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Study on changes of plasmalemma permeability and some primary inorganic ions of Antarctic ice microalgae (*Chlamydomonas* sp. ICE-L) in the low-temperature stress

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Abstract The changes of plasmalemma permeability and some primary inorganic ions of Antarctic ice microalgae (*Chlamydomonas* sp. ICE-L) in the low-temperature stress were examined. The plasmalemma of ICE-L could maintain the stability at the freezing condition of -6 ℃. That signifies that it could maintain the proper function of plasmalemma and stability of the intracellular environment during sea ice formation. The function of inorganic ions on low-temperature adaptation of ICE-L was investigated by using the X-ray microanalysis method. Low temperature (0~ -6 ℃) induces Ca^{2+} concentration increment of cytoplasm, but after 24 h the content decrease quickly to normal value. As a matter of fact, Ca^{2+} plays an important role as the second messenger in the low temperature adaptation of ICE-L. In addition, low temperature also influences on the other primary inorganic ions transfer and the cell maintains activity by keeping ratio balance among different ions. Above all, it is necessary for Antarctic ice microalgae to survive and breed by maintaining the stability of K^+ content and the balance of Na^+ /Cl^-.

Key words Antarctic ice microalga, X-ray microanalysis, inorganic ions, low-temperature adaptation

The pack ice of Antarctic oceans appears to be frozen white desert, devoid of life. However, beneath the snow lies a unique habitat for groups of bacteria and microscopic plants and animals that are encased in ice matrices at low temperatures and light, with the only liquid being pockets of concentrated brines. Antarctic ice microalgae thrive in the ice, and their prolific growth ensures they play a fundamental role in Antarctic ecosystems. Antarctic ice algae were combined in the sea ice and lived in brine channels during sea ice formation, and the environmental conditions under which Antarctic ice algae live are highly variable (Thomas and Dieckmann 2002). Sea ice is also a relatively extreme environment with internal temperatures ranging from -1 ℃ to as low as -50 ℃ in winter. The salinity of sea ice brines within channels and cracks of the sea ice (formed when salt is ejected during freezing) can rise as high as 150‰ (Brown and Bowman 2001). Survival in these conditions requires a complex suite of physiological and metabolic adaptations.

Antarctic ice microalgae adapt well to hostile environments. In plants, the cytoplasm membrane plays an important role in the sensing of environmental conditions such as temperature and salinity. In previous studies, it has been shown that the variation of temperature and salinity could affect cytoplasm membrane permeability (Steponkus 1984; Maurel 1997). In addition, some important inorganic ions play an important role in viability of plant cell. The important role of calcium ion signalling in the transduction of environmental change into plant has been documented (Knight *et al.* 1996; Sedbrook *et al.* 1996; Sanders *et al.* 1999). The environmental conditions under which Antarctic microalgae live characterized by high contents of Na^+ and Cl^- and low temperature, which could affect absorbtion and utilization of inorganic irons by Antarctic ice microalgae (Kottmeier and Sullivan 1988). Therefore, the high salinity and low temperature acclimation of Antarctic ice microalgae should compare with the inorganic irons change of Antarctic ice microalgae. This study describes the effect of temperature on the cytomembrane permeability of *Chlamydomonas* sp. ICE-L and the function of some important inorganic ions in the low temperature and high salinity acclimation of Antarctic ice microalgae.

1 Materials and methods

1.1 Algal strains and experimental design

Antarctic ice microalga *Chlamydomonas* sp. ICE-L was purified from Antarctic sea ice collected in 18th Chinese Antarctic expedition during 2001/2002. ICE-L was cultured to log phase at 4 ℃, and then cultured for 6 h, 12 h, 24 h and 48 h at three temperature grades: − 6 ℃, 0 ℃, 6 ℃. The plasmalemma permeability of ICE-L was assayed respectively.

