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ARTICLE

Effects of Salinity Changes on the Growth of *Dunaliella salina* and Its Isozyme Activities of Glycerol-3-phosphate Dehydrogenase

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Dunaliella salina could survive in media containing a wide range of NaCl concentrations ranging from about 0.05 M to saturation (around 5.5 M). Glycerol is an important osmolyte when *Dunaliella* survive in various salt environments, and G3pdh is a key enzyme in glycerol metabolism. The osmotic response of *D. salina* was investigated by studying its cell growth, glycerol content change, and isozyme activity of glycerol-3-phosphate dehydrogenase (G3pdh) in different salinities. Results showed that 2.0 M NaCl was the optimal salinity for the growth of *D. salina*, in which condition the highest glycerol content of 64.02 ± 3.21 (mean ± SD) μg/mL was detected. *D. salina* could rapidly increase or decrease glycerol contents to adapt to hypoosmotic or hyperosmotic environments. The glycerol content declined 52.05% when salinity was changed from 2.0 to 0.5 M NaCl, and the glycerol content increased 43.61% when salinity was increased from 2.0 to 5.0 M NaCl. In the isozyme electrophoresis assay two kinds of isozymes, G3pdh and superoxide dismutase (Sod), were detected synchronously. Interestingly, it was first found that there are five isozymes of G3pdh in *D. salina*. G3pdh-2 mainly takes effect in moderate to high salinities, whereas the other four isozymes take effect in low salinities, which may provide an important clue for future research on osmoregulation mechanisms.

KEYWORDS: ***Dunaliella salina*; osmotic; glycerol; salinity; isozyme; glycerol-3-phosphate dehydrogenase**

INTRODUCTION

Dunaliella salina, one member of the genus *Dunaliella* (Chlorophyceae, Volvocales), is an extremely halotolerant, unicellular, green, and motile algae. The genus *Dunaliella* is unique in its remarkable ability to survive in media containing a wide range of NaCl concentrations, ranging from about 0.05 M to saturation (around 5.5 M), while maintaining a relatively low intracellular sodium concentration (*1*). In addition, under high salt stress, *D. salina* could accumulate large amounts of β-carotene in cells, which makes it one of the best sources of natural β-carotene (*2*−*5*).

In *Dunaliella*, the synthesis or elimination of glycerol to an intracellular concentration osmotically balancing the external salinity permits the cells to resume growth (*6*). Two different metabolic pathways may be responsible for glycerol formation: one using a photosynthetic product and the other via the metabolic degradation of starch, the storage product in *Dunaliella* (*7*). The pathway of glycerol synthesis is as follows: Glucose, from photosynthesis or hydrolysis from starch, is converted to fructose-1,6-diphosphate and next to dihydroxyacetone phosphate (DHAP), which is converted to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase (G3pdh). Finally, glycerol-3-phosphate is converted to glycerol by glycerol-3-phosphate phosphatase (*8*). In the opposite direction, excess glycerol is removed by oxidation to dihydroxyacetone (DHA) catalyzed by glycerol dehydrogenase (also known as DHA reductase), and then DHA is converted to DHAP catalyzed by DHA kinase (*9*−*11*).

G3pdh is an important enzyme in the pathway of glycerol synthesis. In higher plants and algae, G3pdh is referred to as DHAP reductase, because at physiological pH and substrate, the enzyme is essentially inactive as a dehydrogenase (*11*). The presence of DHAP reductase has been reported in *Dunaliella tertiolecta*, and three isoforms of DHAP reductase have been separated from *D. tertiolecta* (*12*, *13*). The chloroplasts contained the two major isoforms, and the third, minor form was in the cytosol. The first chloroplast form was the major form when the cells were grown on high NaCl, and it has been a form for glycerol production for osmoregulation. The second form increased in specific activity when inorganic phosphate was increased and played roles in stimulating cell growth and glyceride synthesis. He et al. (*11*) have cloned the cDNA encoding a (NAD^+)-dependent G3pdh from *D. salina*. RNA gel blot analysis and detection of glycerol content under different conditions indicated that the cDNA may encode an osmoregulated isoform primarily involved in glycerol synthesis.

