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Identification of osmolytes from a moderately halophilic and amylolytic *Bacillus* sp. strain *TSCVKK*

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ABSTRACT

Compatible solute accumulation by a newly isolated moderately halophilic and alkali-tolerant bacterium, Bacillus sp. strain TSCVKK, was studied when grown with 3.5, 5, 10 and 15% NaCl at 30^{o}C. The organism showed optimum growth with 10% NaCl and failed to grow in a medium deprived of NaCl. The total ethanolic solute content extracted was high at 10 and 15% NaCl compared to 3.5 and 5% NaCl. Results of spectroscopic studies by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and Electron spin ionization mass spectrometry (ESI-Mass spec) showed that ectoine and betaine are the dominant solutes accumulated as a function of osmoadaptation and showed a switch in osmolyte accumulation with betaine accumulated in high proportion with low NaCl and ectoine at high concentration of NaCl.

Key words: Moderate halophile, Osmolytes, ectoine, betaine, amylase.

INTRODUCTION

Halophilic bacteria (salt-lover) can live, grow, and multiply in saline and hypersaline environments. They are classified into non-halophile (<1.2% NaCl), slight halophile (1.2-3%), moderate halophile (3-15%) and extreme halophile (> 15% NaCl) with respect to their salinity tolerance [1]. Ability of moderate halophiles to grow at fluctuating salinities makes them potential candidates for industrial and bioremediation applications [2, 3].

Osmolytes are zwitterionic, non-charged, or anionic and acts without inhibiting normal metabolism of the cell and at the same time providing osmotic balance under osmotic conditions. Cytoplasmic accumulation of osmolytes by uptake or by synthesis is a general phenomenon of osmoadaptation in halophiles. However, uptake is more energetically favorable than synthesis [4]. Osmolytes prevent water efflux from the cell and prevent cell death due to plasmolysis. Microorganisms belonging to archaea, bacteria, yeast, filamentous fungi and algae, rely

exclusively on osmolytes for osmoadaptation except for Halobacteriaceae, Halanaerobiales and the recently discovered bacterium *Salinibacter rubrum*, depends on intracellular accumulation of inorganic salts, mainly KCl.

The existence of osmolytes was studied by using spectroscopic studies like NMR spectroscopy, a powerful tool for the structural determination of organic molecules, conformation and dynamics of biomolecules [5]. Some of the osmolytes identified by NMR include glycine; ectoine [6]; 4-hydroxy ectoine [7]; proline and glutamate [8] and trehalose ([9].

Osmolytes also protects the cell and its components from freezing, desiccation, high temperature and oxygen radicals [10]; used as enhancers in polymerase chain reaction (PCR), skin protection from UVA induced cell damage, and inhibition of β-amyloid formation in Alzheimer's disease. Ectoines (ectoine and its hydroxy derivative, hydroxyectoine) are used as anti-ageing agents in skin creams, as components of shampoos, for oral care and as adjuvants for vaccines.

Till to date ectoines are known to be produced by only microorganisms. With the increasing applications of osmolytes there is a need to isolate novel microbial flora inhabiting extremophilic environments and study the osmolytes synthesized or accumulated by them. Therefore the aim of the present study is focused on identification of osmolytes accumulated by a newly isolated moderately halophilic and amylolytic *Bacillus* sp. strain *TSCVKK* and switching of osmolytes when it was subjected to hypo and hyperosmotic stress using NMR and ESI Mass spectroscopy.

MATERIALS AND METHODS

Ferrous chloride and Deuterated water (D_2O) were purchased from Sigma, St. Louis, USA; starch soluble was purchased from Central Drug House, New Delhi, India; yeast extract and tryptone were purchased from HiMedia, Mumbai, India and all other chemicals used in the study were of analytical grade and were purchased from local agencies.

Culture conditions

Bacillus sp. strain *TSCVKK* was grown for 24 h in Great salt lake-2 (GSL-2) medium as described earlier [11]. One ml of this culture broth was inoculated into 50 ml of GSL-2 medium of pH 7.5 containing 1% soluble starch with different NaCl concentrations ranging from 0 to 15% (w/v) and was grown for 24, 48 or 72 h with agitation at 180 rpm at 30°C. Growth was monitored by measuring the optical density of the broth at 600 nm in a Unicam UV-Vis spectrophotometer for every 6 h.

