As. J. Food Ag-Ind. **2010**, 3(01), 25-34

OPEN ACCESS

Asian Journal of
Food and Agro-Industry
ISSN 1906-3040
Available online at www.ajofai.info

Research Article

Salt stress induced glycine-betaine accumulation with amino and fatty acid changes in cyanobacterium *Aphanothece halophytica*

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This paper was originally presented at the International Conference on the Role of Universities in Hands-On Education, Chiang Mai, Thailand, August 2009.

Abstract

A halotolerant cyanobacterium *Aphanothece halophytica* was grown under various NaCl concentrations from 0.5 – 3.0 M and pH from 6.5 – 10.5. The optimal condition for cell growth was 0.5 M NaCl and pH 9.5. The relationship of cell growth, cell morphology, intracellular solute, and amino acid composition was investigated. Under salt stress conditions, cell growth was reduced when NaCl was increased and cell size was increased with high glycine-betaine accumulation. Ion contents of Na^+, K^+, NH_4^+, and NO_3 were not significantly different. Under both non-stress and salt stress conditions, glutamine was the major amount of amino acid. However, glutamine, aspartate, proline and glutamate were increased under salt stress conditions. Exposure of *A. halophytica* cells to salt stress conditions significantly enhanced saturated fatty acid composition.

Keywords: Cyanobacteria, amino acid, glycine-betaine, salt stress, fatty acid, Thailand

Introduction

Salinity is a physical parameter to determine the ability of organisms to survive in their environment. At high salinity, bacteria, plants and animals adapt to live in their habitats by using two strategies; to exchange ions and/or to accumulate compatible solutes or osmoprotectants. The increase of osmoprotectants is achieved either by increasing of biosynthesis and/or decreasing of degradation or by transporting substances into or out of cells [1, 2].

The compatible solutes, also called osmolytes, include sugars, amino acids and their derivatives, polyols and their derivatives, betaine and ectoines [3]. The compatible solutes in general are low molecular weight organic compounds that accumulate to high intracellular levels under osmotic stress and are compatible with the metabolism of the cell. For example, glycine betaine is the compatible solute in halotolerant bacteria, archaea and cyanobacteria. It is not synthesized by most microorganisms but is taken up from the medium and used for osmoadaptation [1, 4, 5].

The types of organic molecules used for osmotic balance include polyols and derivatives, sugars and derivatives, amino acids and derivatives, betaines, and ectoines as well as peptides altered to remove charges [6]. Osmoprotectants can either be synthesized by the cell or transported into cells from the medium. Osmoprotectants fall into three general chemical categories: (i) zwitterionic solutes, (ii) noncharged solutes and (iii) anionic solutes. [7]. Compatible solutes serve a dual function in living organisms by accumulating them up to molar concentrations; compatible solutes lower the cytosolic osmotic potential and hence make major contributions to the restoration and maintenance of turgor [8, 9].

Glycine betaine was first shown to be the major osmoticum in a halotolerant cyanobacterium *Synechcytis* DUN52 [10]. The unicellular, *Aphanothece halophytica* is a highly halotolerant organism that can grow at high external NaCl concentrations up to 3 M [11, 12]. It was demonstrated that glycine betaine is accumulated as the major osmoticum inside *A. halophytica* cells in response to changes in external salinity [12]. High concentration of salts has been reported to inhibit the activity of many enzymes of both eukaryotic and prokaryotic origins. It has also previously been reported that salt inhibits enzyme activity of RuBisCo from *A. halophytica* and glycine betaine protects the enzyme against salt inhibition [13] and it is not harmful to the metabolic activities of cells even at high concentration.

A. halophytica is a halotolerant cyanobacterium that can grow in a wide range of salinity conditions from 0.25 to 3.0 M NaCl. *A. halophytica* accumulates an osmoprotectant glycine betaine at high salinity. However, the expression of a Na^+/H^+ antiporter from *Vibrio alginolytica* NhaAv resulted in a Na^+-sensitive transformant. Recently, the eukaryotic Na^+/H^+ antiporter gene (*apnha*P) was isolated from the halotolerant cyanobacterium *A. halophytica* and showed that ApNhaP exhibits high Na^+/H^+ exchange activity over a wide range of pH with novel ion specificity [14]. Therefore, it is interesting to examine the effects of expression of ApNhaP and other genes for the synthesis of betaine, catalase and chaperone (DnaK) and their combinations. For this study, the unicellular halotolerant cyanobacterium, *A. halophytica* was selected as a source for an examination of the effects of salt stress on its growth, ion contents and accumulation of amino acids, amino acid derivatives and fatty acid.

