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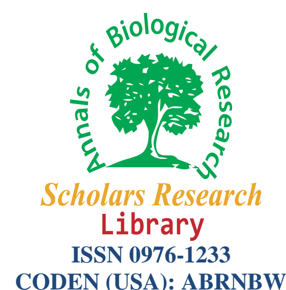

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Modification of fatty acid composition in salt adopted *Synechocystis* 6803 cells

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ABSTRACT

Cyanobacteria are having the potential for the development of effective adaptive mechanism during their three billion years of evolution. They are capable of performing oxygenic photosynthesis which is similar to eukaryotic algae and plants. They are considered to be a good model system for studying plant responses to environmental stress. The strain Synechocystis sp. PCC 6803 which is a salt sensitive mutant was used as a model organism in many laboratories to study the salinity adaptations. The unicellular cyanobacterium Synechocystis sp.PCC 6803 was grown in BG 11 medium as a batch culture with different concentration of NaCl. The results of analyzed various biochemical parameters were conform that the accumulation of chlorophyll a and proline content in salt adopted cells. Furthermore in the fatty acid composition, out of 11 fatty acids eight were saturated and three of them were unsaturated fatty acids in gas chromatography. In salt adopted cells the modification of fatty acid composition was understand in this studies.

Key words: Cyanobacteria, Fatty acids, Response, Salt stress, *Synechocystis 6803 cells*.

INTRODUCTION

Soil is a major constraint for food production because it limits crop yield and restricts use of land previously uncultivated. United National Environment Programme estimates the approximately 20% of agricultural land 50% of crop plant in the world is salt stressed. Salt stress had both osmotic and ionic effects. The osmotic effect decreased the water in that cytosol rapidly increasing the intracellular concentrations of salts. The ionic effects was caused by a influx of Na^+ ions through K^+/Na^+ channels that also increased concentrations of salts in the cytosol. The plant response to salinity consists of numerous process that must function in coordination to alleviate both hyperosmolarity and ion disequilibrium. The transport system that facilitate cellular capacity to utilize Na^+ for osmotic adjustment and the growth and the role of the Salt Overly Sensitive (SOS) signal transduction pathway in the regulation of iron homeostatic and salt tolerance.

Cyanobacteria are a group of photoautotrophic organisms that occur in wide range salinities. They are able to live in almost all ecosystem of the planet. This capability is based on the development of effective adaptive mechanism during their three billion years of evolution. They are considered to be a good model system for studying plant responses to environmental stress. *Synechocystis sp*. PCC 6803 cells is a unicellular fresh water cyanobacterium which naturally transformable and can grow photoheterotrophically [3]. The strain *Synechocystis sp.* PCC 6803 which is a salt sensitive mutant was used as a model organism in many laboratories to study the salinity adaptations.

Lipids are the esters of fatty acids and alcohols that compromise a large group of structurally distinct organic components like fats, waxes, phospholipids, and glycerolipids. Cyanobacteria may contain significant quantities of lipids with compositions similar to those of vegetables oils. The lipids of fatty acids such as C18 linoleic acid, C20 eicosapentaenoic acids. The lipids of cyanobacteria are generally esters of glycerol and fatty acids. They may be either saturated or unsaturated. The fatty acid might be involved in the protection against salt stress. When photosynthesis organisms are exposed to salt stress, the fatty acids become unsaturated. [1] Have acid targeted mutagenesis to alter genes for fatty acid desaturase in *Synechocystis* 6803 and they have produced strains with decreased level of unsaturated fatty acids in their membrane lipids [30] as well as tolerance to salt. The result demonstrate than an increase in the unstauration of fatty acids in membrane lipids enhances the tolerance to salt stress of photosynthetic and Na^+/H^+ antiport system of *Synechocystis* 6803.