Osmolyte extraction

Cells were grown with no NaCl, 1, 3.5, 5, 10 and 15% for 48 h in triplicate flasks and were harvested by centrifugation at 10, 000 rpm for 20 min and were washed twice with Tris buffer, pH 7.5, with 10% NaCl. For determining the effect of time period on solute accumulation, cells were grown up to 24, 48 or 72 h and were used for extraction and identification of osmolytes. In all the cases 500 mg of wet biomass was extracted twice with 70% ethanol at 100°C [12] and the corresponding dry weight was determined after drying the wet biomass at 55^{o}C for 24 h. Ethanolic extracts were concentrated by rotary evaporation under reduced pressure and the residue obtained was dissolved in 1 ml of distill water and lyophilized. Dry weights of ethanolic extracts were quantified gravimetrically and the average of three weights was calculated. Errors were less than 3%.

Identification of osmolytes

a) NMR analysis

Lyophilized extract (30 mg) was dissolved in 0.5 ml of D_2O containing 2,2,3,3-d4-3-(trimethylsilyl) propionic acid sodium salt (TSP) as internal standard and was analyzed in duplicates by ^{1}H NMR, ^{13}C NMR. Spectra were recorded at 23°C on Bruker 400 MHz NMR spectrometer. ^{1}H spectra (400 MHz) of all samples were obtained under the identical conditions (number of scans = 32).

b) ESI-MS analysis

Three milligram of the lyophilized extract was analyzed for mass by ESI-MS using Micromass Q-Tof-Mass Spectrometer. Samples were analysed in duplicates for determining the mass of the solutes.

RESULTS AND DISCUSSION

Bacillus sp. strain *TSCVKK* showed a broad salinity tolerance for growth from 1 to 20% and failed to grow in the absence of NaCl in the growth medium and maximum growth was achieved with 10% NaCl. Growth was decreased when NaCl concentration was decreased or increased from 10% (Fig. 1) which is a prerequisite for an obligate halophile. Weight of ethanol extracted solutes of *TSCVKK* was determined after it was grown with 1, 3.5, 5, 10 or 15% NaCl for 48 h (Fig. 1).

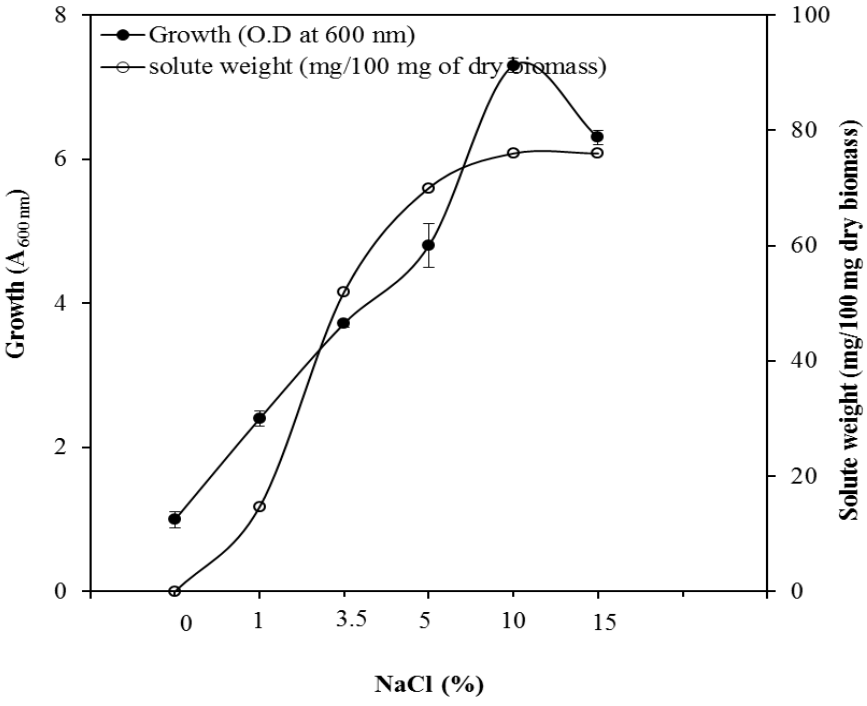

Fig. 1 Growth and solute accumulation by *TSCVKK* grown at different NaCl concentrations in GSL-2 broth containing 1% soluble starch

Salinity dependent increase in the total amount of solutes accumulated was noticed in strain *TSCVKK*. The amount of solute accumulated was high when grown with 10 and 15% NaCl (75 and 76 mg per 100 mg of dry weight of the biomass) and decreased when grown at 3.5 and 5% NaCl (52 and 70 mg). When grown with 10% NaCl, *TSCVKK* cells accumulated 69, 75 and 81

mg of at 24, 48 and 72 h respectively. Halophiles either synthesize or accumulate low molecular weight osmolytes as a survival strategy under high salt environments [10].