Methodology

Culture conditions

A. halophytica is a short, cylindrically shaped cyanobacterium surrounded with a mucous membrane. It multiplies by binary fission. Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution. The NaCl concentration of the culture medium was adjusted to a range from 0.25 to 3.0 M as desired. Cotton-plugged 500-ml conical flasks containing 200 ml of medium were used and shaken on a reciprocal shaker without supplementation of condensed CO_2 gas. The culture flasks were incubated at 28°C under continuous fluorescent white light ($30 \mu E m^2 s^{-1}$) [15]. For the growth experiments, cells at the late logarithmic phase were transferred into fresh medium containing various concentrations of NaCl at pH 7.6. Growth of the cells was determined from the OD_{730}.

Amino acid analysis

Cells were homogenized with 9 volumes of 100% methanol and centrifuged for 5 minutes at 1500xg at 4^{o}C. After centrifugation, the supernatant was transferred to another tube and the remaining pellet was re-extracted with 90% methanol and centrifuged as per the above conditions. 90% methanol extract was pooled with the previous 100% methanol extract and this pooled extract was dried in a vacuum rotary evaporator at 43^{o}C. After drying, dried pellet was re-dissolved in MQ water (500 µl) and the same volume of chloroform was added, mixed on a vortex, and the sample was centrifuged for 5 minutes at 1500xg at 4^{o}C. Upper aqueous phase was taken and filtered with 0.22-µm membrane filter. Filtrate was again dried in a vacuum rotary evaporator at 43^{o}C. Dried samples could be stored at -20 or -80^{o}C until analysis. Before analysis, samples were dissolved in MA solution (pH 2.6, lithium citrate tetra hydrate, methoxy ethanol and perchloric acid) and then injected into the amino acid analyzer by using a shim pack Li column (Shimadzu, Japan).

Lipid extraction, transesterification, and fatty acid analysis

The plasma and thylakoid membrane were extracted by French pressure cell, followed by sucrose gradient centrifugation [16]. Lipids of two fractions, plasma membrane and thylakoid membrane were extracted with chloroform/methanol/water as described by Bligh and Dyer (1959) [17]. The oil sample was then placed in a vacuum oven kept at 60^{o}C for 30 min and then accurately weighed and the percentage yield was calculated. Fatty acid methyl esters were prepared by 15 min incubation at 95°C in boron trifluoride/methanol using the method of Morrison and Smith (1964) [18]. The fatty acid methyl esters were extracted with hexane. Fatty acid methyl esters (Sigma-Aldrich, England) standard solution mixture was used. The fatty acid composition of the esterified oil was characterized and quantified using Shimadzu model GC-2010 gas chromatograph as described below. BP-20 column (30m ×0.25mm) was used and the initial column temperature was 210^{o}C and final temperature was 250^{o}C. The detector and temperature were FID and 300^{o}C, respectively. Hydrogen and air flows were 30 and 300 mL/min, respectively.

Other methods

Cellular ions were determined with a Shimadzu PIA-1000 personal ion (Japan). Betaine was extracted as described previously and measured after esterification with a time of flight mass spectroscopy (KOMPACT MALDI IV tDE, Shimadzu/Kratos, Japan) using d_{11}-betaine as an internal standard [19].

Results

Growth of A. halophytica at various NaCl concentrations

A. halophytica was able to grow in BG$_{11}$ plus 18 mM NaNO$_3$ and Turk Island Salt Solution plus modified BG$_{11}$ medium photoautotropically at 30^{o}C and varied salt concentration from 0.5 M up to 3.0 M NaCl (Figure 1). Cell morphology at 0.5 M NaCl (normal condition) and 2.0 M NaCl (stress condition) were investigated. The changes in the shape of cells during incubation with 0.5 M NaCl were examined and a significant increase in the diameter of cells was observed at 2.0 M NaCl (data not shown). Shape of cells grown under stress conditions was longer and flatter than cells grown under the normal conditions.

