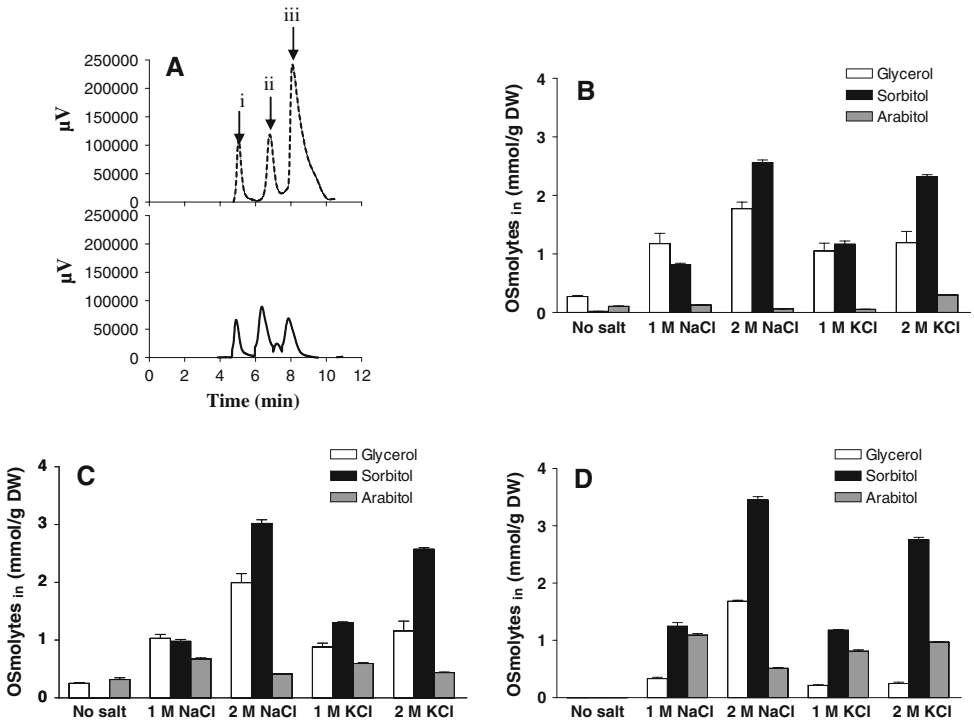

Fig. 5 Intracellular amounts of glycerol and sorbitol produced by salt-adapted cells of *D. nepalensis*. **a** HPLC chromatogram of osmolytes accumulated by the cells under salt stress condition. *Solid and broken lines* corresponds to the chromatogram of one of the sample and standards of glycerol (*peak i*, retention time: 5 min, 5 mg/ml), arabitol (*peak ii* retention time: 6.6 min, 5 mg/ml), and sorbitol (*peak iii* retention time: 8 min, 10 mg/ml). Osmolytes accumulated at **b** exponential phase, **c** stationary phase, and **d** late stationary phase of growth on defined medium containing different concentration of NaCl and KCl. Cell extract was prepared and analyzed as described in "Materials and methods". The data shown are the mean and standard deviation of three replicates

Cells decrease its intracellular osmolyte during hypoosmotic shock

We performed hypoosmotic shock on *D. nepalensis* cells grown in medium containing 1 and 2 M NaCl and KCl, respectively. The intracellular amount of polyols was measured to test their osmotic role and viability of cells after osmotic shock. The mid-exponential and stationary phase cells were incubated in 0.8, 0.5, and 0.2 M salt for cells grown in 1 M salt, 1.6, 1, and 0.4 M salt for cells grown in 2 M to perform hypoosmotic shock.