MATERIALS AND METHODS

The unicellular cyanobacterium *Synechocystis sp*.PCC 6803 was grown through batch culture in BG 11 medium [25]. The culture of *synechocystis sp*.PCC 6803 was treated with different concentrations of NaOH 50mM, 100mM over the control. This culture should grow through continuous agitation in shakers about 60rpm (Fig. 1). The optimum conditions of 20°C were provided for the growth of organisms. The strain which is a salt sensitive mutant was used as a model organism. After 15 days the culture was used for the various biochemical parameter analyses.

Fig. 1 ***Synechocystis sp*.PCC 6803 cell culture grows in agitation shakers at 60rpm**

Estimation of Chlorophyll a: Chlorophyll a was estimated by Mackinney [19]. 10ml of culture was taken and centrifuged at 5000rpm for 10mins. The chlorophyll was extracted with 90% methanol. The content was centrifuged at 5000rpm for 10mins and the supernatant containing chlorophyll a was collected and the process was repeated till the pellet turn non green. The supernatant were pooled together and the volume was made upto 10ml and absorption was measured at 665nm by using spectrophotometer. The chlorophyll content was expressed in ug/ml of culture.

Estimation of Proline: Proline content was estimated according to Bates [4]. Culture was centrifuged and the harvested cells were ground in 10 ml of 3% aqueous sulfosalicylic acid. The proline content was calculated from the standard curve plotted with authentic proline and the proline content was expressed in nmol/ml of culture.

Analysis of fatty acid in Gas Chromatography

Extraction of Fatty Acid: 10ml of homogenous algal suspension was centrifuged at 5000 rpm for 10 mins. Then the supernatant was discarded, and the pellet was washed twice with distilled water. Sonicate or grind with glass powder a known weight of the algal pellet in a known volume of chloroform: methanol (2:2) (v/v). Then the extracts filter through filter paper and ensure the filter paper is free of lipids. Then 1/3 of volume of distilled water was added to the filtrate and vortex thoroughly to remove the water soluble impurities (upper layer) finally the water content was removed by the filtrate. Then little amount of sodium sulphate crystals was added and vortex. (Non-

clumping of crystals indicates that the filtrate is free of moisture). Then transfer the filtrate to a pre-weighted bottle (A). Dry the filtrate under the rotator evaporator. Reweigh the bottle (B). To a known volume of sample 1 ml of saponification reagent (dissolve 45g of NaOH in 300ml of meathanol water mixture (1:1) was added and tightly close the tube with Teflon lined screw cap. Vortex the content and boil the contents for 30 min (bubbling in the tube during saponification indicates leakage). Then 2ml of methylation regent (325ml of 6N HCL with 275ml of CH_3OH) was added and mixed thoroughly. Then it should keep in water bath at 80ºC for 20 minutes. Then cool in to the room temperature and add 1.25ml of extraction solvent (200ml of hexane with 200ml of anhydrous diethyl ether) tightly close the tube and rotate for 10 min end over end. Discard the aqueous lower phase. Finally add the base wash (dissolve 10.8 g of NaOH in 900ml of distilled water) to the tube. Rotate for 5 mins end over end tightly closing the tube. Transfer 2/3 of the extract (upper phase) to a Gas Chromatography vial.

RESULTS

Synechocystis 6803 cells were grown in BG 11 medium in the presence (50 and 100mM) and the absence of NaCl for 15 days and on the 15th day various biochemical parameters were analyzed.

Table 1 Chlorophyll a content of *Synechocystis* 6803 cells under saline conditions

S.No	Concentration of NaCl (mM)	Chlorophyll a content (ug.ml-1)
1	0	7.269 (100)
2	50	10.321 (142)
3	100	9.086 (125)

(Values are the average of at least three independent experiments)

The analysis of chlorophyll a content in *Synechocystis* 6803 showed that the chlorophyll a content increased with increased NaCl concentration (Table 1). The drastic increases (>300%) in the proline content in saline stressed cells suggest that these cells are under osmotic stress. (Table 2).

