



Increase in intracellular proline content in *Anabaena variabilis* during stress conditions

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Abstract: The present study deals with the effects of stress due to varying concentrations of sodium chloride, cadmium, pH and exposure to pesticides on intracellular proline accumulation in *Anabaena variabilis* in laboratory conditions. The stresses induced due to these changes in their growth media were expressed as decrease in growth (by 40% at pH 5; 30% at 50 mM NaCl; 80% at 50 μ M endosulphan and 55% at 10 μ M CdSO₄) as measured in terms of chlorophyll a concentrations in the test organism. Low pH (5, 6) had stronger negative effect on growth than increasing alkaline pH. The cyanobacterium showed varying degree of susceptibility to increasing salt, cadmium and endosulphan concentrations. Under all these altered conditions, their intracellular proline concentration was found to increase linearly in the test organism with increase in its amount of stress inducing substances in its vicinity. Proline appears to be a stress provoked substance in *A. variabilis*. However, there seemed to be a breakdown in the proline synthesis mechanism when concentrations of such compounds reached toxic proportions.

Keywords: Cyanobacteria, NaCl, Cadmium, pH, Endosulphan, Intracellular proline

INTRODUCTION

Microbes in their natural environment are constantly challenged by various stresses in diverse forms *viz* changes in temperature, moisture (droughts and floods), salinity and so forth. Salinity is now known to be involved in remarkably reducing the growth and productivity of plants, eukaryotic microorganisms and bacteria (Inabha *et al.* 2001) and affects agricultural productivity in areas with saline soils. In addition, manmade pollutants further add to the stresses encountered by these organisms. Increasing industrial activities release heavy metals in to the environment which cannot be further transmuted to totally innocuous forms. Many heavy metals are readily taken up by crops and fruits, which subsequently results in movement through the food chain and their ultimate accumulation in the highest trophic level in humans. One such heavy metal contaminant is cadmium (Cd). Agricultural soils are mainly contaminated with Cd from the extensive use of fertilizers, dispersal of sewage sludge and atmospheric deposition. Cd accumulates in the body replacing zinc in the liver and kidneys leading to serious diseases. It is also known for its phytotoxicity to germinating rice seeds resulting in adverse effects on RNA levels, ribonuclease activity, phosphorolytic and proteolytic enzymes, and thus compromising productivity (Shah and Dubey, 1998).

Soil is a dynamic system in which the physical, chemical and biotic components are in a state of equilibrium. Application of insecticides such as endosulphan without

taking care of the other soil constituents disturbs this equilibrium which adversely affects the productivity of the soil. Pesticides reaching the soil affect non-target organisms and their activities severely limiting beneficial soil microorganisms such as cyanobacteria (Abdel-Raouf and El-Shafey, 2009).

Modern agriculture finds increasing use of herbicides and pesticides in crop cultivation in a bid to enhance yield. Even though cyanobacteria are not the target organisms, application of insecticides (many of which interfere with the process of photosynthesis) has adverse effects on them as they are actively engaged in carbon fixation via photosynthesis. However, cyanobacteria are resilient organisms and combat such stresses in their environment with elaborate protective mechanisms to ensure their survival. Previous researchers have shown proline accumulation in plants, bacteria, protozoa, algae and marine invertebrates in response to stress in their surroundings (Delauney and Verma, 1993; Fatma *et al.*, 2007).

Proline has been known as important indicator for stress tolerance and functions as stabilizer (Shah and Dubey, 1998), a metal chelator (Farago and Mullen, 1979), as an inhibitor of lipid peroxidation (Mehta and Gaur, 1999), a hydroxyl radical (Smirnoff and Cumbes, 1989), and a singlet oxygen scavenger (Alia *et al.*, 2001). Not many reports are available on role of proline in stress tolerance in cyanobacteria except for a report on impact of environmental pollution on cyanobacterium *Westiellopsis prolofica* by Fatma *et al.* (2007).

One of the potential biotechnological applications of cyanobacteria is their role as bioremediator in polluted aquatic systems. However, polluted environments present stress to the cyanobacteria which prompts the organisms to develop protective strategies. The aim of the present study was to find out the accumulation of intracellular proline in cells of *Anabaena variabilis* and its role when presented with stress in their surroundings.