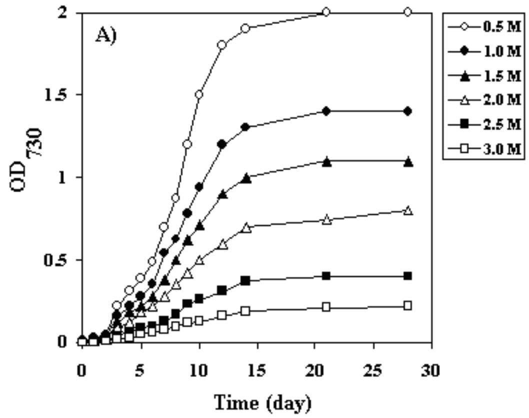

Figure 1. Growth curve of *A. halophytica* grown in medium containing various NaCl concentrations from 0.5 – 3.0 M.

Chemical and betaine contents

Cells were grown in BG$_{11}$ containing 0.5 M NaCl (normal condition) or 2.0 M NaCl (stress condition) for 7 day**s** and betaine, Na^{+}, K^{+}, NO$_3^{-}$, and NH$_4^{+}$contents were determined, data as shown in Figure 2. At stress condition; betaine content was highest whereas Na^{+}, K^{+}, and NH$_4^{+}$ were slightly increased. NO$_3^{-}$ was slightly reduced to about 30% of the normal condition.

Amino acid contents at normal and stress conditions

Cells were grown in BG11 containing 0.5 M NaCl (normal condition) or 2.0 M NaCl (stress condition) for 7 days and amino acid contents were determined, data as shown in Figure 3. The result showed that glutamine was the highest amount of amino acid at both conditions. The small figure inside Figure 3 indicates that glutamine slightly increased at stress condition to compare with the normal condition. Aspartate, proline and glutamate were induced to increase about two-fold, while glycine and arginine were reduced at stress conditions when compared with the normal condition.

Fatty acid composition at normal and salt stress conditions

The fatty acid composition of the plasma and thylakoid membrane was analyzed by a gas chromatograph and the results showed that the dominant fatty acids found in the oil of plasma and thylakoid membranes represented equal data as unsaturated fatty acid (C16:1 and C18:1). The investigated fatty acids of samples are shown in Table 1.

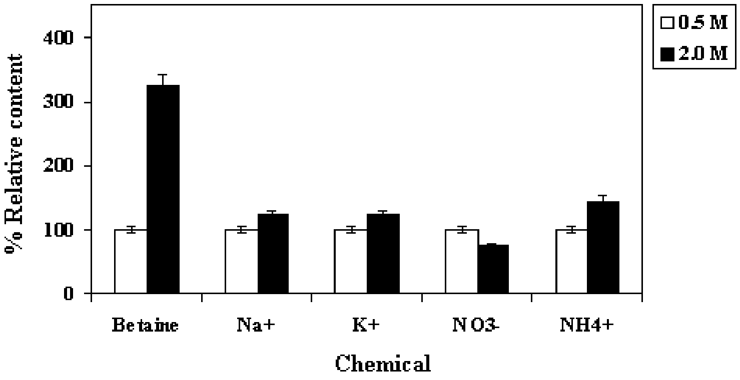

Figure 2. Chemical contents of *A. halophytica* grown under normal and stress conditions.
A. halophytica was cultured in medium containing 0.5 M or 2.0 M NaCl and contents of betaine, Na^+, K^+, NO_3^-, and NH_4^+ were determined. Data are means from three independent experiments with vertical bars representing standard errors of the means.