Previous report with strain *TSCVKK* showed that maximum amylase was produced when grown in 10% NaCl [11]. The maximum amylase production at high NaCl could be explained by the fact that higher amount of osmolytes accumulated might have contributed for osmoprotection of the organism and also stabilized the amylase produced when grown in presence of 10% NaCl which otherwise would have been denatured. Osmolytes induced protein stability has been well studied [13].

Identification of osmolytes by NMR spectroscopy

^{13}C NMR spectra of the solute extracts of *TSCVKK* cells grown in medium containing 3.5, 5, 10 and 15% NaCl are shown in Fig. 2. Chemical shift values for the signals in the ^{13}C NMR spectrum are given in Table 1. These values were compared with chemical shift values in the NMR spectra available in literature for these osmolytes [12]. As a result betaine and ectoine were identified as key osmolytes present in the ethanolic extracts of this strain. Strain *TSCVKK* might have synthesized ectoine where as betaine could have been taken up from nitrogen source provided in the growth media. Although two solutes were identified at all salinities tested, the signals for ectoine were dominant at higher salinities of 10 and 15% than that of betaine. Fig. 3 shows ^{1} H NMR spectra of whole cell ethanolic extract from 3.5, 5, 10 and 15% NaCl grown culture. The chemical shift values for signals in the ^{1}H NMR spectrum are given in Table 1.

Table 1 Chemical shift values of compatible solutes from *TSCVKK* by ^{13}C & ^{1}H NMR and identification of ectoine and betaine by ESI-MS

Solute	^{13}C-NMR (400MHz,D_2O) δ value	^{1}H-NMR (400MHz, D_2O) δ value	ESI-MS	Solute identified
1	176.7 (C1), 160.6 (C5), 53.5 (C2), 37.4 (C4), 21.5 (C3), 18.3 (C6)	δ 4.07 (t, J = 5.6 Hz, 1H, 2-H), 3.51-3.25 (m, 2H, H-4), 2.20-2.05 (m, 2H, H-3), 2.24 (s, 3H, -CH_3)	Calculated for $C_6H_9O_2N_2$ ($[M+Na]^+$): 165, ($[M+H]^+$): 143	Ectoine
2	δ 174.49 (C1), 70 (C2), 54 (C3)	δ 3.91 (s,-CH_2-), 3.27 (s, 9H, 3X – CH_3)	Calculated for $C_5H_{11}O_2N$ ($[M+Na]^+$): 140, ($[M+H]^+$): 118	Betaine

Spectra were recorded at 23°C on Bruker 400 MHz NMR spectrometer. Identification of solutes was done based on comparison of chemical shift values described by Xu *et al*., [12]

The spectrum shows presence of two components: the major one, ectoine, appeared from δ2.0 to 5.0 ppm regions, and another one, glycine betaine, was identified from two peaks at δ3.3 and 3.9 ppm. Similar peak pattern was found in all the spectra at different salinities of growth and at different times of growth in presence of 10% NaCl. The other signals in both ^{13}C and ^{1}H NMR needs to be studied further for the identification of other osmolytes if any.

Determination of molecular mass of osmolytes by ESI-MS

The presence of betaine and ectoine was confirmed by ESI-mass spectrometry. All spectra showed the presence of betaine (*m/z* 118 $[M+H^+]$ and *m/z* 140 $[M+Na^+]$) and ectoine (*m/z* 143 $[M+H^+]$ and *m/z* 165 $[M+Na^+]$) (Fig. 4). There was a salinity-dependent "osmolyte switch" of strain *TSCVKK* cells where, at lower salinity, betaine was dominant and when grown at higher salinity ectoine was the dominant one. At low salinity (3.5%) the percentage abundance of betaine was higher in the cell extract and in 5, 10 and 15% NaCl; ectoine was accumulated to a higher percentage. This was similar to *Halobacillus halophilus*, where the cells accumulated glutamate and glutamine when grown in 1 M NaCl compared with cells grown in 2 or 3 M NaCl

where proline was the dominant osmolyte [8] and to *Virgibacillus pantothenticus* where at lower NaCl concentration proline was the dominant osmolyte and when NaCl concentration increased ectoine was the dominant one [14]. However the other peaks present in the spectrum were not identified and warrants further study.

