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Molecular identification of *Dunaliella viridis* Teod. strain MSV-1 utilizing rDNA ITS sequences and its growth responses to salinity and copper toxicity

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A B S T R A C T

In addition to biochemical, physiological and morphological analysis, molecular studies provide additional information for establishing phylogenetic relationships among different species and strains of the genus *Dunaliella*. In the present study, based on neighbor- joining analysis of the nuclear rDNA ITS sequence, a novel strain of the green algae *Dunaliella viridis* was identified from Maharlu salt lake in Shiraz, Iran. The phylogenetic tree shows that the new strain is part of a clade containing several strains of *D. viridis*. The new strain was designated *Dunaliella viridis* MSV-1 and submitted to the GenBank under the accession number HQ864830. The optimum salinity for MSV-1 growth is between 1.0 to 1.5 M NaCl and does not turn red up to 4.5 M NaCl, confirming identity of the isolated strain. With respect to growth response to copper toxicity, increase in Cu^{2+} concentration from 1 to 30 µM, caused progressive increase in cell number ml^{-1} of culture over time, whereas reduction in cell number occurred at 100 and 200 µM Cu^{+2}. Nano copper (colloidal copper with 40 nm dimensions) showed less toxicity compared to the ionic form. Cell number ml^{-1} of culture did not change up to 200 µM nano copper but decreased at 500 µM. In conclusion, the analysis of the ITS sequence is a reliable basis for establishing evolutionary relationships among species and strains of the genus *Dunaliella* and due to rapid growth at 1.5 M NaCl and high cell density, *D. viridis* MSV-1 is a good candidate for biofuel production from microalgae.

Keywords: *Dunaliella viridis* MSV-1; ITS sequences; nano copper; biofuel

INTRODUCTION

The unicellular green algae *Dunaliella viridis* has been described as a hypersaline species with optimum salinity range of 5.8 to 8.9% NaCl [1, 2]. *Dunaliella viridis* from an athalassic lake in Spain was reported to grow over 23.3% (4M) NaCl with optimum salinity for growth and

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reproduction at about 5.8% NaCl [3]. This species is widely distributed throughout the word and based on morphology several strains have been described from geographically distant locations [4]. DNA-based molecular methods have provided new tools for studying evolutionary relationships among various species and strains of the genus *Dunaliella* [5]. The internal transcribed spacer regions 1 and 2 (ITS-1+ 5.8s rDNA+ ITS-2), which separate the nuclear ribosomal genes, have been used for studying genetic relatedness at species and strain levels [6, 7].

With respect to the effects of copper on living organisms, it is well known that this trace element is required for activity of several enzymes and is a component of the electron transport chain in mitochondria and chloroplast [8]. However, when present in excess, it is one of the most toxic trace metals to organisms including algae [9]. Copper ion exerts its toxicity on cellular metabolisms, growth and development mainly by increasing production of reactive oxygen species (ROS) [10]. The mechanism of copper toxicity to photosynthetic electron transport was suggested to be the inhibition of the donor and acceptor side of photosystem II [11].

The chemical form of copper in water is very important during copper toxicity investigation, as different copper species differ in their degree of toxicity to algae [12]. Due to current and future widespread usage of nanomaterial, their release into the environment is inevitable. At present, over 1000 nanotechnology products are on the market. Therefore, ecotoxicity of nanoparticles in natural water has attracted the attention of many investigators [13].

In the present study, we report the isolation and phylogenetic relationship of a new strain of *D. viridis* from Maharlu salt lake in Shiraz, Iran. Then, growth responses of the isolated strain to salinity, ionic form of copper and nano copper are presented. Finally, the potential of the new isolate as raw material for biofuel production is discussed.

MATERIALS AND METHODS

Microalgal isolation: *Dunaliella viridis* was isolated from Maharlu salt lake in Shiraz (latitude 29.26 N, longitude 52.48 E), Iran, purified as described before [6] and identified according to morphological description presented by Borowitzka and Siva [4].