Some *Dunaliella* species generated reactive oxygen species (ROS), including superoxide ($^{\bullet}O_2^-$), singlet oxygen ($^1O_2$), and

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hydrogen peroxide (H_2O_2) in the chloroplast under osmotic stress (*14*). ROS interact with a wide range of molecules causing pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation, and DNA mutation (*15*). To mitigate and repair the damage initiated by ROS, *Dunaliella* accumulated antioxidants including low molecular mass nonenzymatic free radical scavengers and enzymes such as superoxide dismutase (Sod, EC 1.15.1.1), peroxidase (Pod, EC 1.11.1.7), and catalase (Cat, EC 1.11.1.6) (*16*). Sod is a major scavenger of $^{\bullet}O_2^-$ and converts it into H_2O_2 and O_2. The H_2O_2 is then scavenged by Cat and a variety of Pod into H_2O and O_2 (*17*).

The experiment investigated the effects of salinity changes on the growth of *D. salina*, the role of glycerol for *D. salina* grown at various salinities, and the relationship between salinity and activity expression of G3pdh isozyme. The aim of the research was to provide further important clues for the fields of glycerol regulation mechanism of *D. salina*, and it is hoped that the results would be helpful for the applied research by the use of the unique osmotic regulation ability of *D. salina*.

MATERIALS AND METHODS

Cultivation of *D. salina* under Salt Stresses. *D. salina* strain 435 (UTEX 200) was obtained from Institute of Hydrobiology, Chinese Academy of Sciences. Cells of *D. salina* were cultivated in the defined medium (*18*) containing 2.0 M NaCl at 26 °C and 8000 lx provided by cool-white fluorescent lamps, under a 14/10 h light/dark cycle with shaking at 96 rpm. Cells in log phase or late log phase were harvested by centrifugation at 4500*g* for 10 min at room temperature. Then the algal pellets were transferred respectively to fresh medium containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 M NaCl, and the inoculum concentration was 1% (w/v). These cultures were also cultivated at 26 °C and 8000 lx provided by cool-white fluorescent lamps, under a 14/10 h light/dark cycle with shaking at 96 rpm.

To check the cells' growth under different salt stresses, the absorbance of each NaCl concentration of cultures was read at 630 nm in a spectrophotometer (*19*), and a corresponding concentration of blank medium without algae was used as the control sample. From the relationship curve between OD_{630} and cell number regression equation, which was constituted in our previous works (*19*), the cell number was obtained by determining OD_{630}: $y = 899.08x - 12.544$, $R^2 = 0.9992$, where y = cell number ($\times 10^4$) and $x = OD_{630}$ value.

Determination of Glycerol. According to the method of Zhou et al. (*20*) with some modifications, the glycerol contents in *D. salina* cells under different salt stresses were measured. At the 6th, 9th, 12th, and 22nd days, cells were used for measurement of glycerol content. The cultures of *D. salina* containing from 0.5 to 5.0 M NaCl were transferred respectively to centrifuge tubes (5 mL/tube) and centrifuged at 4500*g* for 10 min at room temperature. The algal pellets were washed with fresh iso-osmotic medium, and centrifugation was repeated. The washed algal pellets were resuspended in 1.5 mL of distilled water and 0.2 mL of chloroform. The suspensions were sonicated at 200 W for 3 min (5 s working time and 10 s interval in a cycle) in an ultrasonic cell disruptor at room temperature, and the supernatants were collected by centrifugation at 10000*g* for 10 min at room temperature. Thirty microliters of supernatant of each sample was put in a test tube and made up to 0.2 mL by distilled water; then 1 mL of sodium periodate reagent (3 mmol/L sodium periodate and 100 mmol/L ammonium acetate in 100 mL 6% acetic acid) was added with mixing to each sample. Five minutes after the addition of sodium periodate reagent, 2.5 mL of acetyl acetone reagent (acetyl acetone/isopropanol = 1:99) was added to each sample with mixing. All tubes were placed in a water bath at 60 °C for 30 min. After the tubes had cooled at room temperature, the absorbance of each sample was read at 410 nm in a spectrophotometer (*20*). The corresponding concentrations of blank samples without algae were run in each test. Finally, the absorbance of each sample was converted to glycerol content by the 0−50 μg/0.2 mL standard curve of pure glycerol.