Antarctic ice microalgae ICE-L and mesophile microalgae *Platymonas* sp. (Control) were cultured to the late log phase. ICE-L were cultured by three temperature grades: 6 ℃, 0 ℃ and − 6 ℃, − 6~ 0 ℃ counted as low temperature; *Platymonas* sp. were cultured by three temperature grades: 15 ℃, 6 ℃ and 0 ℃. ICE-L and Platymonas sp. were cultured for 6, 12, 24, 48 h in temperature grades, and content of some inorganic ions were assayed.

1.2 Assay of relative permeability of plasmalemma

Antarctic ice microalgae ICE-L were cultured in the f/2 medium of Guillard and Ryther (1962). ICE-L cells were collected by centrifugation (6 000 × g for 5 min), and washed three times using distilled water to eliminate salinity. Then the pellets were put into tubes with quantificational distilled water, shaken for 15 min, and assayed the conductance by conductometer after being placed for 15 min. This was low temperature treated conductance (E_1). Then the tube was placed in boil water and placed for 10 min, cooled to room temperature and the total conductance (E_2) was assayed. The conductance of distilled water was E_0. Relative permeability of plasmalemma (P) was calculated using the following formula:

$$P = [(E_1 - E_0)/(E_2 - E_0)] \times 100\%$$

The experiments were performed three times.

1.3 Preparation of samples for X-ray microanalysis

Microalgae cells were collected by centrifugation (6 000 × g for 3 min), and washed with ice-cold distilled water(4 ℃) to remove the culture medium and prevented the extracellular medium from contributing to the intracellular elemental content. After washing, specimens were placed in a aluminium netty bags and frozen immediately in liquid iso-pentane / propane (V/V = 1∶3) cooled by liquid nitrogen for 1-2 min, then put into freeze-drier at (-106 ℃ placed for 4 d, and then placed at room temperature for 24 h. Specimens were transferred to T vacuum osmotic tubes and filtered with ethyl ether at 27 ℃ vacuum for 24 h and then were embedded in Spurr's resin (Spurr 1969). After embedment, ultrathin (1 μm) sections were made with an ultramicrotome. The sections were then fixed to copper nets and coated with a conductive carbon layer.

1.4 X-ray microanalysis

Prepared samples were subjected to electron microscopy and the X-ray microanalysis was conducted using a HITACHI H-800 transmission electron microscope equipped with an EDAX 9100 series energy dispersive X-ray microanalyzer. The microscope was running at 120 kV. X-ray spectra were analyzed for the peak-to-background area ratios (P/B) of elements (Ca, Na, K, Cl). The elemental composition parameters P and P/B of slices were duplicated and averaged over the results of seven replicated measurements. The intensity was expressed as the number of counts per second (cps).

2 Results

2.1 Effect of temperature on the plasmalemma permeability of ICE-L

The effect of temperature on the plasmalemma permeability of ICE-L is shown in Figure 1. The plasmalemma permeability of ICE-L showed similar variation trends in response to different culture temperature. Cultured at -6 ℃, it increased with the treating time prolonged at beginning from 63.33% for 6 h to maximum 72.21% for 24 h, then declined slightly. The plasmalemma permeability of ICE-L cultured at -6 ℃ was relatively higher than that at 0 ℃ and 6 ℃, which increased to maximum at 24 h from begin, then declined slightly. But The plasmalemma permeability of ICE-L at 0 ℃ was lower than that at 6 ℃.

2.2 Effect of temperature on Ca^{2+} contents of ICE-L

The effect of temperature on Ca^{2+} contents of ICE-L is shown in Figure 2a. The Ca^{2+} contents of ICE-L cultured at -6 ℃ increased to maximum 105.03 cps at 12 h and decreased to minimum 2.58 cps at 48 h. The Ca^{2+} contents of ICE-L cultured at 0 ℃ and 6 ℃ showed similar variation trend, but the Ca^{2+} contents of ICE-L cultured at -6 ℃ were highest at three temperature grades.