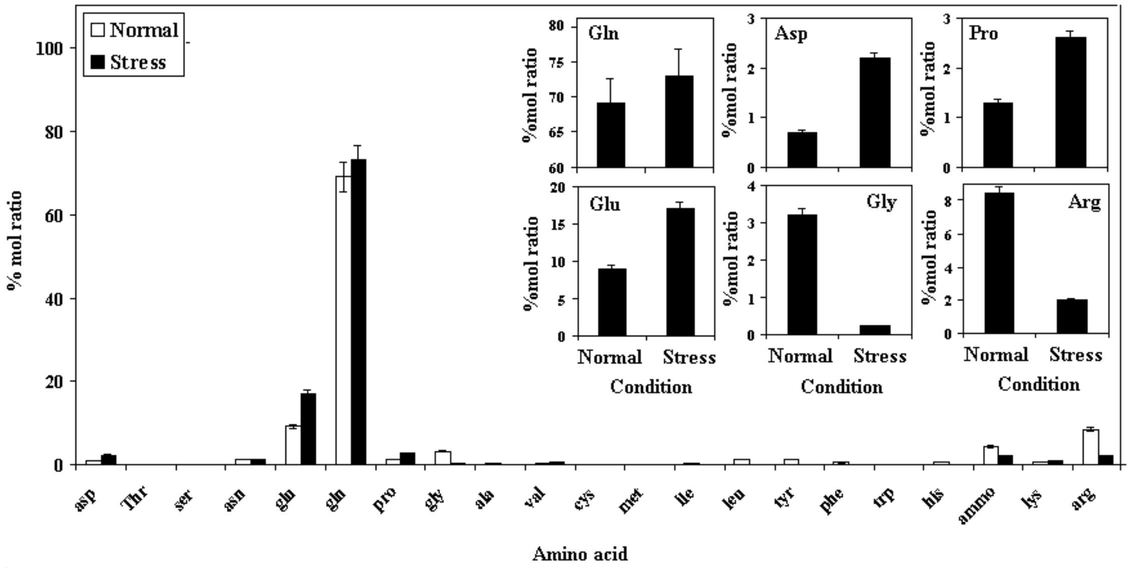

Figure 3. %mol ratio of amino acids at normal and stress conditions.
A. halophytica was cultured in medium containing 0.5 M or 2.0 M NaCl and amino acid contents were determined. Data are means from three independent experiments with vertical bars representing standard errors of the means.

Table 1. Comparison of the fatty acids (C16 and C18) in plasma and thylakoid membrane of Aphanothece halophytica under normal and salt stress conditions.

Membrane fraction	Fatty acid composition* (%)							
	Normal condition				Salt stress condition			
	C16:0	C16:1	C18:0	C18:1	C16:0	C16:1	C18:0	C18:1
Total membrane	23	13	4	60	45	13	5	37
Plasma membrane	25	15	5	55	50	14	6	30
Thylakoid membrane	23	11	5	68	40	12	4	44

*Data are means from three independent experiments with vertical bars representing standard errors of the means.

Discussion and Conclusions

Living organisms must be able to adapt to changes in high osmolarity and salinity of their environment. Basic schemes of living organisms to high salinity are exchange of ions and to accumulate some substances, named compatible solutes or osmoprotectants. Almost all bacteria and halotolerant cyanobacteria accumulate glycine betaine to protect cells against osmotic stress.

Growth rate of cyanobacterium *A. halophytica* was investigated in various NaCl concentrations. The growth rate was decreased when the cells were transferred to the medium with higher NaCl concentration. The results showed that *A. halophytica* could adapt to a broad range of salt concentrations from 0.5 – 3.0 M NaCl (Figure 1).

The cessation of growth due to salt stress probably occurred as a result of energy being diverted to the initial adjustment of the cells by regulating the influx and efflux of certain ions. The influx of Na^+ and K^+ in *A. halophytica* in 100% sea water medium has previously been shown to occur mostly in the first 24 hours [12, 20]. Furthermore, uptake and extrusion of Na^+ in *Synecocystis* PCC 6714 have also been shown to occur during the initial period of hypersaline treatment [20, 21]. It remains to be clarified how the movement of various solutes in and out of *A. halophytica* affects the osmoregulation of the cells. Results similar to those observed in *A. halophytica* have been recently reported for the effect of salt stress on growth of salinity-adapted cyanobacterium, *Spirulina platentis*.