D. nepalensis accumulated significant amounts of glycerol and sorbitol as osmolyte within the cell during growth in medium containing 1–2 M of NaCl and KCl. When subjected to hypoosmotic shock, cells released its intracellular polyol to withstand decrease in osmotic pressure in its environment. Intracellular concentration of glycerol for cells grown in 1 M NaCl was not affected when exposed to moderate hypoosmotic shock (1–0.8 M) (Fig. 6a). However, cells grown in 2 M NaCl had to release 25% of its accumulated glycerol when incubated with 1.6 M NaCl (Fig. 6b). On drastic hypoosmotic shock (1–0.2 or 2.0–0.4 M NaCl), cell could conserve 40–50% of its accumulated glycerol (Fig. 6a, b). Cells grown in the presence of 1 and 2 M NaCl released approximately 35% (Fig. 6c) and 50% of accumulated sorbitol, respectively, on exposure to hypoosmotic shock (Fig. 6d). However, only 25% of accumulated sorbitol was conserved by the cells grown on 1 and 2 M NaCl after drastic hypoosmotic shock (Fig. 6c, d). Cells at mid-log and exponential phase of growth showed similar behavior on hypoosmotic shock.

When cells grown in medium containing 1 and 2 M of KCl were exposed to moderate hypoosmotic shock, cells lost approximately 10% of glycerol (Fig. 7a, b). However, cells grown in 1 and 2 M KCl lost 15–20% of accumulated sorbitol (Fig. 7c, d). On exposure to drastic hypoosmotic shock, cells grown in KCl showed similar behavior as that of NaCl and it conserved approximately 50–60% of accumulated glycerol on exposure to drastic hypoosmotic shock which is similar to that of NaCl. In general, significant release of osmolyte was not observed when cells were moved from isoosmotic to moderately hypoosmotic condition (1–0.8 or 2.0–1.6 M). However, drastic hypoosmotic shock (1–0.2 or 2–0.4 M) caused a significant decrease in intracellular amount of glycerol for both exponential and stationary phase cells. Cells suffered significant loss of viability after hypoosmotic shock. In both the cases of KCl and NaCl (Table 3), % viability of cells diminished steeply with increase in hypoosmotic stress with the effect being less severe for stationary phase cells grown in the presence 2 M NaCl. This might be correlated with high amount of osmolyte accumulated by the cells during growth.

Cells synthesize and accumulate osmolyte during hyperosmotic shock

Cells grown on medium with less salinity (0.5 M) were subjected to hyperosmotic shock by incubating the cells in