MATERIALS AND METHODS

Soil samples were collected from various rice fields during the month of May. For isolation of cyanobacteria, 1 g soil was vortexed in 99 ml of sterile water (Jurgensen and Davey, 1968). The mixture was used to inoculate Petri-dishes containing 2.5 % nutrient agar made in BG 11₀ medium (Rippka *et al.*, 1979). All Petri-dishes were incubated on a 14 / 10 h light / dark cycle with a light intensity of 50 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ at $25 \pm 2^\circ\text{C}$. Individual colonies developed were picked up and transferred to liquid BG 11₀ medium and further purified by repeated pour plate method. The cyanobacterium *Anabaena variabilis* was selected for the study as this strain was isolated from almost all of the fields undertaken for study and thus is perceived as widely present in most rice field ecosystems. Cultures were maintained in BG 11₀ medium. For the experiments, axenic *Anabaena variabilis* was grown in BG 11₀ medium with varying pH (5, 6, 7, 8 and 9); NaCl concentrations (0, 50, 100, 200, 300, 400 and 500 mM); CdSO₄ concentration (0, 5, 10, 15, 20, 25, 30 and 40 μM) and endosulphan concentration (5, 10, 20, 30, 40, 50, 60 μM). The cyanobacterial biomass was harvested on eighth day after inoculation by centrifuging at 10,000 g for 5 min. The pellets obtained were used for estimating chlorophyll *a* (Mackinney, 1941) and proline content (Bates *et al.* 1973) using a UV-VISIBLE spectrophotometer (Variance Cary 50 Bio).

RESULTS AND DISCUSSION

Relation between increase in intracellular proline and heavy metal stress in plants has been reported by many researchers (Alia and Saradhi, 1991; Hanson and Burnet, 1994; Mansour *et al.* 2005). The role of proline has also been indicated in adjusting osmotic stress (Watanabe *et al.* 2000) and in reducing oxidative stress resulting from enhanced level of salinity in the vicinity (Hare *et al.* 1998). Not many reports have focussed their attention towards the role of intracellular proline in stress management in cyanobacteria. Fatma *et al.* (2007) have reported impact of environmental pollution on the cyanobacterium *Westiellopsis prolofica* where they have identified intracellular proline as one of the metabolites that helps alleviate stress in the experimental organism. Taking cue from their work the present study had investigated increase in intracellular proline content in the cyanobacterium *A. variabilis* in presence of frequently

occurring undesirable elements in its vicinity in an attempt to further understand and establish proline as a common metabolite that comes into play during stress management in cyanobacteria.

The experimental organism was isolated from various rice fields of Meghalaya during a diversity study. Meghalaya presents unique geographical and climatic challenges to its faunal and floral population. Among these is acidic pH of soil and water that is often recorded in the rice fields of the state (Syiem *et al.*, 2010). Salinity is another common stress experienced by these microbes. Other common unwanted compounds are herbicides and pesticides used during cultivation and heavy metals due to mining activities in the state. Thus, in this study changes in pH, NaCl, pesticide endosulphan and CdSO₄ concentrations had been used as stresses on the cyanobacterium, *A. variabilis*.

A study of this organism in varying pH was intriguing as it was isolated from acidic rice fields. Cyanobacterial growth was susceptible to pH change in the medium. The effect was more pronounced in acidic pH (5, 6) compared to cultures growing in higher pH (8, 9). The growth of *Anabaena variabilis* was reduced by 26% in pH 6 while a further drop in pH by another unit of 1 (pH 5) reduced the growth by 40% compared to the controlled cultures (pH 7.6). However, in alkaline pH the reduction in growth was much milder in nature (12% in pH 8 and 14% in pH 9). Intracellular proline content increased in all experiments when pH deviated from the ideal value of 7.6 (Fig. 1). This increase in proline content was comparable in all range of pH change even though growth was visibly compromised in pH 5 and 6. Further on comparison, proline accumulation was not found to be proportional to the changes in chlorophyll *a* content brought about by variation in pH values of the media. Thus, defence mounted by the cyanobacterium in form of increased levels of intracellular proline may not distinguish the differential severity of the adverse condition that arises because of change in hydrogen ion concentration of the growth media.

