Impact of Salinity and pH on Several Species of *Anabaena* (Nostocaceae, Nostocales) Isolated from Rice Fields in Iran

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Received: 2017-11-15 Revised and accepted: 2017-12-20

Abstract

The purpose of this study is to develop a biofertilizer based on filamentous nitrogen-fixing cyanobacteria selected from rice fields and to generate a technological package compatible with its use for the rice crop in Iran. Cyanobacteria was isolated and purified from rice fields in Kalate Naderi. In this research we studied the effect of salinity (NaCl, 0, 1, 2 and 4%) and pH (5, 7, 9 and 11) on growth and chlorophyll-a contents in six species of *Anabaena*. Results showed that *Anabaena sphaerica* Bornet & Flanault possessed the best adaptation to pH changes. It could be more active in 5-11 pH values. *A. vaginicola* F.E. Fritsh & Rich and *A. variabilis* Kutzing ex Bornet & Flanault were remarkable for salinity tolerance. They adapted to salinity stress up to 2% salt concentration in the medium. Our results indicated that the growth of all strains decrease by 4% salt concentration and pH 11. Indeed, *Anabaena* is a cyanobacterium with nitrogen fixation ability and high potency of adaptation to environmental stress. So, it can be a useful candidate for biofertilizer in agriculture, particularly in rice fields.

Key words: Biofertilizer, Heterocyst cyanobacteria, pH stress, Rice field, Salinity stress.

Introduction

It is known that cyanobacteria supply more nitrogen in wetland rice fields in tropical regions (Singh, 1985, 1988) than in dryland fields (Yamaguchi, 1979), and this is attributable to the unique characteristics of wetland rice fields: along with water, there is a natural supply of plant nutrients, especially nitrogen, which encourages general plant growth when soil pH is neutral. Additionally, there is considerable literature indicating that in rice-flooded soils of temperate regions, cyanobacteria increase soil nitrogen through nitrogen fixation (Vaishampayan et al., 2001), such as, for example, in Japan and in most Southeast Asian countries (Watanabe, 1973). However, this behavior is not shown in all rice soils in temperate regions, since the environmental conditions such as moisture, soil pH, combined nitrogen levels and temperature are factors that can play an ecologically determinant role on the abundance of cyanobacteria and nitrogen fixations (Pereira et al., 2009). On the other hand, salinity of soil is an important ecological variable and a serious problem in agriculture. The widespread distribution of cyanobacteria indicates that they can tolerate a wide spectrum of global environmen-

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tal stress such as temperature, pH, desiccation. Salt stress is one the limiting factors on growth and productivity of microorganisms. They have developed a number of mechanisms by which cyanobacteria defend themselves against environmental stress (Rajendran et al., 2007). The physiological basis for the adaptation to high salinities in several cyanobacteria species includes three main sub processes: 1) active extrusion of inorganic ions, leading to relatively unchanged internal salt concentrations; 2) accumulation of large internal amounts of organic osmo-protective compounds; and 3) expression of a set of salt stress proteins (Soltani et al., 2007). Iranian rice fields are a source of enormous biological diversity-which is scarcely studied. Characterization of biotechnology value in cyanobacteria has been reported by Soltani et al. (2005, 2006). In this study, we investigated six species of *Anabaena* that were isolated from four rice fields as biofertilizer in the salinity or acidic/alkaline agriculture soils in Iran. Growth rate was measured under salinity and pH stress.

Materials and Methods

Sampling and cyanobacterial culture

This study is focused on Kalat Naderi district of Khorasan Razavi Province. The Kalat Naderi town is located in the north-east of Iran between 59° 9′ to 60° 27′ E longitude and 36° 24′ to 37° 17′ N latitude. Soil samples were collected from four sites (Amirabad, Sarrood, Qaleno and Kalat). The depth of sampling was 5 cm. The collected soil samples were transferred to sterilized nitrate free BG-11 medium (Stanier et al., 1971). The Petri dishes were placed in a culture chamber (Model SB5520) at 25±5°C and 12/12 h light/dark cycle under fluorescent illumination of 40μmol photon m^{-2} s^{-1} for two weeks. After colonization, the cyanobacterial strains were studied by optical microscopy (Olympus, Model BM-2) and different taxa were identified based on morphological characteristics using standard key books and articles (Desikachary, 1959; Prescott, 1970; Komárek and Anagnostidis, 1989). Morphology of filaments, shape and dimensions of vegetative cells, heterocyst and akinetes were some of the characters used for identification of these taxa.