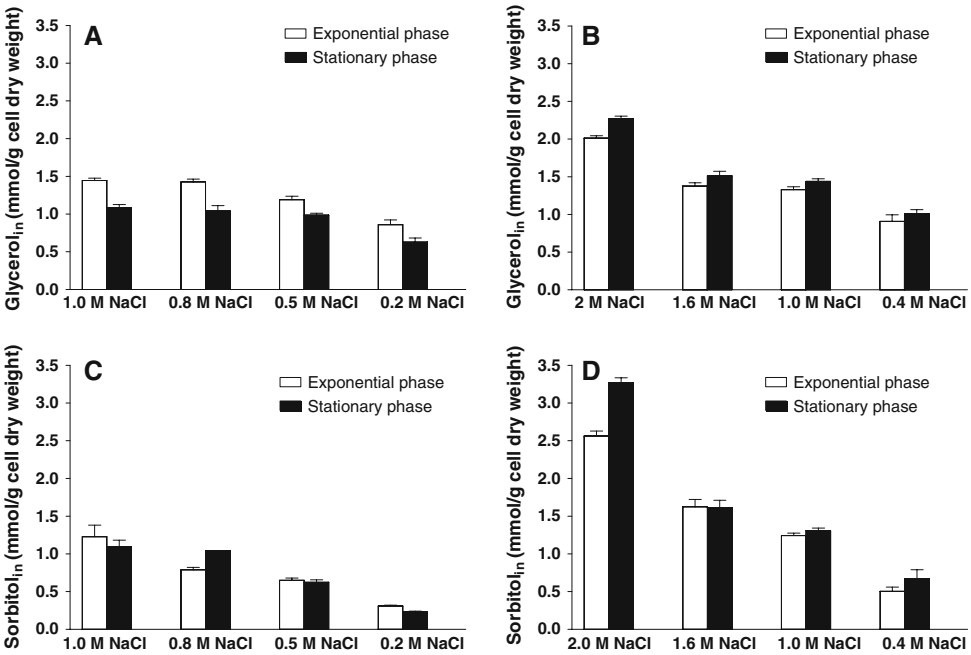

Fig. 6 Intracellular polyol concentration in cells of *D. nepalensis* subjected to hypoosmotic shock by incubating the cells with decreasing concentration of NaCl. Glycerol concentration after hypoosmotic shock in cells grown in the presence of **a** 1 M NaCl and **b** 2 M NaCl. Sorbitol concentration after hypoosmotic shock in cells grown in the presence of **c** 1 M NaCl and **d** 2 M NaCl. The samples were prepared and analyzed as described in "Materials and methods". The data shown are the mean and standard deviation of three replicates

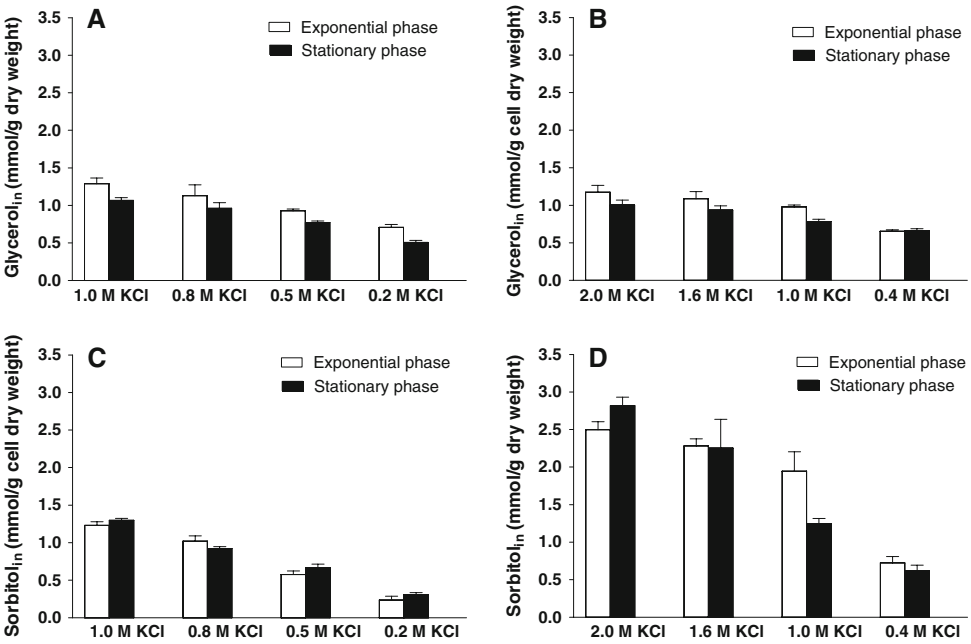

Fig. 7 Intracellular polyol concentration in cells of *D. nepalensis* subjected to hypoosmotic shock by incubating the cells with decreasing concentration of KCl. Glycerol concentration after hypoosmotic shock in cells grown in the presence of **a** 1 M KCl and **b** 2 M KCl. Sorbitol concentration after hypoosmotic shock in cells grown in the presence of **c** 1 M KCl and **d** 2 M KCl. The samples were prepared and analyzed as described in "Materials and methods". The data shown are the mean and standard deviation of three replicates

medium with high salinity (2 M). Cells were harvested at mid-log phase and stationary phase of growth and samples collected at different period of time were checked for accumulation of osmolytes after hyperosmotic shock (Fig. 8).

Exponential phase cells grown in the presence of 0.5 M KCl showed approximately 15% increase in its glycerol and sorbitol content after 15 min of hyperosmotic shock with 2 M KCl. However, 3 h after the shock, cells accumulated almost twice the concentration of glycerol and 1.7 times the concentration of sorbitol as accumulated by it before shock (Fig. 8a). However, after 15 min of hyperosmotic shock, stationary phase cells showed only 10% increase in its glycerol content and sorbitol increased four times as accumulated by it before shock. Thereafter, it was found to enter steady state and significant increase in intracellular osmolyte content was not observed even after 3 h of hyperosmotic shock (Fig. 8b). As compared to that of KCl, exponential phase cells grown in the presence of 0.5 M NaCl showed similar increase in glycerol on hyperosmotic shock. However, after 15 min of hyperosmotic shock, it accumulated 2.5 times sorbitol and after 3 h