Hypoosmotic or Hyperosmotic Stress Culture. To estimate glycerol content changes of cells subjected to hypoosmotic or hyperosmotic stress, 50 mL of culture containing 2.0 M NaCl was put into 10 centrifuge tubes (5 mL/tube), and algae cells in these tubes were harvested by centrifugation at 4500*g* for 10 min at room temperature. Then algae pellets were respectively resuspended in isovolumetric fresh medium, which contained 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 M NaCl. After 1 h, resuspended algae cells were harvested again by centrifugation at 4500*g* for 10 min at room temperature, the algal pellets were washed with fresh iso-osmotic medium, and centrifugation was repeated. The washed algal pellets were resuspended in 1.5 mL of distilled water and 0.2 mL of chloroform for the measurement of glycerol content.

Enzyme Extraction. Cells of *D. salina* at log phase in different salinity cultures (100 mL) were harvested by centrifugation at 4500*g* for 10 min at room temperature, the algal pellets were washed with fresh iso-osmotic medium, and centrifugation was repeated. The washed algal pellets were resuspended in 2 mL of enzyme extraction buffer (100 mmol/L Tris, 20 mmol/L ascorbic acid, pH 6.9) (*21*). The suspensions were sonicated at 200 W for 3 min (5 s working time and 10 s interval in a cycle) in an ultrasonic cell disruptor at 4 °C, and the supernatants were collected by centrifugation at 13000*g* for 30 min at 4 °C. The supernatants were used for isozyme electrophoresis directly or diluted with glycerol by 50% (w/v) to maintain its activity at −20 °C until electrophoresis.

Isozyme Electrophoresis. G3pdh in supernatants were electrophoresed with vertical polyacrylamide gel electrophoresis (PAGE) as described by Jiang and Xiong (*22*) with some modification. PAGE (30 μL of each sample) was carried out at 4 °C and 80 V, increased to 160 V after 1 h. The separating gel consisted of 15% (acrylamide+bisacrylamide), 10% ammonium persulfate (AP), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and 1.5 mol/L Tris-HCl, pH 8.8. The stacking gel consisted of 5% (acrylamide + bisacrylamide), 10% AP, TEMED and 0.5 mol/L Tris-HCl, pH 6.8. The buffer was 0.05 mol/L Tris−glycine buffer, pH 8.3. The G3pdh activity staining method was according to that of Bai et al. (*21*). Immediately after electrophoresis, gel was washed and stained at 37 °C in the dark for 3 and 12 h by soaking in 50 mL of staining solution, containing 25 mg of nicotinamide adenine dinucleotide (NAD^+), 1 mg of phenazine methosulfate (PMS), 15 mg of nitro blue tetrazolium (NBT), 0.9 mM NaCl, 5 mM sodium α-glycerophosphate, and 0.15 mM phosphate-buffered saline (PBS), pH8.0. Then the stained gel washed again, fixed, and photographed. Acetic acid (7.5%) was used as stationary phase.

Statistical Analyses. Each result shown was the mean of three replicated studies. Statistical analysis of the data was performed using the program SPSS-13, and significance was determined at a 95 or 99% confidence limit.

RESULTS

Cell Growth. A correlation test showed that the growth of *D. salina* cells was significantly correlated with the salinity and incubation time ($p < 0.01$). Three obvious groups can be seen from the growth situations in different NaCl concentrations (**Figure** 1). One-way ANOVA test showed the growth of the three groups had significant difference at the 0.05 level ($p < 0.05$), and the culture containing 2.0 M NaCl showed a higher growth rate compared to other cultures. The optimum growth salinity range of *D. salina* was 1.0−2.0 M NaCl, and cells grew rapidly after 7 days (**Figure** 1**A**). Cultures containing high salinity (3.5−5.0 M NaCl) showed poor growth, which cells begun to grow even after 10 days and the growth rate decreased gradually with the augmentation of salinity (**Figure** 1**B**). Although the growth rates of other cultures (0.5, 2.5, and 3.0 M NaCl) (**Figure** 1**C**) were lower than those of cultures grown in the optimum growth salinity range, these cultures showed better growth than cultures containing 3.5−5.0 M NaCl.