In conclusion, strain *TSCVKK* found to secrete industraily important amylase and also biotechnologically important low molecular weight compatible solutes namely ectoine and betaine which have tremendous biotechnological applications. Quantification of compatible solutes and identification of genes involved in the synthesis of these solutes is under progress.

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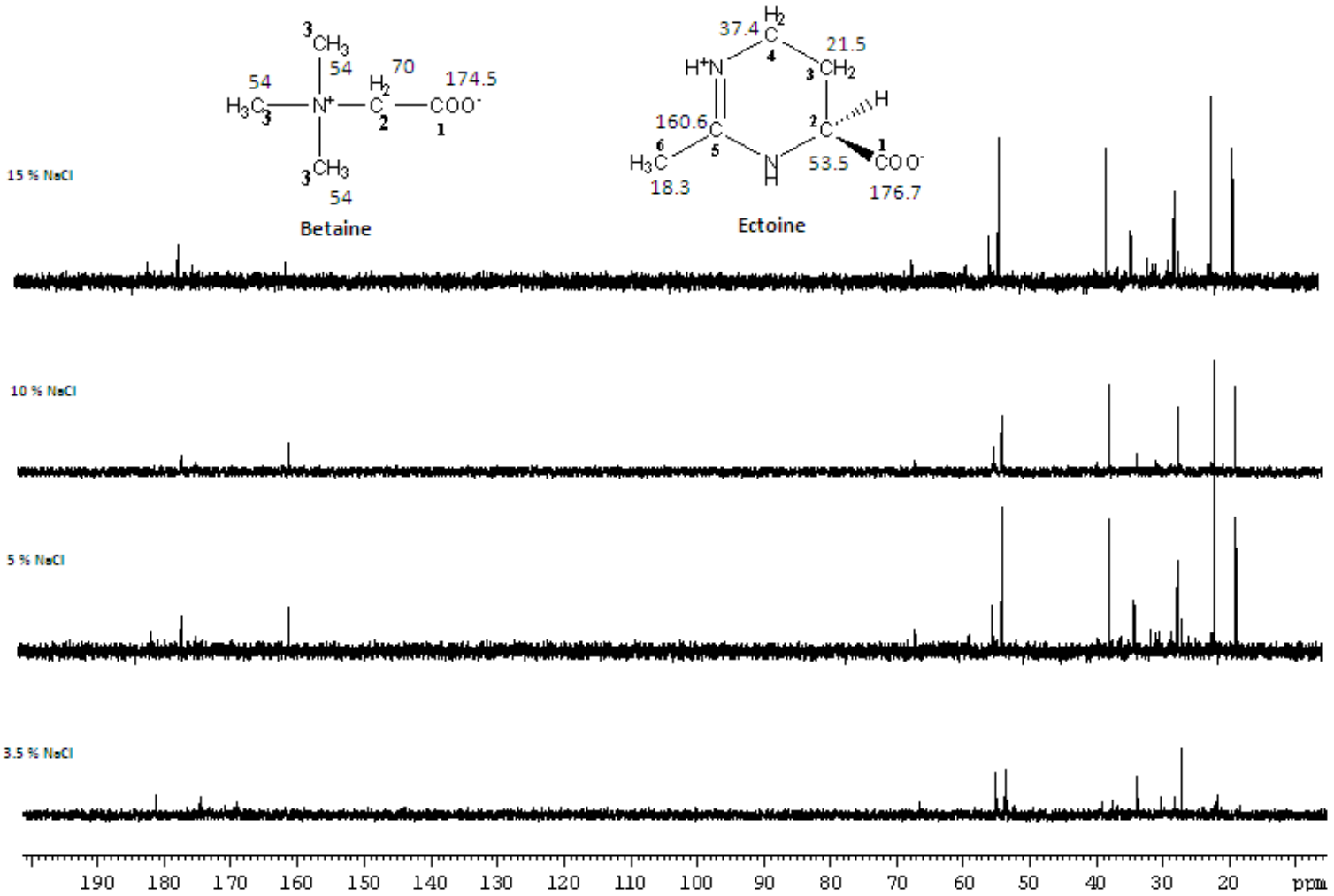