Table 2. Proline content of *Synechocystis* 6803 cells under saline conditions

S.No	Concentration of NaCl (mM)	Chlorophyll a content (umoles.ml-1)
1	0	27.80 (100)
2	50	66.72 (240)
3	100	97.30 (350)

(Values are the average of at least three independent experiments)

Many higher plants are known to accumulate proline during osmotic stress [5]. The proline was accumulated in salt/osmotic stressed conditions in higher plants. The capacity to accumulate proline is being taken as criteria for the development of stress tolerant in plants. Even though the proline accumulation continued till 100mM NaCl treatment. There was 350% increase in proline level in 100mM NaCl treated cells. These results unequivocally suggest that proline could be an important osmoprotectant compound in *Synechocystis* 6803 cells. Oxidative stress occurs when plant are exposed to various forms of environmental stress. Plant can be produce antioxidants for protection against the cytotoxic species or activated O_2, such as super oxide (O_2-), H_2O_2, and the hydroxyl radical (OH-)[12].

Lipids are the most effective source of storage energy, functions of insulators of delicate internal organs and hormone and play an important role as the structural constituents of most of the cellular membranes. Analysis of fatty acids in control and NaCl adopted *Synechocystis* 6803 showed interesting variations in the fatty acids composition of NaCl adopted *Synechocystis* 6803 cells (Tables I3,4 and 5). Alterations in the lipid content of membranes of an organism are of major importance in response to environmental stresses [23, 27 and 29]. Fatty acid analysis of *Synechocystis* 6803 cells showed the presence of 22 fatty acids in control cells. With the available standards could identify only 11 fatty acids of them eight were saturated and three of them were unsaturated fatty acids (Table.3).

Table 3 Fatty acid profile of *Synechocystis* 6803 cells in the absence of NaCl

S.No	Retention time (min)	Fatty acid	Area (uV.s)	Area (%)
1	5.286	Butyric acid methyl ester (C4:0)	56302.77	0.11
2	8.633	?	25834.88	0.05
3	10.773	Caproic acid methyl ester (C6:0)	32781.22	0.06
4	12.920	?	24386.48	0.05
5	13.548	?	102300.83	0.20
6	15.550	?	48110.56	0.10
7	16.693	Capric acid methyl ester (C10:0)	12871.51	0.03
8	16.786	?	38078.94	0.08
9	17.461	?	22944.87	0.05
10	18.908	Lauric acid methyl ester (C12:0)	460334.75	0.91
11	20.508	Tridecaonic acid methyl ester (C13:0)	29399.91	0.04
12	25.033	Pentadeconic acid methyl ester (C15:0)	251349.09	0.50
13	27.641	Palmitic acid methyl ester (C16:0)	327614.11	0.65
14	31.805	?	43182.75	0.09
15	33.132	?	97893.18	0.019
16	33.998	?	44210.96	0.09
17	36.907	Linoleic acid methyl ester (C18:2n6c)	68170.48	0.14
18	44.423	?	49816.85	0.10
19	445.224	Eurcic acid methyl ester (C22:1n9)	12835.55	0.03
20	48.639	Cis- 13,16, docosadienoic acid methyl ester (C22:2)	21486.12	0.04
21	49.578	?	49608.71	0.10
22	50.688	Lignoceric acid methyl ester (C24:0)	130605.78	0.26

(?) – Unknown/standard reference not available

The smallest fatty acid identified was Butyric acid (C4:0) and the longest one was Lingoceric acid (C24:0). The major constituent fatty acid was Lauric acid (C12:0), trace amounts of Docosadienoic acid (C22:2) and Tridecanonic acid (C13:0) were also present in the *Synechocystis* 6803 cells. Significant amount of Linoleic acid was also present in these cells (Table 3).

Table 4 Fatty acid profile of *Synechocystis* 6803 cells in 50mM NaCl concentrations.