Physiological analysis

The axenic culture of *Anabaena* was prepared by repeated subculture on solid medium. Stock cultures were grown in nitrate free BG-11 (BG-110). Cells in logarithmic phase of growth were collected from stock cultures and inoculated. BG-110 medium in different salinity and pH was made for inoculation of *Anabaena* species.

The required salinity was obtained by adding sodium chloride (NaCl). The six strains of *Anabaena* were grown in a salinity gradient from 0, 1, 2 and 4%. The pH gradient was prepared by KOH and HCl. The pH values 5, 7, 9, 11 were adjusted. The flasks were maintained for 10 days at 25±5°C and 12/12 h light-dark cycle under fluorescent illumination of 40 μmol photon $m^{-2}s^{-1}$. Growth rate of each strains in different treatments was measured as the content of chlorophyll-*a* according to Soltani et al. (2007).

Each treatment consists of three replicates; the results presented are mean values. Moreover, the morphological character's variation and changes due to stress conditions, salinity and pH were investigated.

Results

Six nitrogen-fixing species of *Anabaena* were isolated from paddy fields in Iran (Table 1). In order to select a suitable candidate as bio-fertilizer, we investigated their tolerance to salinity and pH stress. The growth curves for six species under salinity stress were obtained (Fig. 1). The results showed that *A. torulosa* grows in no salt and 1% salinity. *A. variabillis* exhibited good increasing growth with no salt, cells became colorless and the filament was degenerated in higher than 2% salt concentrations. *A. vaginicola* was relatively resistant, its growth rate continued up to 2% of salinity. Although it could tolerate 2% salt in the medium, finally the cells died. *A. sphaerica* was able to grow up to 1% of salinity. *A. ambigua,* at the beginning of the growth phase, showed delay. After a few days, adaptation was found and became resistant to 1% salt concentration. *A. oscillarioides* in the control treatment (0% salinity) showed the maximum growth rate and resistant to 1% salinity (Fig. 1). *A. oscillarioides* in the acidic pH 5 and alkaline pH 11 became deformed; the cells were destroyed and the filaments changed. The maximum growth rate of *A. torulosa* obtained in pH 9. *A. variabillis* was able to grow in pH 5 and 7 but the best condition was in pH 9. *A. sphaerica* adapted to pH 11. *A. vaginicola* showed the maximum grow rate in pH 9. *A. ambigua* became completely deformed in pH 5, optimum pH value for this strain was 9 (Fig. 2).

Discussion

Industrial-scale microalgae cultivation, needs selection of algae strain with high production of target biochemical and tolerance to a wide range of environmental conditions, such as salinity, temperature, pH and nutrient or pollutant loads. Such algal 'super-species' should also show high biochemical productivity, which would be considerably simple production regarding

Table 1. List of *Anabaena* species identified from the soil of rice fields of Kalat-Naderi

	Locations			
Species	Amirabad	Qaleno	Sarrood	Kalat
A. vaginicola	-	+	+	+
A. torulosa	-	+	+	+
A. oscillarioides	-	+	+	+
A. ambigua	-	-	+	-
A. sphaerisca	-	-	+	-
A. variablis	-	-	+	-