Reduced photosynthetic activity and growth has earlier been reported in cyanobacteria due to increase in salinity (Schubert *et al.* 1993). 50 mM NaCl in the growth medium reduced chlorophyll *a* content by about 30% (Fig 2). Beyond that the decline was gradual with increasing NaCl concentration up to 400 mM. The decrease in chlorophyll *a* content was only 18% between 50 mM and 400 mM concentration of NaCl. But the percent decrease in chlorophyll *a* content was close to 30% between 400 and 500 mM. Intracellular proline in cells under NaCl stress recorded a gradual increase with increasing NaCl concentration up to 300 mM (11-45%). However the increase in proline concentration was comparatively less in NaCl concentration beyond 300 mM (Fig. 2). This may

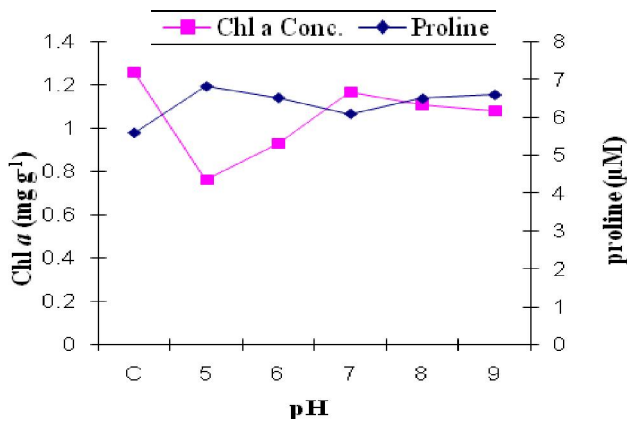


Fig. 1. Effect of varying pH on the chlorophyll a and proline content of *Anabaena variabilis*.

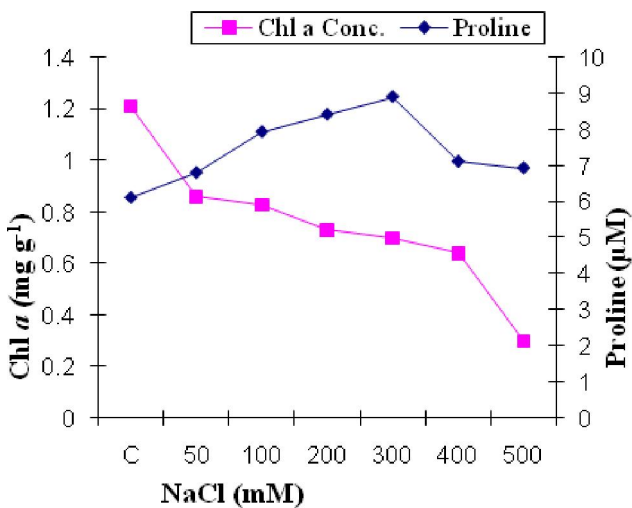


Fig. 2. Effect of different concentrations of NaCl on the chlorophyll a and proline content of *Anabaena variabilis*.

be due to the fact that at such high concentrations of sodium chloride, many of the vital metabolic activities are compromised by ionic imbalance that may also affect proline synthesis.

The pesticide endosulphan is often used in rice cultivation. However, effect of endosulphan application has not been studied in detail on the microbes that exist in the rice field ecosystem and are beneficial to crop plants and help maintain the soil quality and fertility. A study involving increasing concentrations of endosulphan in the vicinity of the test organism showed negative effect on their growth (Fig. 3). Micro molar concentrations of endosulphan in the medium could visibly reduce the growth in terms of chlorophyll a content.

A. variabilis could tolerate an increase up to 30 μM endosulphan. This tolerance level could be attributed to sharp rise in intracellular proline in these organisms as the concentration of endosulphan increased in the medium. However as in the case with NaCl, beyond a certain concentration of endosulphan, the cells showed

severe drop in their capacity to synthesize proline (Fig. 3). The fall in proline synthesis is in tune with decline in their growth. Chlorophyll a content was only ~20 and 10% of that of control culture in media that contained 50 and 60 μM concentration of endosulphan. Proline concentration had reached a value ~ 250% of that of control in presence of 20 μM endosulphan in the medium, where growth was recorded to be 83% of the control. However proline synthesis seems to have been affected by increased concentration of endosulphan in the medium. This may be the cause of visible decline seen in growth as intracellular proline was not made in sufficient amount to provide protection against endosulphan toxicity. The proline concentration has fallen by 20% below control value in medium containing 60 μM endosulphan.