A. halophytica cells increased significantly in size. Thus, salt stress or osmotic stress had different effects on the proliferation of *A. halophytica*. To date, little attention has been paid to the morphological changes that occur in *A. halophytica* cells under stress conditions. The result of this research suggests that NaCl might arrest the formation of the shape of cells (data not shown). The results demonstrated clearly that *A. halophytica* cells under salt stress were highly heterogeneous with respect to their size and shape. The effects of salt stress on cell size of the halotolerant bacterium *Staphllococcus aureus* was investigated in detail and demonstrated that salt shock due to 2.5 m NaCl increased the cell size [22, 23].

The content of ions, betaine and amino acids in *A. halophytica* under normal and stress condition was determined. Na^+, K^+, and NH_4^+ slightly increased but NO_3^- slightly decreased when cells were transferred and grown under stress conditions. High salt concentration imposes both hyperosmotic and hyperionic stress. *Halobacterium salinarum* was grown in the medium containing Na^+ (3.3 M) and K^+ (0.05 M) but Na^+ and K^+ inside the cells were 0.8 M and 5.3 M, respectively. *H. salinarum* used K^+ as the compatible solute. High intracellular K^+ was required for ribosomal stability and for activity of various enzymes [24]. To resist the salt stress *A. halophytica* used ions for osmotic adjustment. Ion accumulation or ion exchanging in the cell keeps sodium away from the cytoplasm, while at the same time there is not much difference in the ions inside the cell under both conditions. To test directly, the cellular compartmentalization of sodium in cells resulted in higher sodium uptake, measured by an ion analyzer.

Of particular interest in this experiment, amino acid contents under normal and stress conditions were determined. Glutamate and glutamine were major amino acids found at both conditions. Glutamine was increased a little at the stress condition when compared with the normal condition. Aspartate, proline and glutamate were enhanced about two-fold, while glycine and arginine were decreased at the stress condition. Glycine is a substrate for glycine betaine synthesis, thus the decrease of glycine is probably related to the salinity response of cells by increasing betaine content [14]. Exogenously supplied proline is osmoprotective for bacteria, facilitating growth in highly saline environments [1, 25, 26]. Accumulation of proline in the cytoplasm is accompanied by a reduction in the concentrations of less compatible solutes and an increase in cytosolic water volume [8, 27]. Proline is an allosteric inhibitor of glutamate kinase and dehydrogenase [29]. Inhibition of enzymes which catalyze the conversion of glutamic acid to glutamic acid semialdehyde could prevent proline accumulation beyond a certain concentration. It is also possible that osmoregulatory control via potassium accumulation requires less energy than de novo synthesis of free amino acids.

It has been shown that in *A halophytica*, the ratio of saturated to unsaturated fatty acids in both the plasma and thylakoid membranes increases when NaCl concentration was incresed. At high salinity or osmotic condition, the cell also showed that an increase of membrane lipids enhances activity of Na^+/H^+ antiport of *Synechococcus* sp. [30, 31, 32, 33].

In *A. halophytica*, it is indicated that tolerance to high salinity is associated with intracellular accumulation of free neutral amino acids and, under conditions of extreme salt stress, with selective internal concentration of potassium. The exception was a slight increase of Na^+, K^+ and NH_4^+ and a great increase of some amino acids (asparagine, proline and valine; as compatible solutes from many reports) [7]. Betaine was found to increase three-fold under salt stress when compared with the normal condition. This result showed that *A. halophytica* accumulates betaine at high salinity. Previous research detailed that *A. halophytica* synthesized betaine by two steps of enzyme GSMT and DMT at high salinity [14]. The strategy used by most extremely halophilic bacteria is to synthesize or uptake the organic osmolytes called compatible solutes which are highly soluble and compatible with cellular metabolism and the most important compatible solute is glycine betaine [9].

Acknowledgement

This work was financially supported by the Fund of Research Department of Srinakharinwirot University (027/2552 and 312/2551) and The Commission on Higher Education, Thailand (The university staff development consortium). The authors would also like to thank the Faculty of Science, Srinakharinwirot University for their support to attend the International Conference in Chiang Mai.

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