Glycerol Content. The glycerol contents of *D. salina* grown at a range of salinities were measured on the 6th, 9th, 12th, and 22nd days (**Figure** 2). According to **Figures** 1 and 2, the accumulation of glycerol in *D. salina* grown at different salinities is connected not only to the growth of *D. salina* but also to the salinity (correlation test, $p < 0.01$). On the 6th day, glycerol could be detected only in the four cultures (1.0−2.5 M NaCl) with higher growth rates than the others. With the growth of *D. salina*, the glycerol was accumulated

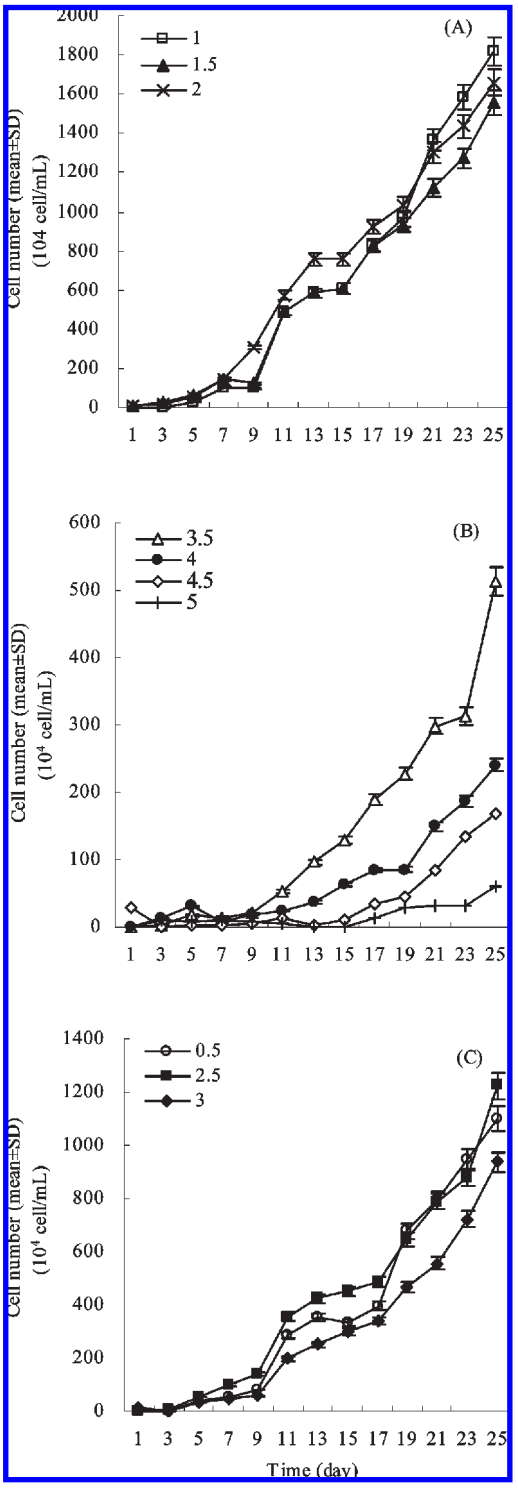

Figure 1. Cell growth curves of *D. salina* grown in a range of NaCl concentrations. The culture conditions and testing method were described under Materials and Methods. All cultures were divided into three groups: (**A**) cultures containing 1.0−2.0 M NaCl; (**B**) cultures containing 3.5−5.0 M NaCl; (**C**) cultures containing 0.5, 2.5, and 3.0 M NaCl. Data points represent the means of three replicated studies in 10 independent cultures, with the SD of the means (*t* test, $p < 0.05$). Correlation test showed the growth of *D. salina* cell was significantly correlated with the salinity and incubation time ($p < 0.01$). One-way ANOVA test showed the growths of the three groups had significant difference at the 0.05 level ($p < 0.05$).

gradually. On the 9th day, glycerol was also detected in two cultures (3.0 and 3.5 M NaCl) besides the cultures containing