Fig. 2 ^{13}C NMR spectra of osmolytes from 48 h grown cells at different NaCl concentration

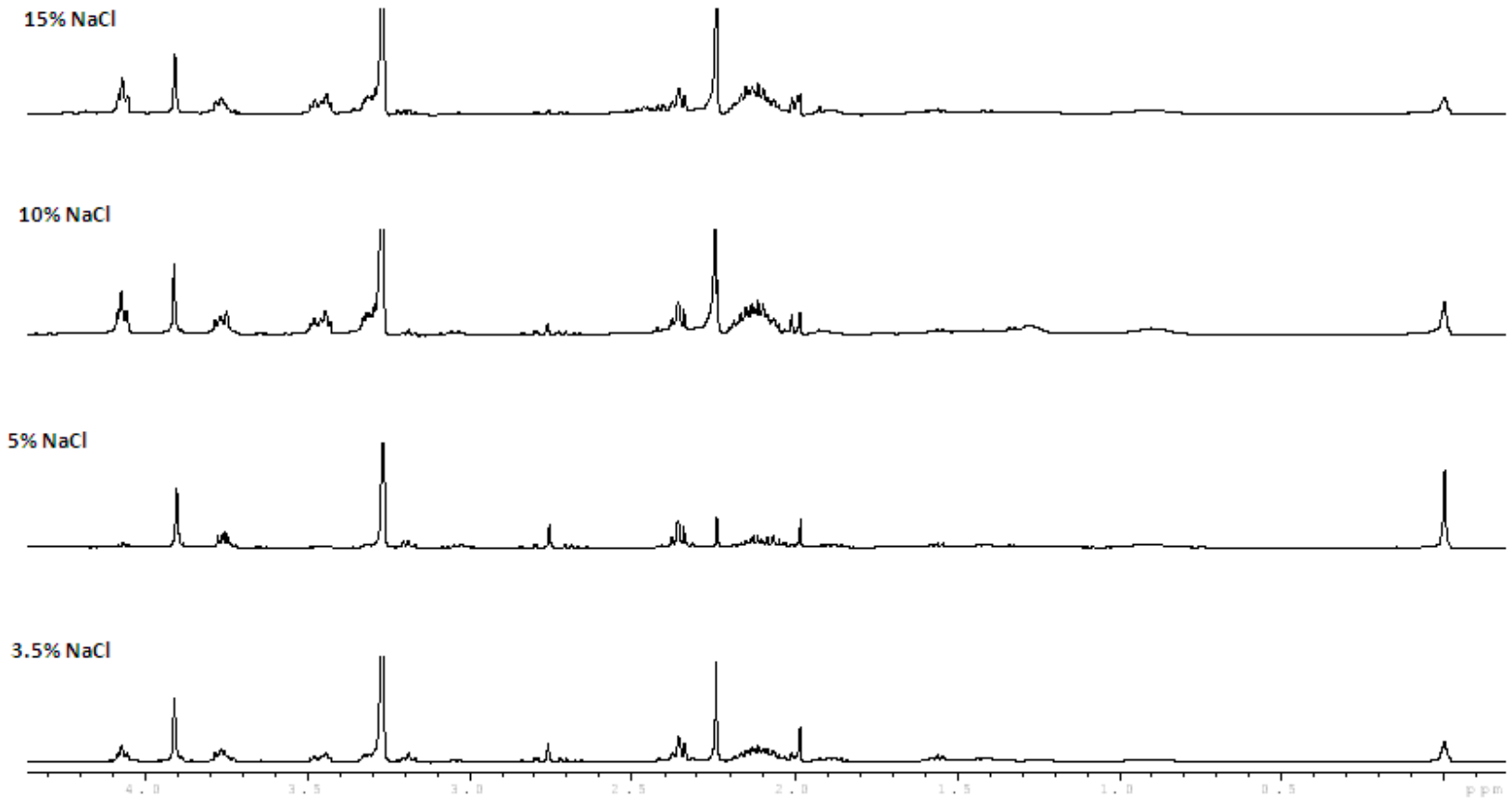


Fig. 3 ^{1}H NMR spectra of ethanolic extracts of cells grown for 48 h at different NaCl