Algal growth conditions: *Dunaliella viridis* was cultured in 100 ml flasks with 50 ml modified growth medium as described previously [6]. To investigate the effect of salinity on growth (expressed as the number of cells ml^{-1} of solution culture) final concentration of NaCl in the growth media was adjusted to 0.5 up to 4.0 M. Using $CuCl_2$, $2H_2O$, ionic copper concentration in the growth media was set to 1.0, 10, 30, 50, 100 and 200 µM. Appropriate volumes of nano copper (colloidal particle with 40 nm diameter, determined by transmission electron microscopy) were delivered from a 2000 ppm (2 mg ml^{-1}) stock solution to give the final concentrations of 0, 50, 100, 200 and 500 µM nano copper in the growth media. Samples were taken at indicated time intervals and numbers of cells ml^{-1} of culture were determined.

Growth determination: Cell number was determined by cell count using a hemacytometer and reported as the number of cells ml^{-1} of the growth culture.

DNA extraction and PCR amplification: Total DNA extraction was carried out as described by Gomez and Gonzalez [14]. The primer used for amplification of the ITS region (ITS-1+ 5.8s

rDNA+ ITS-2) were AB28 (5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3') and TW81 (5'-GGGATCCGTTTC CGTAGGTGAACCTGC-3') described by Goff et al. [15]. The amplification and PCR program were performed according to the previously described methodology [6].The amplified DNA was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. After purification of ITS fragments by the Roche agarose gel DNA extraction kit, the purified PCR product was sequenced in both directions by SeqLab Company, Germany.

Data analysis: The ITS sequences were aligned with the sequences available in the GenBank database by the alignment tool of the MEGA software package. By using different methods integrated in the software (including maximum-likelihood, maximum parsimony, UPGMA and neighbor-joining), a phylogram was constructed with MEGA 4.0 software programs [16]. Statistical analysis was carried out using SPSS version 13.0 and reported as mean ± standard error (SE).

RESULTS

Strain molecular characterization: *Dunaliella viridis* co-exists with *Dunaliella salina* in hypersaline environments. Based on this information and morphological descriptions presented by several authors [2, 4, 17], a strain of *Dunaliella viridis* was isolated from Maharlu salt lake in Shiraz, Iran. To confirm the identity of the isolated strain, the internal transcribed spacer (ITS) regions separating nuclear ribosomal RNA genes (ITS-1+ 5.8s rDNA+ ITS-2) of Chlorophytes were amplified by PCR. The amplification resulted in a single band (Figure 1) on the electrophoretic gel with 651 bp in length. The sequence was submitted to the GenBank under the accession no. HQ864830. A phylogram based on neighbor-joining analysis of the entire ITS-1+ 5.8s rDNA+ ITS-2 sequences of several species and strains of the genus *Dunaliella* was constructed (Figure 2). *Chlamydomonas leiostraca* (FR865590) was included as outgroup. The tree shows that the new isolate is part of a major clade containing several strains of *D. viridis* and was designated as *D. viridis* MSV-1. The closest relative of the isolated strain MSV-1 were *D. viridis* DCCBC3 and *D. viridis* CONC 002. The presence of *D. parva* UTEX 1983 in this clade confirms its re-indentification as *D. viridis* by Borowitzka and Siva [4].

Growth response to salinity: Optimum salinity for the growth of the isolated strain, expressed as number of cell ml^{-1} of growth culture, was between 1.0 to 1.5 M NaCl (Figure 3). Although cell density at 1.5 M NaCl was about the same as 1.0 M, the growth rate, as evident by the slope of the curves at logarithmic phase of growth, was higher at 1.0 M NaCl. Cell densities at other NaCl concentrations (0.5, 2.0, 2.5, 3.0, 3.5 and 4 M) were lower compared to 1.0 and 1.5 M NaCl.

Growth responses to copper toxicity: The growth responses of *D. viridis* MSV-1 to different concentrations of Cu^{2+} and nano copper are presented in Figures 4 and 5. Cells at about mid-logarithmic phase of growth were exposed to $CuCl_2$ or nano copper and sampled 24, 48 and 72 hrs after exposure. Cell density expressed as cell number ml^{-1} of culture increased with time at 1, 10 and 30 µM $CuCl_2$, stayed relatively unchanged at 50 µM and decreased at 100 and 200 µM Cu^{+2} (Figure 4). It is possible that at 50 µM Cu^{+2}, the cell lysis is balanced with cell division, while at higher Cu^{+2} concentrations cell lysis outnumbers cell division.