S.No	Retention time (min)	Fatty acid	Area (uV.s)	Area (%)
1	10.614	Caproic acid methyl ester (C6:0)	60616.52	0.22
2	15.391	?	372022.07	1.38
3	16.101	Capric acid methyl ester (C10:0)	28891.26	0.11
4	18.698	Lauric acid methyl ester (C12:0)	113738.65	0.42
5	19.829	?	61644.59	0.23
6	27.296	Palmitic acid methyl ester (C16:0)	164433.11	0.61
7	36.563	Linoleic acid methyl ester (C18:2n6t)	91362.81	0.34

(?) – Unknown/standard reference not available

Imposition of salt stress to *Synechocystis* 6803 cells brought in drastic changes in their fatty acid profile (Table 4 and 5). Salt stressed *Synechocystis* 6803 cells showed only seven out of the 22 fatty acids observed in control cells. Except Linoleic acid (C18:2) all other unsaturated fatty acids disappeared even at 50mM NaCl treated *Synechocystis* 6803 cells. These results indicate the susceptibility of unsaturated fatty acid to salt stress in *Synechocystis* 6803 cells. In contrast to the unsaturated fatty acids, there was a rapid accumulation of saturated fatty acids in NaCl treated *Synechocystis* 6803 cells (Table 5).

Table 5 Fatty acid profile of *Synechocystis* 6803 cells in 100mM NaCl concentrations

S.No	Retention time (min)	Fatty acid	Area (uV.s)	Area (%)
1	10.339	Caproic acid methyl ester (C6:0)	49882.39	0.34
2	15.376	?	529460.04	3.57
3	16.089	Capric acid methyl ester (C10:0)	28034.86	0.19
4	18.709	Lauric acid methyl ester (C12:0)	169569.52	1.14
5	27.334	Palmitic acid methyl ester (C16:0)	170438.98	1.15
6	37.005	Linoleic acid methyl ester (C18:2n6t)	100365.73	0.38
7	50.375	Lignoceric acid methyl ester (C24:0)	125748.82	0.85

(?) – Unknown/standard reference not available

It is to be noted that under saline conditions cyanobacteria are known to synthesize increased amount of unsaturated fatty acids[1]. There was upto six fold increase in the population of Caproic (C6:0) and Capric acids (C10:0) in NaCl treated *Synechocystis* 6803 cells. There was also 1.25 fold increase in the Lauric acid content in salt stressed *Synechocystis* 6803 cells (Table 5). There was a 2 fold increase in the Palmitic acid content in 100mM NaCl treated *Synechocystis* 6803 cells. It was interesting to note that under salt stressed conditions there was an increased accumulation of an unidentified fatty acid in *Synechocystis* 6803 cells (Table 4 and 5). In 100mM NaCl treated *Synechocystis* 6803 cells there was a 35 fold increase in this unidentified fatty acid. The specific accumulation of fatty acids has been reported in number of cyanobacteria [25, 14, and 17]. Since this unidentified fatty acid was seen only in NaCl treated cells, we suggest that this fatty acid is probably expressed only under saline conditions in *Synechocystis* 6803 cells. This unidentified fatty acid could be a marker for salt stress in *Synechocystis* 6803 cells. Salt stress also brought in a specific structural change in Linoleic acid. The double bond in the sixth position has changed from cis configuration to trans configuration in NaCl treated *Synechocystis* 6803 cells (Table 5). Similar observations were also made by other workers [22].

DISCUSSION

Analysis of *Synechocystis* 6803 cells adapted to different NaCl concentration revealed the accumulation of chlorophyll in different concentration NaCl tested (Table 1). Other workers also made similar observation in salinity adapted *Synechocystis* 6803 cells [28]. In photosynthetic organisms the chlorophylls bind to specific proteins and are organized into chlorophyll protein complexes, which are associated with the photosystems of the photosynthetic membranes [19 and 7]. Proline has been shown to accumulate during salt stress in higher plants and in some algae [5 and 10]. Being a small molecular weight compound (M.Wt. 115.13) with hygroscopic proline has been showed to be an ideal molecule to act as an osmoprotectant in salt stressed cells. Accumulation of proline in NaCl adapted *Synechocystis* 6803 cells suggests that these cells are under osmotic stress (Table 2). Salinity tolerant/insensitive cells are known to accumulate more proline than the normal cells [9]. Since higher amount of proline accumulation was observed only in salt adapted cells, it is proposed that the *Synechocystis* 6803 cells have acclimatized themselves to the saline environments.