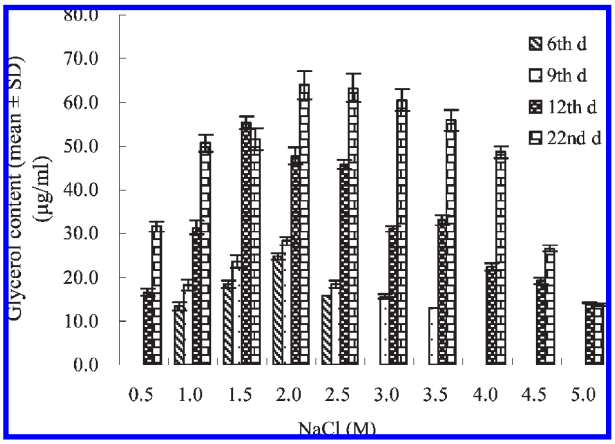

Figure 2. Glycerol content of *D. salina* grown in a range of NaCl concentrations. On the 6th, 9th, 12th, and 22nd days, the glycerol contents of cultures containing various salinities were detected as described under Materials and Methods. Columns represent the means of three replicated studies in 10 independent cultures in each day, with the SD of the means (*t* test, $p < 0.01$). Correlation test showed the accumulation of glycerol at different salinities significantly correlated with the growth of *D. salina* and salinity ($p < 0.01$) (For the growth of *D. salina*, see **Figure** 1.) One-way ANOVA test showed glycerol contents of cultures at various salinity on each day had significant difference at the 0.05 level ($p < 0.05$).

1.0−2.5 M NaCl. On the 12th and 22nd days, glycerol could be detected in all cultures.

One-way ANOVA test showed glycerol content of cultures at various salinities on each day had significant difference at the 0.05 level ($p < 0.05$). The largest glycerol concentration on the 6th, 9th, and 22nd days was always detected in the culture containing 2.0 M NaCl, and the largest glycerol concentration on the 12th day was detected in the culture containing 1.5 M NaCl. The largest glycerol concentration detected in cultures grown at various salinities was 64.02 ± 3.21 (mean ± SD) μg/mL. The culture grown at optimum growth salinity (2.0 M) was treated by hypoosmotic or hyperosmotic shock to estimate glycerol content changes of cells subjected to osmotic stress.

Treated by hypoosmotic shock, the glycerol content declined and achieved the minimum detected when salinity was diluted to 0.5 M, which was 30.27 ± 2.88 (mean ± SD) μg/mL and declined 52.05% from than the glycerol content before treatment. Treated by hyperosmotic shock, the glycerol content increased. The detected maximum glycerol content was 90.66 ± 3.64 (mean ± SD) μg/mL when the salinity was increased to 5.0 M NaCl, which increased 43.61% from than the glycerol content before treatment (**Table** 1). It is supposed that *D. salina* responds to salt stress by synthesis or elimination of intracellular glycerol. Therefore, glycerol may play an important role in osmotic adjustment to enable *D. salina* to survive at various salinities.

Isozyme Electrophoresis. The G3pdh appeared as dark bands in the isozyme electrophoresis zymogram (**Figure** 3). Four G3pdh loci were detected. Loci *G3pdh-1*, *G3pdh-2*, and *G3pdh-3* respectively encoded one homodimer enzyme G3pdh-1, G3pdh-2, and G3pdh-3. Locus *G3pdh-4* was a heterozygosis producing two homodimer enzymes G3pdh-4 and G3pdh-5. Loci *G3pdh-1*, *G3pdh-3*, and *G3pdh-4* worked only under conditions of low salinity from 0.5 to 1.5 M NaCl. G3pdh-1 existed in 0.5−1.5 M NaCl, G3pdh-4 and G3pdh-5 existed in 0.5−1.0 M NaCl, and G3pdh-3 existed in only the 0.5 M NaCl. Locus *G3pdh-2* worked in moderate and high salinities. Its product, G3pdh-2, existed from 1.5 to 4.0 M NaCl. G3pdh-2 activity was not detected at high salinities of 4.5 and 5.0 M NaCl.

Table 1. Variations of Glycerol Contents Treated by Hypoosmotic or Hyperosmotic Shock[a]

original salinity (M NaCl)	treatment salinity (M NaCl)	glycerol content, mean ± SD (μg/mL)	variation of glycerol content (%)
2.0	0.5	30.27 ± 2.88	−52.05
	1.0	49.66 ± 1.92	−21.34
	1.5	55.28 ± 1.75	−12.43
	2.0	63.13 ± 3.87	
	2.5	64.76 ± 1.07	+2.58
	3.0	69.35 ± 2.62	+9.85
	3.5	76.30 ± 3.31	+20.86
	4.0	85.78 ± 3.89	+35.88
	4.5	88.00 ± 5.32	+39.39
	5.0	90.66 ± 3.64	+43.61

[a] The *D. salina* cell in culture containing 2.0 M NaCl was treated by hypoosmotic or hyperosmotic shock from 0.5 to 5.0 M NaCl as described under Materials and Methods. The testing method of glycerol content was also described under Materials and Methods. Data are the means and SD of the means of three replicated studies (*t* test, $p < 0.01$).