δ for Ectoine: 4.07 (t, J = 5.6 Hz, 1H, 2-H), 3.51-3.25 (m, 2H, H-4), 2.20-2.05 (m, 2H, H-3), 2.24 (s, 3H, -CH_3); for Betaine: 3.91 (s,-CH_2-), 3.27 (s, 9H, 3X –CH_3)

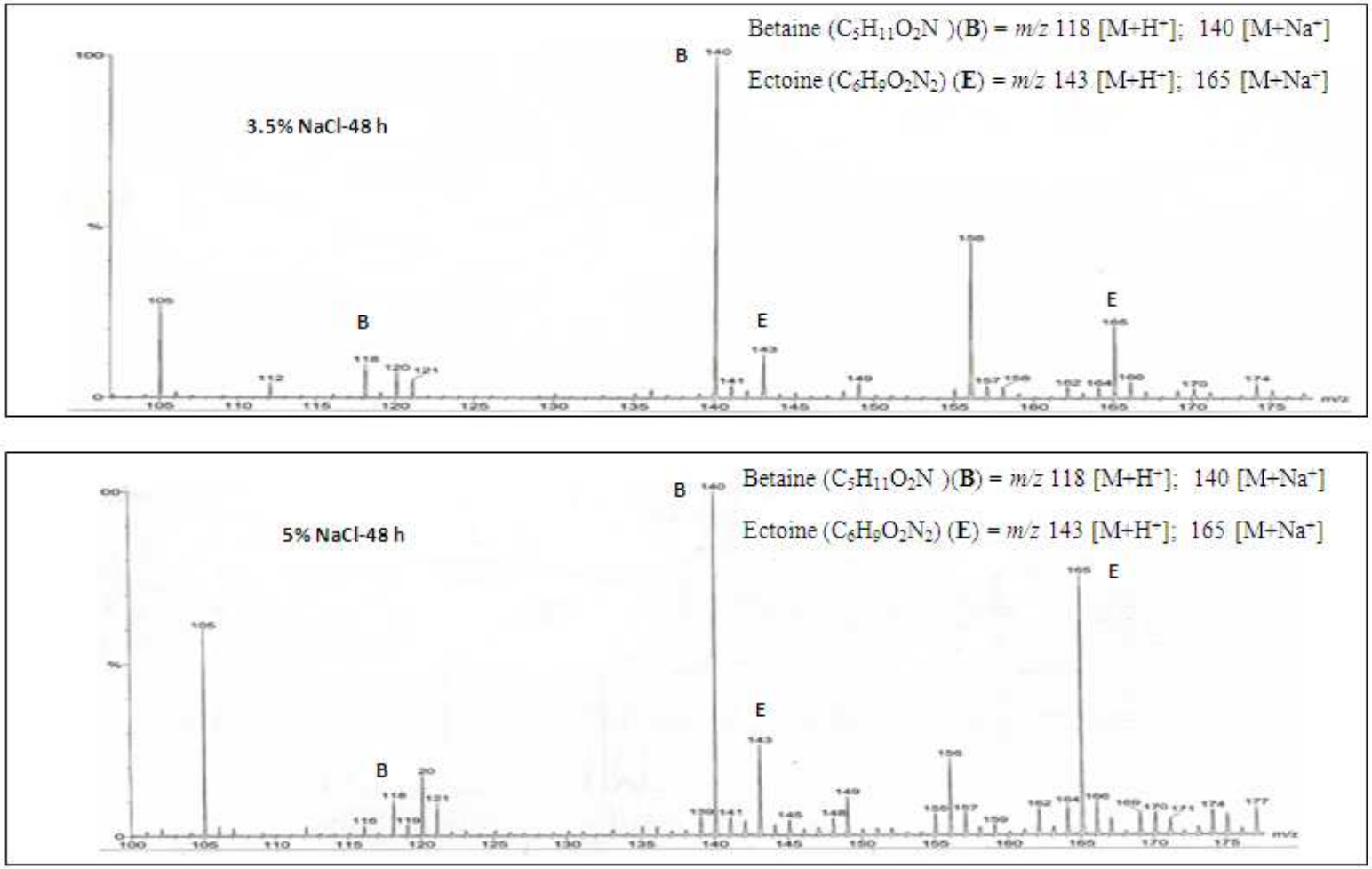
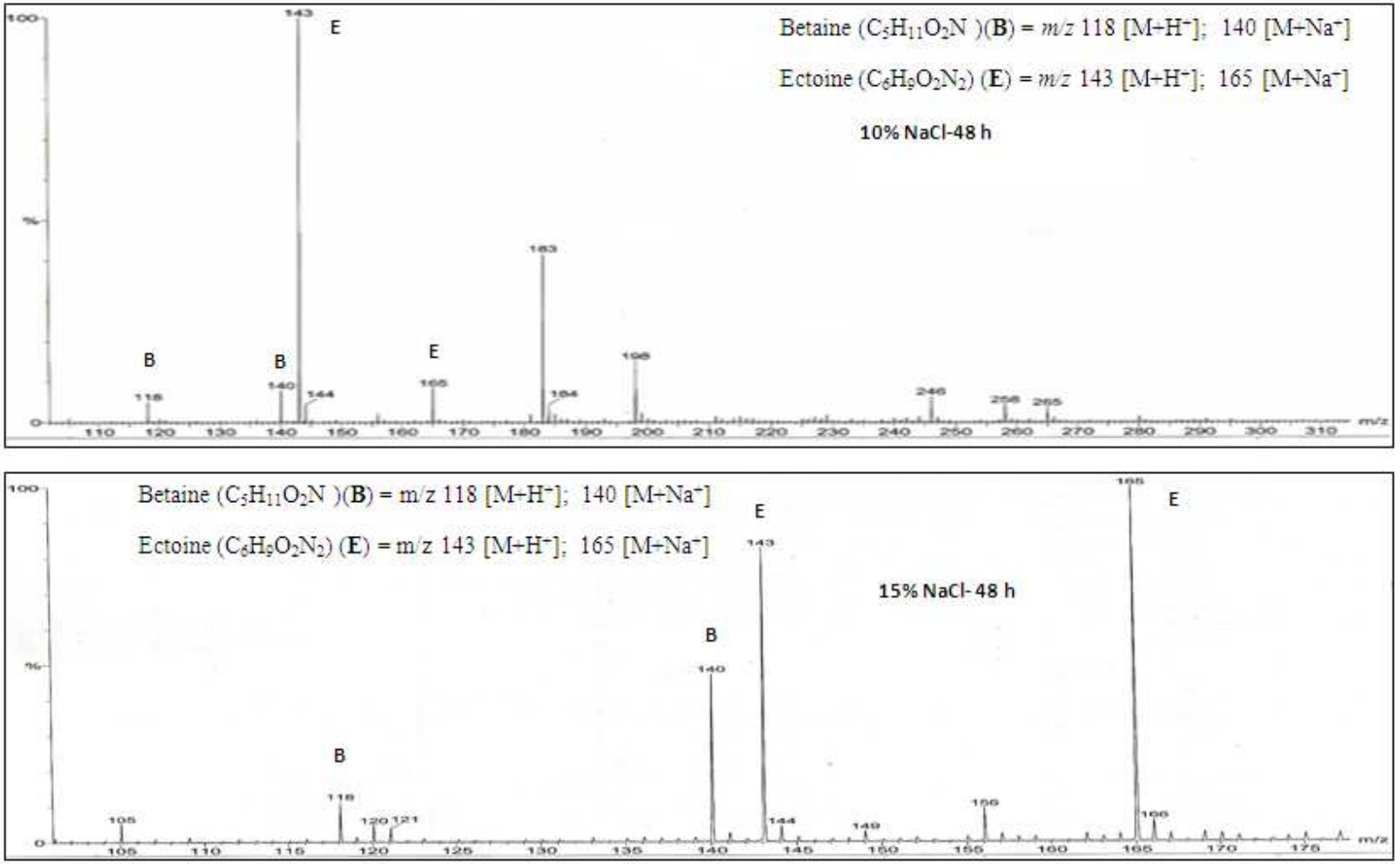


Fig. 4 ESI-MS analysis of osmolytes grown at different salinities



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