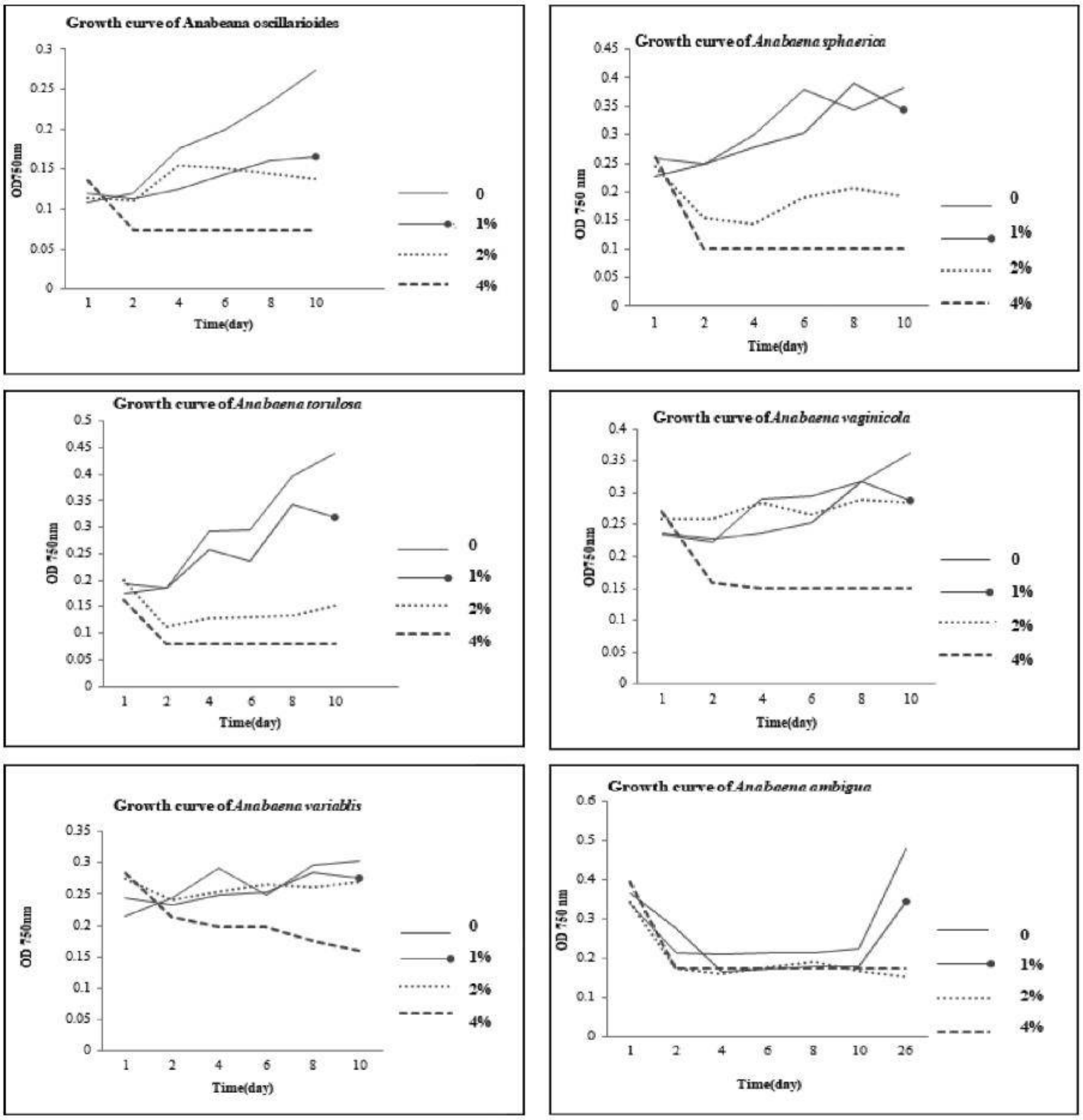

Fig. 1. Growth curve of six species of *Anabaena* studied under salinity stress.

standardization of product quality, across a range of production sites (Alvensleben et al., 2013). Two potentially important factors that can regulate growth and biomass of cyanobacteria are salinity and pH. This study revealed six species of *Anabaena* species, isolated from different salinity and pH in rice fields. Although metabolic requirement of sodium for physiological activities like nitrogen fixation, growth, photosynthesis and intracellular pH regulation, transport of carbon and energy, transductions and maintenance of RuBisCo are documented in cyanobacteria, hyper-salinity is found to induce a number of adaptive responses (Sekar and Subramanian, 1999). The halotolerant cyanobacteria are known to synthesize a variety of osmolites to balance hypersaline stress in response to the concentration of NaCl in the external media (Reed et al.,1986). Many agricultural ecosystems increasingly become salt affected, thus rendering them inhospitable to crops. Such salt affected soils cover an estimated 7 million hectares of potential crop land in India (Roychoudhury et al.,1985). So, indication of halotolerance cyanobacteria can be important for use as a biofertilizer and improvement of salty soils conditions.