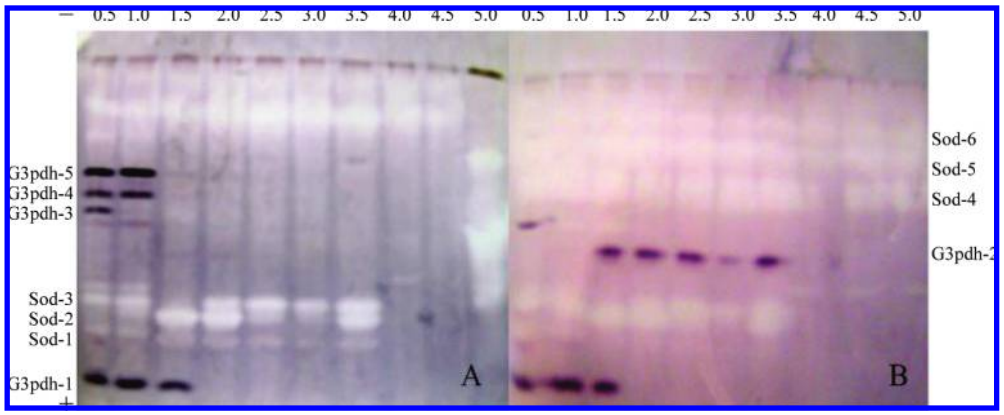

Figure 3. Two kinds of isozyme expression in *D. salina* in a range of NaCl concentrations: (**A**) staining time is 3 h; (**B**) staining time is 12 h. Preparation of enzyme extracts, vertical PAGE, and G3pdh activity staining were carried out as described under Materials and Methods. G3pdh appeared as dark bands and Sod appeared as colorless bands in gels. Symbols "+" and "−" represent the anode and cathode. Numbers 0.5−5.0 indicate the enzyme extracts were respectively prepared from cultures containing 0.5−5.0 M NaCl. The figure is representative of three replicated studies with similar findings.

Buth and Murphy (*23*) indicated that staining time is an influential factor on the score of enzyme activity. Staining a set of tissues from a single specimen on a gel was stopped when overstaining began to occur in one or more tissues. This introduces a bias against very low levels of enzyme activity that may have been expressed if staining had been allowed to proceed further. It was tested whether if the staining reaction were allowed to proceed for a longer time, more loci would be observed (*24*, *25*). We extended the time for staining to study the impact of the length of staining time. Panels **A** and **B** of **Figure** 3, respectively, show the zymograms of 3 and 12 h staining. With the extension of staining time, it was found that the activities of G3pdh-4 and G3pdh-5 disappeared, but G3pdh-2 expressed higher activity in 1.5−3.5 M NaCl. It is supposed that G3pdh-2 could express activity persistently for a long time in a large range of salinity and may play an important role in *D. salina* to tolerate high-salt environment.

Zhou et al. (*26*) have found some colorless bands besides the dark bands of triosephosphate isomerase (Tpi) in the same gel, and they have confirmed that the colorless bands were Sod, so it was possible to identify the Tpi and Sod in the same gel without a separate Sod staining. In the present research, the colorless bands in **Figure** 3 were Sod, and G3pdh and Sod were identified in the same gel. In **Figure** 3, Sod of *D. salina* is heterodimers and encoded by two loci; locus *Sod-1* encoded three isozymes, Sod-1, Sod-2, and Sod-3. Locus *Sod-2* encoded Sod-4, Sod-5, and Sod-6, which did not show a relationship with the changes of salinity. Similar to locus *G3pdh-2*, locus *Sod-1* activity was not detected at high salinities (4.0, 4.5, and 5.0 M NaCl).