Table 3 Viability test of the cells grown under isoosmotic and hypoosmotic stress condition of 1.0 and 2.0 M of KCl

Salt (M)	Salt concentration in diluted medium (M)	Viability (%)			
		KCl		NaCl	
		Mid-log	Stationary	Mid-log	Stationary
1.0	1.0	100[a]	100[a]	100[b]	100[b]
	0.8	44.0	69.0	81.0	64.0
	0.5	15.0	51.0	30.0	24.0
	0.2	5.0	7.0	13.0	7.0
2.0	2.0	100[c]	100[c]	100[d]	100[e]
	1.6	26.0	20.0	75.0	79.0
	1.0	16.0	14.0	16.0	38.0
	0.4	2.0	2.0	12.0	26.0

Viability test was done as described in "Materials and methods"

[a] Corresponds to 180 CFU/ml of cell culture at 10^{10} dilution

[b] Corresponds to 150 CFU/ml of cell culture at 10^{10} dilution

[c] Corresponds to 47 CFU/ml of cell culture at 10^{10} dilution

[d] Corresponds to 120 CFU/ml of cell culture at 10^{10} dilution

[e] Corresponds to 170 CFU/ml of cell culture at 10^{10} dilution

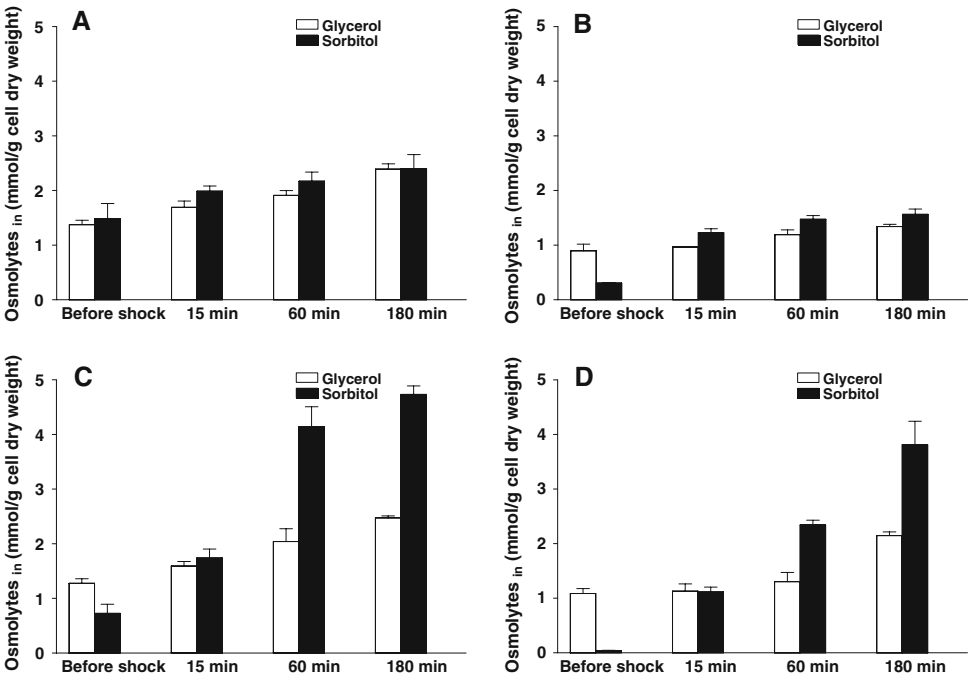

Fig. 8 Intracellular polyol concentration in cells of *D. nepalensis* subjected to hyperosmotic shock. Glycerol and sorbitol concentration after hyperosmotic shock in cells grown to **a** mid-exponential and **b** stationary phase of growth in medium containing 0.5 M KCl. Glycerol and sorbitol concentration after hyperosmotic shock in cells at **c** mid-exponential and **d** stationary phase of growth in the presence of 0.5 M NaCl. The samples were prepared and analyzed as described in "Materials and methods". The data shown are the mean and standard deviation of three replicates