Regarding physiological responses of *Anabaena* species to NaCl, as shown in figures 1, 3 and 4, growth rate decreased with salinity increase in but it continued up to 2%. These results also reported for *Anabaena aphanizomenoide* which grew in NaCl up to 15 gL^{-1} and 20 gL^{-1} had inhibiting effect (Moisander et al., 2002). These results confirm the variation of chlorophyll content in different salinity. Figure 2 shows a similar growth rate pattern. It confirms the role of chlorophyll in cyanobacteria growth which changes by varied environmental factors.

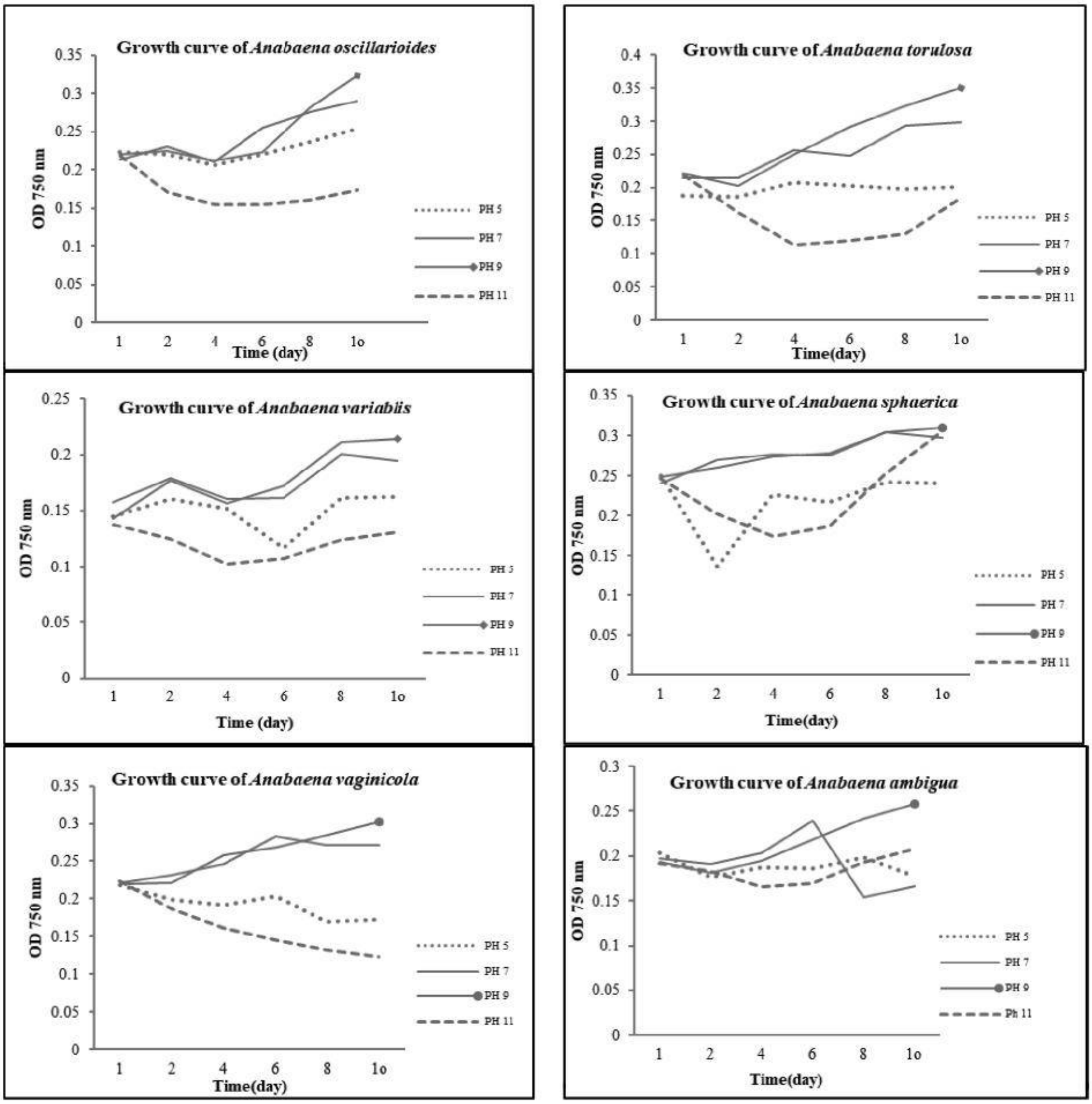

Fig. 2. Growth curve of six species studied of *Anabaena* under pH stress.