Figure 1: Agarose gel electrophoresis of amplified ITS sequences of *D. viridis* MSV-1 isolated from Maharlu salt lake. Lane 1 represents 250 bp DNA ladder and lane 2 is amplified ITS sequences.

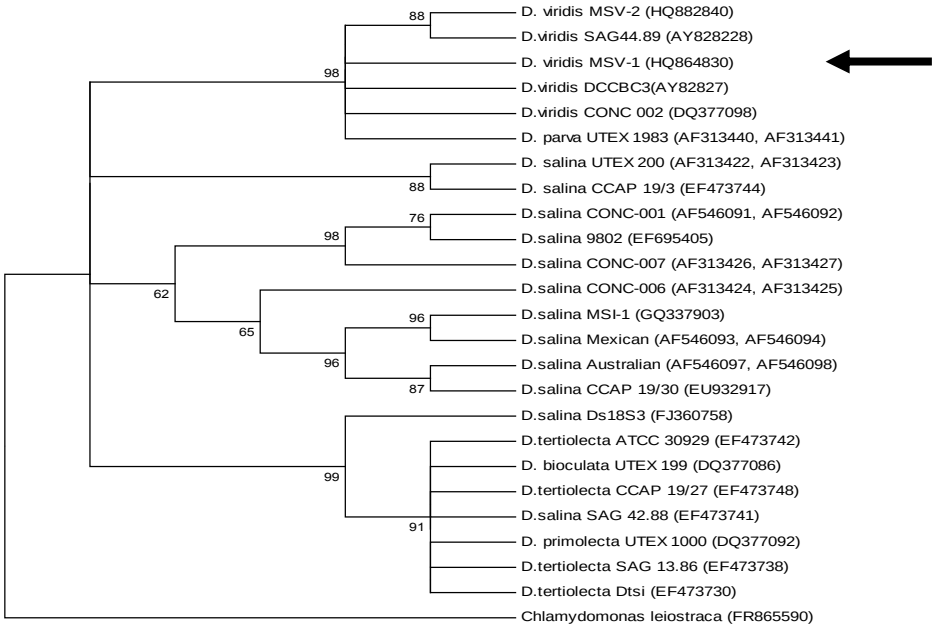

Figure 2: Phylogram based on neighbor-joining analysis of *Dunaliella* rDNA ITS sequences. The phylogenetic tree shows the position of *D. viridis* MSV-1 relative to other species and strains of *Dunaliella*. *D. parva* UTEX 1983 in this clade is misnamed and should be designated as *D. viridis* [4]. The optimal tree with the sum of branch length = 0.453 is shown. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Bootstrap values greater than 50% are indicated.