of incubation, 7 times sorbitol was accumulated by it before shock (Fig. 8c). For stationary phase cells grown in the presence of 0.5 M NaCl, cells accumulated approximately 1.1 mmol/g cell dry weight of glycerol and trace amount of sorbitol. However, its intracellular sorbitol increased to 1.2 mmol/g cell dry weight and glycerol content was unaltered after 15 min of hyperosmotic shock. After 3 h of incubation, intracellular glycerol increased 2 times as accumulated by it before shock and intracellular sorbitol content was raised to 4.2 mmol/g cell dry weight (Fig. 8d). During incubation after hyperosmotic shock, exponential and stationary phase cells released approximately 1.8 and 1 mM of glycerol, respectively, in its surrounding medium (data not shown). This might be attributed to the passive diffusion of glycerol across the cell membrane. In all the cases, approximately 50% of cells were viable even after 180 min of hyperosmotic shock (Table 4).

Table 4 Viability test of the cells grown under isoosmotic and hyperosmotic stress condition of 2.0 M NaCl and KCl

Salt	Duration of hyperosmotic shock	Viability (%)	
		Mid-log	Stationary
2.0 M NaCl	Before shock	100[a]	100[b]
	15 min	79.0	69.0
	180 min	45.0	47.0
2.0 M KCl	Before shock	100[c]	100[d]
	15 min	62.0	76.0
	180 min	57.0	64.0

Viability test was done as described in "Materials and methods"

[a] Corresponds to 504 CFU/ml of cell culture at 10^{12} dilution

[b] Corresponds to 1,050 CFU/ml of cell culture at 10^{12} dilution

[c] Corresponds to 237 CFU/ml of cell culture at 10^{12} dilution

[d] Corresponds to 1,353 CFU/ml of cell culture at 10^{12} dilution

Discussion

Tolerance for osmotic stress is an important determinant of survival of organisms. *Debaryomyces* sp. is a group of rare extremophilic yeast species which are ubiquitous in nature and found in different habitats. It includes *Debaryomyces vanriji* that are thermophilic in nature (Rikhanov et al. 1999), whereas *D. hansenii* (Prista et al. 2005) and *D. nepalensis* (Gummadi et al. 2007) are halotolerant in nature. The main goal of this study was to unravel the osmoadaptation strategy of *D. nepalensis*. Growth of *D. nepalensis* in the absence or presence of different concentrations of NaCl and KCl was quantified on minimal medium. This is in agreement with our previous finding in the sense that *D. nepalensis* grows better in the absence of salt or in the presence of moderate (0.5 M) concentration of NaCl and KCl as compared to higher salt concentration (Gummadi et al. 2007). The decrease in monitored growth parameter (X_{max} produced per unit time) and increase in lag phase with increasing salt concentration can be correlated with toxic effects of high salt concentration. In this study, it has been found that cell size decreases with increase in osmotic stress (Fig. 2). Interestingly, it has also been found that for a given salt concentration, the cell size reduction for different salts is in the order of LiCl > NaCl > KCl (Fig. 2). This observation was in accordance with our earlier report that LiCl was more toxic than NaCl followed by KCl (Gummadi et al. 2007). Hence, the decrease in cell size of *D. nepalensis* can be correlated with the osmotic stress with the largest decrease in cell size found with cells grown on medium with maximum salinity and toxicity induced by salt. A similar decrease was observed for halotolerant yeast *Z. cerevisiae* (van Zyl et al. 1991) and *Yarrowia lipolytica* (Andreishcheva et al. 1999) on exposure to osmotic stress condition. The shrinkage of cells could be a signal for osmoregulatory responses in microorganisms.