Maximum photosynthesis rate was seen in control. Our results confirm the salinity has a significant effect on growth and photosynthesis in some of the species. These cyanobacteria need more light for survive in saline environment. There have been relatively few studies showing how cyanobacteria tolerate acidic or alkaline stress, while cyanobacteria are found in nearly all ecosystems. Ecological observations have shown that cyanobacteria have a preference for alkaline conditions. Steinberg et al. (1998) showed that cyanobacteria were completely absent in picoplanktons when the pH fell below 4.5 in ten lakes. Acidic pH is remarkable considering the physiological changes caused by increasing in external proton concentration. Changes in the proton concentration of a cell's environment can affect the dissociation rate of CO_2, the electrical charge of the cell-wall surface, ion transport systems and membrane potentials (Jean et al., 2002). Acidic pH can interfere with the function of outer-surface cell components such as pili, chemoreceptors, cell walls, exopolysaccharide, periplasmic proteins and flagella. The pH changes can cause disruption of the plasma membrane, protein denaturation and loss of enzyme function, as well as damage to macromolecules or ionization of nutrient molecules, which affects the availability of these compounds to the cell (Park et al., 1996). The dissociation of protein functional groups is directly affected by pH stress and changes; also pH can interfere with enzyme activity. Chakraborty et al. (2011) showed the effect of pH on Chl a concentration in the freshwater system. Concentration of Chl a gradually increased from acidic to neutral and reached at its maximum level at the pH 8.15. They also reported that salinity resistance range in cyanobacteria was attributed to enhance synthesis of zeaxanthin as a protective xanthophyll against the osmotic stress. However, the effect of pH on green algae and cyanobacteria growth rate was not as dramatic as salinity and they showed a considerable acclimation towards fluctuating pH. Cyanobacteria are not only able to fix atmospheric nitrogen, but also have an important role in rice fields by their frequency. Soltani et al. (2007) reported that nitrogenase activity varies depending on the strain. Accordingly, *Anabaena* has higher nitrogen fixation than *Nostoc* in saline water. According to our results in this study, *A. vaginicola*, *A. variabilis* and *A. sphaerica* are more capable of tolerating environmental stress. We suggest that mentioned *Anabaena* species as suitable candidates for biofertilizer in saline and alkaline stressed soils.

Acknowledgment

The authors wish to thank University of Shahid Beheshti for funding this project. Thanks are also due to Dr. Mehri Seyed Hashtroudi for her valuable suggestions and help in chemical analysis.