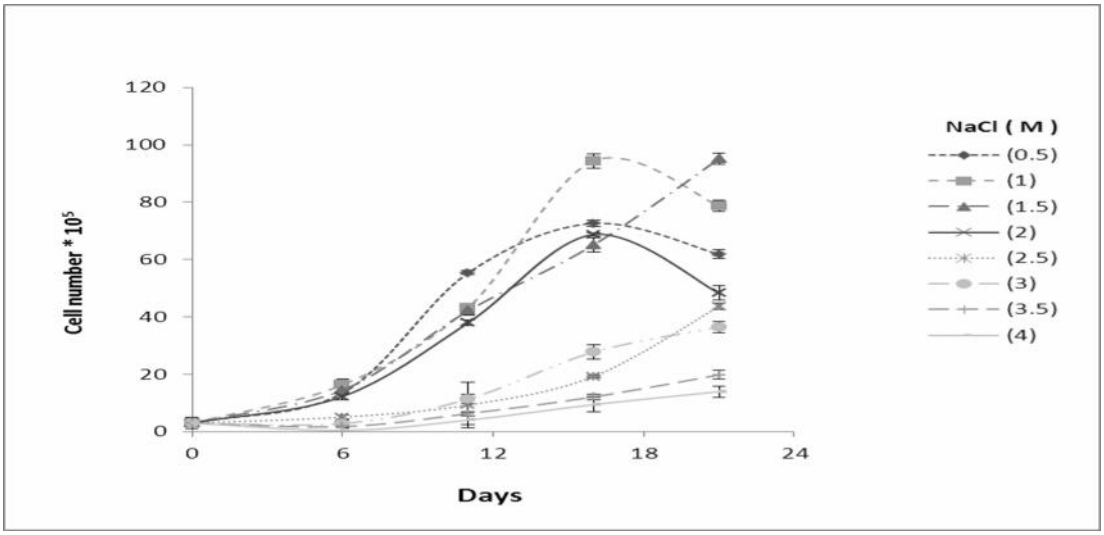

Figure 3: Effects of salinity on growth of *Dunaliella viridis* MSV-1. Each value is mean ± SE.

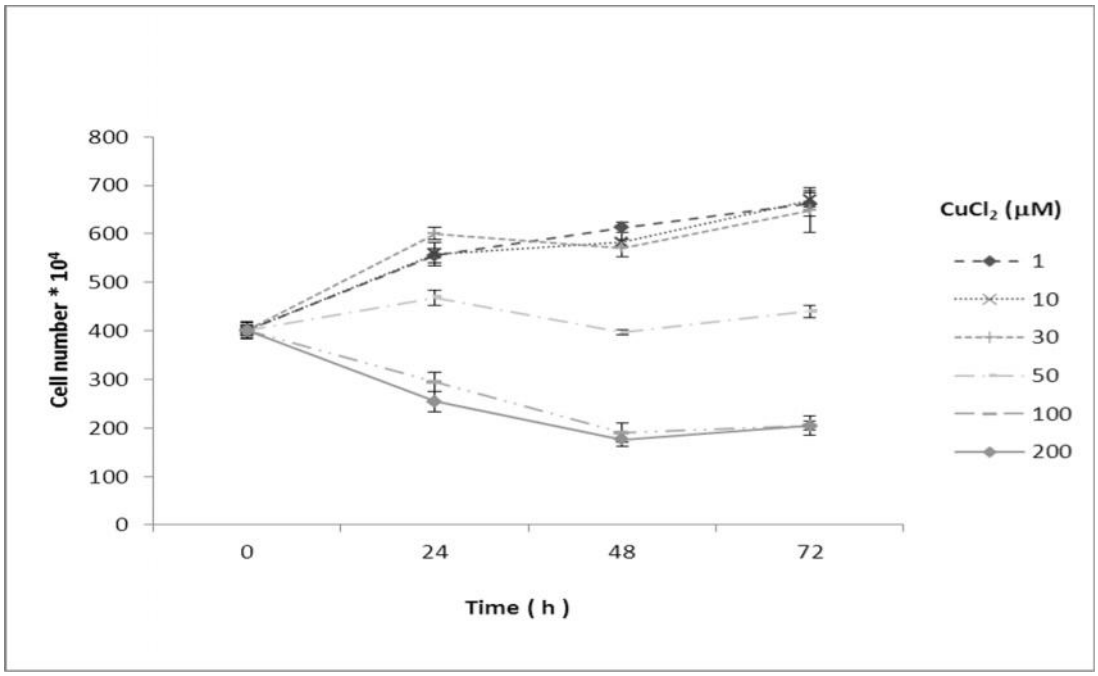

Figure 4: Growth response of *Dunaliella viridis* MSV-1 to different concentrations of ionic form of copper (Cu^{+2}). Each value is mean ± SE.

As shown in Figure 5, the number of cells in samples taken 24 hrs after the nano treatment was relatively equal in the presence of 0.0, 50, 100 and 200 µM nano copper. The same results were observed with samples taken at 48 and 72 hrs after the start of the experiment. At 500 µM nano copper, cell number was reduced at all sampling intervals. The reduced cell number could be the result of both increased cell lysis and reduced cell division in the isolated strain.