Table 2 shows the osmotic adjustments of sodium and potassium in response to osmotic stress. *D. nepalensis* accumulated high amounts of K^+ inside the cell, when grown in the presence of high concentration of NaCl in the medium. This is in agreement with our results on cell viability and decrease in cell size (Fig. 2), suggesting that NaCl is more toxic than KCl (Gummadi et al. 2007). This gives evidence of cell's ability for extrusion of sodium to decrease the continuous inflow of Na^+ at the cost of uptake of K^+ inside the cell for its survival. In contrast to this, another halotolerant yeast, *D. hansenii*, grown in the presence of high concentration of NaCl accumulated Na^+ 2–4 times higher than that of K^+ showing its behavior as Na^+ includer yeast (Gonzalez Hernandez et al. 2004; Prista et al. 2005). However, *D. hansenii* grown in the presence of KCl accumulated more KCl similar to *D. nepalensis* reported in this study. In this study, *D. nepalensis* was exposed to both Na^+ and K^+ during growth either in the presence or absence of salts. Under these conditions, the cell maintains low concentration of Na^+ and accumulates 3–4 times higher K^+ inside the cell, although Na^+ is present in excess outside the cell (Table 2). Based on this behavior, we suggest that *D. nepalensis* is a Na^+ excluder yeast.

Interestingly, this Na^+ exclusion property of *D. nepalensis* was lost when this yeast was grown in the absence of salt and incubated with 2 M NaCl (non-growth conditions). Under these conditions, cells are suddenly exposed to high concentration of Na^+ ion. A rapid uptake of Na^+ was observed accompanied by decrease in accumulated K^+ for shorter period of time ($\sim$5 min). This gives the evidence for rapid activation of a transporter such as cation/cation antiporter which regulates the rapid osmotic adjustments in *D. nepalensis* because cells grown without salt accumulate sufficient K^+ inside the cell. Further, rapid uptake of K^+ was observed by *D. nepalensis* grown in the absence of salt on incubation with 2 M KCl. This suggests that K^+-specific transporter is involved because cation/cation antiporter requires sufficient amount of Na^+ to exchange with K^+ (Table 2). Ramos et al. (1990) described transporters Trk1p and Trk2p in *S. cerevisiae* to be responsible for potassium and sodium influx, which has much higher affinity for K^+ when both are present.

To reevaluate the existence or involvement of cation/cation antiporter in *D. nepalensis*, cells were grown in the presence of high NaCl concentration followed by incubation with KCl of same osmolarity and vice versa. An immediate efflux of abundant ion (Na^+/K^+) accumulated by the cell was observed. This was accompanied by simultaneous uptake of ion present in the medium. These finding suggests the activation of transporters such as

Na^+/K^+ antiporter or cation/H^+ transporter in the *D. nepalensis* which plays a vital role in osmotic adjustments owing to sudden exposure to high salt stress condition. Thomé-Oritz et al. (1998) hypothesized the existence and role of similar transporters while studying the cation fluxes in *D. hansenii*.

To cope with high salinity, osmotic adjustment of cation works together with intracellular accumulation of polyols. Our results shows that *D. nepalensis* accumulates glycerol, arabitol, and sorbitol as osmolyte to compensate for the salinity of the medium. The concentration of these osmolytes (glycerol and sorbitol) was dependent on the external salinity in addition to the age of the cells. The arabitol level was unaffected by the salinity of the medium and found to be dependent on growth phase of cells. This might be correlated with the constitutive nature of the metabolism of arabitol. Non-adapted cells of *D. nepalensis* also accumulated 0.28 mmol/g cell dry weight of glycerol during exponential phase of growth owing to the constitutive nature of glycerol synthesis. Glycerol is known to be the primary osmolyte accumulated by most halotolerant yeasts such as *Y. lipolytica* (Andreishcheva et al. 1999), *D. hansenii* (Larsson et al. 1990), *Z. rouxii* (van Zyl et al. 1991), and *Hortea werneckii* (Kogej et al. 2007). In *D. nepalensis*, amount of glycerol accumulated during growth under salt stress condition was drastically reduced in late stationary phase of growth. This was accompanied by increase in sorbitol. A similar shift in polyol pool has been observed in case of *Aspergillus niger* which accumulates glycerol in exponential phase and mannitol and erythritol in stationary phase (Witteveen and Visser 1995), whereas *D. hansenii* replaces glycerol with arabitol in stationary phase of growth (Adler and Gustafsson 1980).