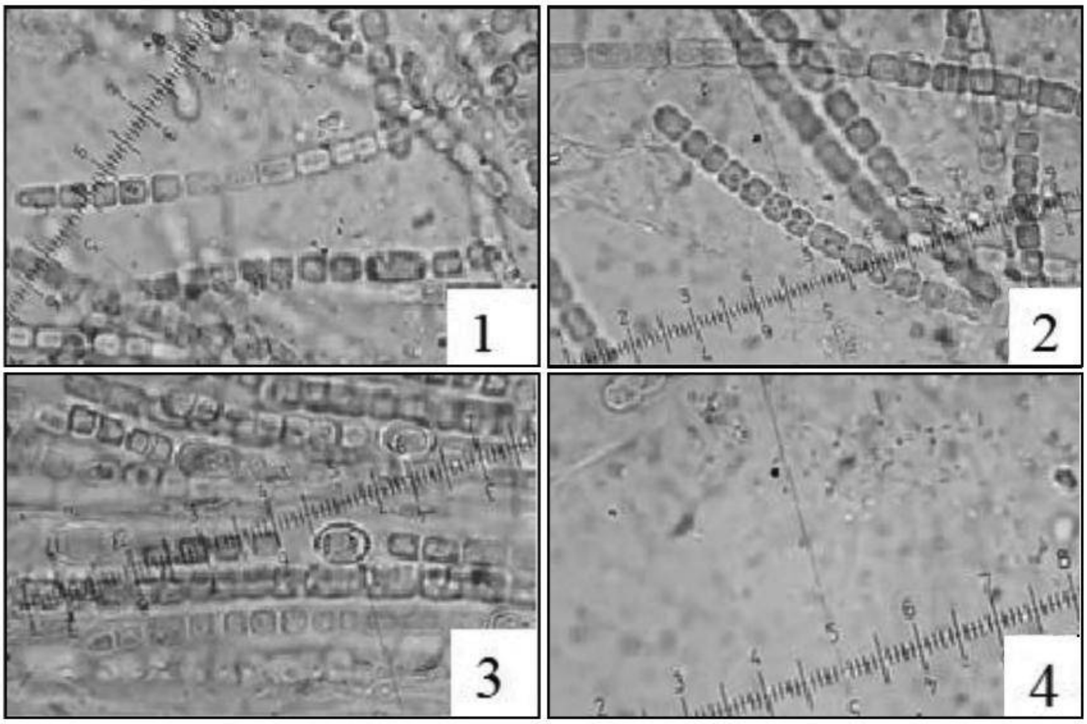

Fig. 3. Morphological changes in *Anabaena variablis* after 2 days under :(1) without salinity, (2) 1% salinity, (3) 2% salinity, (4) 4% salinity.

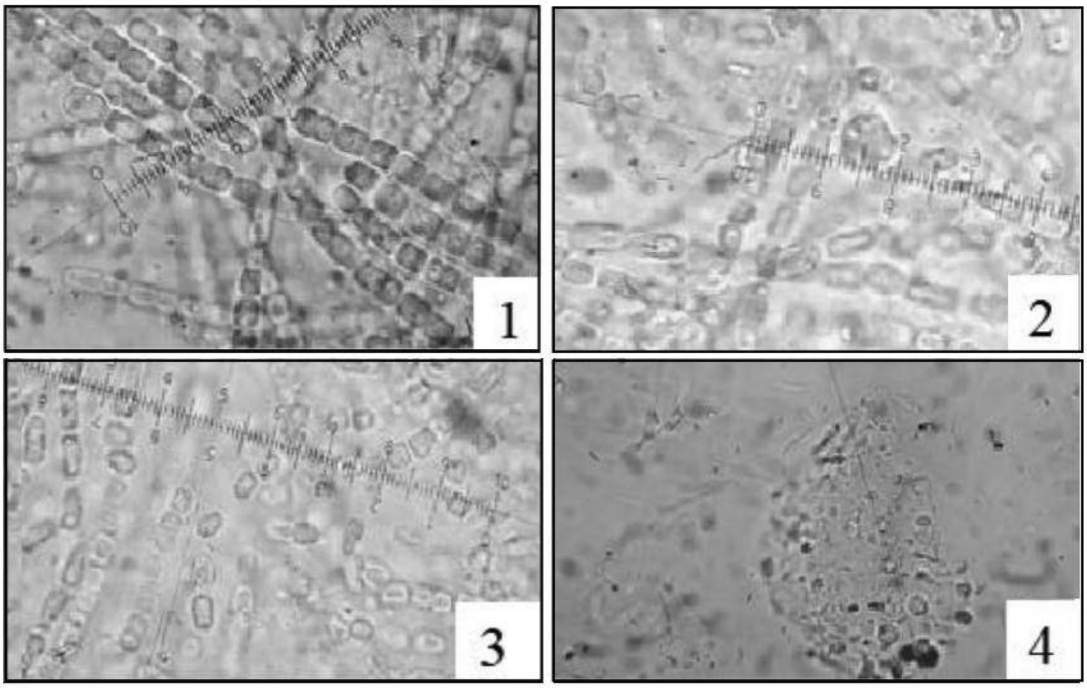

Fig. 4. Morphological changes of *Anabaena variablis* after 10 days under: (1) without salinity, (2) 1% salinity, (3) 2% salinity, (4) 4% salinity.

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