There are many environmental factors that limit the growth and productivity of micro organisms; salt stress is one of them. Cynobacterial systems which have a wide range habitat have shown excellent adaptive mechanisms to various environmental conditions and hence they were widely used as model system of various environmental adaptation studies [14]. Several studies have suggested that lipids might be involved in the protection against salt stress [16, 18, 27 and 30]. When photosynthetic autotrophs were exposed to salt stress, the fatty acids of membrane lipids are desaturated. Increased level of unsaturated fatty acids has been shown to enhance the salt tolerance in *Synechocystis* 6803 cells [2]. In our studies, *Synechocystis* 6803 cells produced more amounts of saturated fatty acids in comparison with that of unsaturated fatty acids (Table 3). However it is to be noted that there was 2.4 fold increase in the Linoleic acid (an unsaturated fatty acid-C18:2) content in NaCl treated cells (Table 5).

Biochemical analysis of salt stressed *Synechocystis* 6803 cells showed drastic accumulations of proline (Table 2). Proline is known to accumulate in higher plants under saline conditions [7 and 10]. Salt stresses known to induce oxidative stress in several organisms including Cyanobacteria [6]. Oxidative stress is known to induce peroxidation of lipids in salt stressed organisms [25]. Photosynthetic systems produce several free radical scavengers such as carotenoids to ameliorate the peroxidation of lipids [13]. Proline is also proposed to be an antioxidant in plants and bacterial systems [11]. *Synechocystis* 6803 cells have been shown to produce tocopherol as antioxidant which has been shown to protect the *Synechocystis* 6803 cells from peroxidation of lipids [21]. Hence, the observed fatty acids changes in *Synechocystis* 6803 cells could be explain only in terms of possible oxidative stress induce by salt stress in them. The very high amount of proline observed in salt stressed *Synechocystis* 6803 cells unequivocally suggest that these cells are under oxidative stress (Table 2). Salt stressed *Synechocystis* 6803 cells have been shown to be have an increased level of peroxidase activity. Higher level of peroxidase activity is required to scavenge the free radicals produced during saline conditions. Increased level of peroxidase and catalase activity oxygen species (ROS)[21]. These ROS are known to target the unsaturated fatty acids [25] and propagate a chain reaction of lipid peroxidation. Hence, the observed decrease in the amount of unsaturated fatty acids in NaCl treated *Synechocystis* 6803 cells could be attributed to the possible increase in the amount of ROS in these cells. Elevated levels of proline in these cells (Table 2) also substantiate our claim.

Contrary of the earlier findings, there was a general decrease in the amount of unsaturated fatty acids in salt stressed *Synechocystis* 6803 cells needs to be probed carefully. The specific increase in the unsaturated fatty acid Linoleic acid content in salt adapted cells of *Synechocystis* 6803 is an interesting observation. Since all other identified unsaturated fatty acids except Linoleic acid (C18:2) have disappeared we would like to propose that under our conditions Linoleic acid may be more crucial for salt tolerance in *Synechocystis* 6803 cells. Salt induced conformational changes (cis → Trans) in Linoleic acid are an interesting observation made with *Synechocystis* 6803 cells. This conformational change requires specific enzymes. It would be interesting to analyse the activity of these enzymes under saline conditions in *Synechocystis* 6803 cells. In the similar line 34 fold increases in an unidentified fatty acid in salt stressed cells of *Synechocystis* 6803 cells warrants a detail investigation which may throw light on a possible salt stress marker in *Synechocystis* 6803 cells.

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