In natural habitat, yeasts cells are exposed to various changes in osmolarity. Lot of studies has been done on effect of osmotic stress on halotolerant yeasts. However, effect of hypoosmotic and hyperosmotic shock on salt-adapted cells has received less attention. When exposed to hypoosmotic shock, rapid in flow of water occurs. This leads to cell swelling and if allowed to continue, the cells may disrupt. This can be prevented by lowering the intracellular osmolytes by rapidly releasing the solutes to the surrounding medium or by metabolizing them (Goyal 1989). Cells of *D. nepalensis* exposed to hypoosmotic shock released their osmolytes (glycerol and sorbitol) to balance the sudden change in osmotic pressure. Similar phenomenon was also observed in cells of osmotolerant yeasts such as *Z. rouxii* and *Pichia sorbitophila* (Kayingo et al. 2001). Upon hypoosmotic shock, they also released glycerol into the surrounding medium. In *S. cerevisiae*, release of intracellular glycerol upon hypoosmotic shock was shown to be controlled by a membrane channel protein, Fps1p. It opens upon hypoosmotic shock to release osmolytes (Tamas et al. 1999). Later on, Kayingo et al. (2004) reported that accumulation and release of osmolyte is independent of this glycerol channel protein in *S. pombe*. Therefore, at present, we have no definitive explanation for how *D. nepalensis* is able to lower the amount of both polyols. Further studies are required to elucidate involvement and the role of this channel protein in *D. nepalensis* under hypoosmotic shock. Cells of *D. nepalensis* encountered significant loss in its viability on exposure to hypoosmotic stress condition. Previous studies on *S. cerevisiae* showed loss of cells viability after hypoosmotic shock in cells that were defective in channel protein Fps1p (Tamas et al. 1999).

When cells of *D. nepalensis* grown in low salt condition were exposed to hyperosmotic shock by incubating it under high salt stress condition, rapid increase in intracellular osmolyte content was observed. This might be responsible for the ability of cell to balance sudden increase in osmotic pressure. We hypothesize that sudden increase in osmotic pressure induces the synthesis of osmolytes and membrane channels such as Fps1p are closed thereby conserving the loss of osmolytes through membrane. On hyperosmotic stress, polyol synthesis (glycerol and sorbitol) was induced. Therefore, cells accumulated greater amount of sorbitol and glycerol after hyperosmotic shock. In addition, cells exposed to hyperosmotic shock by 2 M NaCl were found to accumulate osmolyte at high concentration as compared to that of 2 M KCl. This finding further supports the toxicity of Na^+ to *D. nepalensis* as compared to that of K^+. After 3 h of incubation, around 50% loss in cell viability was observed. This might be correlated to the inability of those cells to maintain the turgor pressure owing to hyperosmotic shock.

In conclusion, the results show that *D. nepalensis* is halotolerant yeast and Na^+ is more toxic to *D. nepalensis* in high concentration as compared to that of K^+. *D. nepalensis* use the strategy of keeping intracellular Na^+ at low concentration. And, hence it can be regarded as Na^+ excluder yeast. This is in contrast to the study on *D. hansenii* which have been reported as 'sodium includer yeast' owing to its ability to accumulate high intracellular Na^+ to withstand salt stress condition (Prista et al. 2005). The activation of uptake K^+ in the environment where K^+ is in scarce and Na^+ is in abundance is advantageous for *D. nepalensis* to overcome the toxic effect of Na^+. Based on our result, we present the evidence for involvement of cation/cation and cation-specific transporters involved in osmoregulation in *D. nepalensis*. Glycerol and sorbitol are the main compatible solutes of *D. nepalensis* which help them to withstand fluctuations in osmolarity of the medium. There also exists a balanced efflux and synthesis of osmolytes when *D. nepalensis* was exposed to hypoosmotic and hyperosmotic stress condition, respectively. The result obtained in this study provides information underlying the

physiological mechanism of osmoadaptation of novel food spoiling yeast, *D. nepalensis*.

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