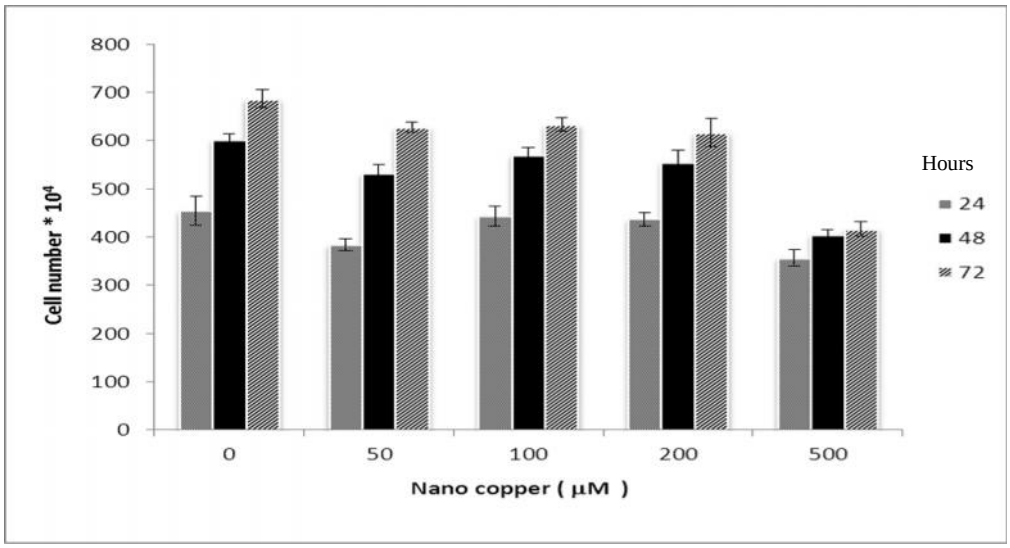

Figure 5: Growth response of *D. viridis* MSV-1 to different concentrations of nano copper measured at 24, 48 and 72 hrs after treating cells with nano copper. Each value is mean ± SE.

DISCUSSION

Dunaliella viridis has been described as a hyperhaline species with optimum growth in the 6 to 10% NaCl range and tolerating up to 23.2% NaCl [2]. These values are lower than the NaCl concentrations reported for the *D. viridis* isolated in Yucatan with optimum growth range of 15 to 20% NaCl [16]. Considering the available data on growth responses to salinity, it seems that optimum NaCl concentration for *D. viridis* growth varies with geographical location of the isolated strains.

Ambient concentrations of dissolved Cu^{2+} in water bodies are low but increases as a result of anthropogenic sources [19]. In addition to ionic form, due to increased production and, therefore, release of nanoparticles into the environment, the effects and toxicity of nano copper on living systems have recently been explored [20]. Growth inhibition of *Phaseolus radiatus* and *Triticum aestivum* seedlings exposed to different concentrations of Cu nanoparticles was reported by Lee et al. [21]. Copper nanoparticle was highly toxic to *Cucurbita pepo* which caused 60 to 70% growth reduction compared to control plants [22]. In the experimental mice, it was shown that micro-copper particles did not have any toxic effect, but nano copper particles caused severe injuries to kidney, liver and spleen. It seems that particle size of elemental copper influences its degree of toxicity to organisms [23]. Although the mechanisms of ionic copper-induced toxicity have been extensively studied and increased production of reactive oxygen species (ROS) in the presence of high levels of ionic copper have been established [24], the mechanism underlying the toxicity of nanoparticles is not clear yet. It is proposed that nano copper exerts its toxicity partly by increased ROS production, therefore, inducing oxidative stress and apoptosis in organisms [25].

With respect to biofuel production, due to high photosynthetic efficiency, fast growth rate and high biomass and oil production, microalgae may soon be one of the earth's most important

renewable biofuel sources [26]. Cell density produced by *D. viridis* is among the highest reported in the halophilic *Dunaliella* species. High salinity requirement of this microalgae minimizes contamination by other organisms in mass cultivation and due to moderate resistance to copper toxicity, and probably to other transition metals, it can be cultivated in non-potable water. Since, in addition to high growth rate and biomass production, high oil and starch content are very important in strain selection for biofuel production, determination of oil and starch content and genetic manipulation of strain MSV-1 need to be investigated.

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