DISCUSSION

Brock (*27*) suggested that the optimal growth salinity of *D. salina* in a laboratory was 2.04 M NaCl, although he observed that *D. salina* grows in >200 g/L salinity (about 3.4 M NaCl), which indicated that *D. salina* can withstand higher salinities than other species. Zhou et al. (*20*) noted that the optimal growth salinity for breeding *D. salina* was 2.0 M NaCl. Our experiment with 10 different salinities also showed that the optimal growth salinity of *D. salina* is about 2.0 M NaCl (**Figure** 1).

Glycerol is massively accumulated in *Dunaliella* to counterbalance the osmotic pressure due to the hypersalinity of the surrounding medium (*10*). The level of intracellular glycerol in *Dunaliella parva* was found to be proportional and osmotically equivalent to the external NaCl concentration, and the maximum glycerol content was detected at saturated NaCl (*7*). In the present research, glycerol contents on the 6th and 9th days were not detected in cultures grown at several salinities, especially at high salinity (4.0−5.0 M NaCl), and the maximum glycerol content was detected at optimum growth salinity (2.0 M) on the 22nd day (**Figure** 2). The difference of detected glycerol contents in the two studies may be related to the growth of cells. In our study, the growth of cells in cultures at high salinity was worse than in other cultures; the glycerol level was lower correspondingly at high salinity.

In the present research, the glycerol contents increased 43.61% when the salinity increased from 2.0 to 4.0 M, whereas the glycerol contents declined 52.05% when the salinity was diluted from 2.0 to 0.5 M (**Table** 1). The results are different from the changes of glycerol contents with osmotic shocks in the paper by

Zhou et al. (*20*), who observed that the glycerol content of the *D. salina* cell quickly rose to 2.36 times the original content when the NaCl concentration rose from 2.0 to 4.0 M and that the glycerol content was down to 11.75% of the original content when the NaCl concentration was diluted from 2.0 to 0.6 M, but all of the results showed that the glycerol content of the *Dunaliella* cell is closely related to the salinity in the medium.

As the key enzyme of glycerol synthesis, three isoforms of G3pdh have been separated from *D. tertiolecta*. The first chloroplast form was the major form when the cells were grown on high NaCl, and it has been a form for glycerol production for osmoregulation (*12*, *13*). In the present research five isozymes produced by four gene loci were detected; among those, G3pdh-2 existed in the widest range of salinity from 1.5 to 4.0 M NaCl (**Figure** 3). Therefore, G3pdh-2 is the main form of G3pdh in *D. salina*. Other isozymes of G3pdh play a role mainly in the low-salinity environment. It should be noted that the activities of all the isozymes of G3pdh did not show the process of gradual changes with the increase or decrease of salinity. For example, the activities of G3pdh-2 were almost the same among the salinities of 1.5−4.0 M NaCl. In addition, there was no G3pdh activity detected in the samples with 4.5−5.0 M NaCl, but cells can survive at these salinities. Therefore, further experiments are needed to prove how these isozymes regulate the synthesis of glycerol to adapt the osmotic stress.

As described by Haghjoua et al. (*14*), high light, low temperature, and combined high-light−low-temperature treatments increased Sod activity in *D. salina*. It was concluded that *D. salina* can partially adjust to photooxidative conditions by increasing Sod activity. In the present research, we found it was possible to identify G3pdh and Sod in the same gel (**Figure** 3), and Sod isozymes did not show significant changes with changes of salinity, except that the activities of Sod-1, Sod-2, and Sod-3 were not detected at high salinities (4.0, 4.5, and 5.0 M NaCl) (**Figure** 3). The results showed that Sod did not play a significant role in *D. salina* under various salt stresses.

In conclusion, in the present research it was shown that 2.0 M NaCl was the optimal salinity for *D. salina* and that *D. salina* could rapidly increase or decrease glycerol contents to adapt to hypoosmotic or hyperosmotic environments. Especially, we identified five isozymes of G3pdh working respectively in different salinities, and Sod had no significant relationship with the changes of salinity. The study on the G3pdh related to glycerol metabolism is a significant work for enhancing the salt tolerance of other plants and microorganisms by molecular biotechnology. To develop the unique biological characteristics of *D. salina*, future works should be conducted on the G3pdh-related genes and other salt-tolerance genes of *D. salina* to explain the mechanism of osmotic adjustment on the level of cell metabolism.

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