Exposure to heavy metals is known to affect cyanobacterial growth (Leena and Fatma, 2000; Fatma *et al.*, 2007). Metal stress in form of increasing concentration of CdSO₄ was also expressed as reduction in Chlorophyll a content. At 10 μM CdSO₄ in the medium, chlorophyll a concentration declined by 55% which further went down to 98% in presence of 35 μM CdSO₄ (Fig. 4). The effect of CdSO₄ was most severe among the four different stresses tested on the cyanobacterium. Again, a rise in intracellular proline content was noted with increasing concentration of CdSO₄ in the growth medium that decline as concentration reached higher values of 30 μM and above. (Fig.4). In other words, as adverse effect was expressed as reduction in chlorophyll a concentration, increase in intracellular proline synthesis with rise in unwanted compounds in the vicinity could be looked at as the cyanobacterium's strategy to combat such undesirable conditions. However at higher toxicity levels even proline synthesis itself seemed to be compromised. As we compare the different stresses presented to the cyanobacterium *A. variabilis*, the toxic effect on chlorophyll a concentration was in the order of CdSO₄ >

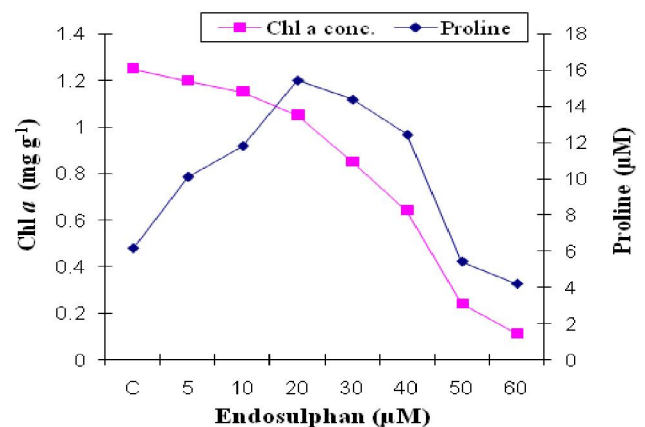


Fig. 3. Effect of different concentrations of endosulphan on the chlorophyll a and proline content of *Anabaena variabilis*.

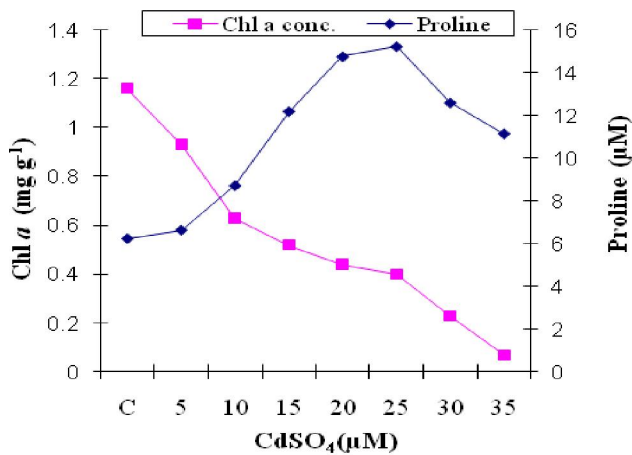


Fig. 4. Effect of different concentrations of Cadmium sulphate on the chlorophyll a and proline content of *Anabaena variabilis*.

endosulphan > NaCl > changes in pH. CdSO₄ and the pesticide endosulphan seem to be more toxic as their toxicity was enunciated at micro molar concentrations. Endogenous proline accumulation was always enhanced whenever the cyanobacterium was subjected to any form of adversity in growth medium. There seems to be an inverse relationship between decrease in chlorophyll a content due to adverse environment and increase in intracellular proline accumulation in *A. variabilis*. Thus proline seems to be a stress induced metabolite in this cyanobacterium. However, beyond a certain concentration of stress compounds, the increase in proline content did not follow this linear relationship. It has been suggested at least in the case of heavy metal exposure that heavy metal ions bind to sulfhydryl groups of enzymes essential for catalysis that leads to death of cyanobacteria (Rosko and Rachlin, 1975). This may be the reason for reduced proline synthesis at toxic levels of these stress compounds (eg. in endosulphan concentration beyond 40 μM; CdSO₄ beyond 20 μM and NaCl beyond 300 mM). Increased in intracellular proline concentration was highest in presence of endosulphan. An increase of 147% was recorded in presence of 20 μM endosulphan in the growth medium. The order of increase in intracellular proline accumulation was 20 μM endosulphan > 25 μM CdSO₄ > 300mM NaCl > pH 5.

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