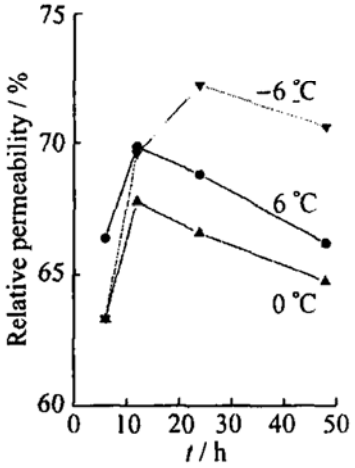

Fig 1 Effect of temperature on the cytomembrane permeability of ICE-L

The Ca^{2+} contents of *Platymonas* sp. cultured at 0 ℃ were 99.79 cps at 6 h, then decreased remarkably to 2.58 cps at 12 h, and maintained 1.96 cps (24 h) and 2.43 cps (48 h). The Ca^{2+} contents of *Platymonas* sp. cultured at 6 ℃ were increased to maximum 102.05 cps at 12 h, and then decreased to minimum 2.4 cps at 48 h. The Ca^{2+} contents of *Platymonas* sp. cultured at 15 ℃ increased slightly from 2.57 cps (6 h) to 11.52 cps (48 h).

The variation trend of Ca^{2+} contents of ICE-L cultured at – 6 ℃ was similar to that of *Platymonas* sp. cultured at 6 ℃. The Ca^{2+} contents of both microgalgae reached maximum at 12 h, and then decreased to minimum remarkably.

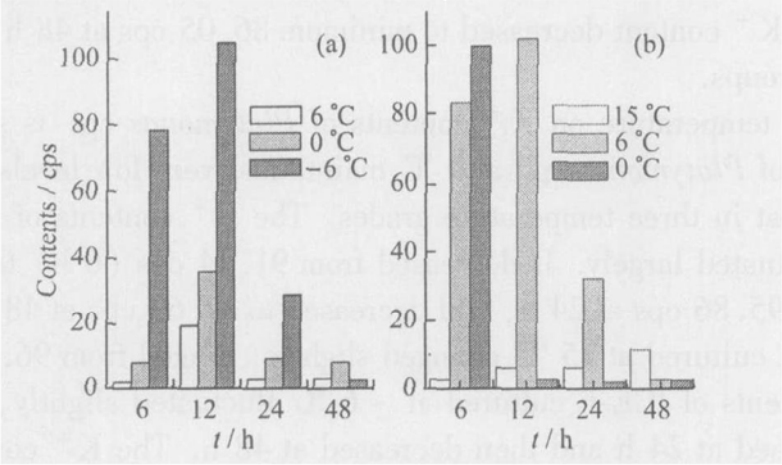

Fig 2 Effect of temperature on Ca^{2+} content of ICE-L (a) and *Platymonas* sp. (b)

2.3 *Effect of temperature on Na^+ contents of ICE-L*

The effect of temperature on Na^+ contents of ICE-L is shown in Figure 3a. The Na^+ contents of ICE-L cultured at – 6 ℃ increased remarkably from 2.86 cps (6 h) to 38.42 cps (48 h). Both Na^+ contents of ICE-L cultured at 0 ℃ and 6 ℃ were low and increased slightly in 48 h, and kept relative balance. The Na^+ contents of ICE-L cultured at – 6 ℃ were much higher than those cultured at 6 ℃ and 0 ℃.

The effect of temperature on Na^+ content of *Platymonas* sp. is shown in Figure 3b. The Na^+ contents of *Platymonas* sp. cultured at 0 ℃ increased from 28.17 cps (6 h) to 41.10 (48 h), which even higher than those of ICE-L cultured at – 6 ℃. The Na^+ con-

tents of *Platymonas* sp. cultured at 6 ℃ were 2.66 at 6 h and 2.03 cps at 12 h, then increased remarkably and reached 36.27 cps at 24 h and kept relative stable. The Na^+ contents of *Platymonas* sp. cultured at 15 ℃ were very low, increased slightly and kept relatively stable (2.19–8.12 cps).

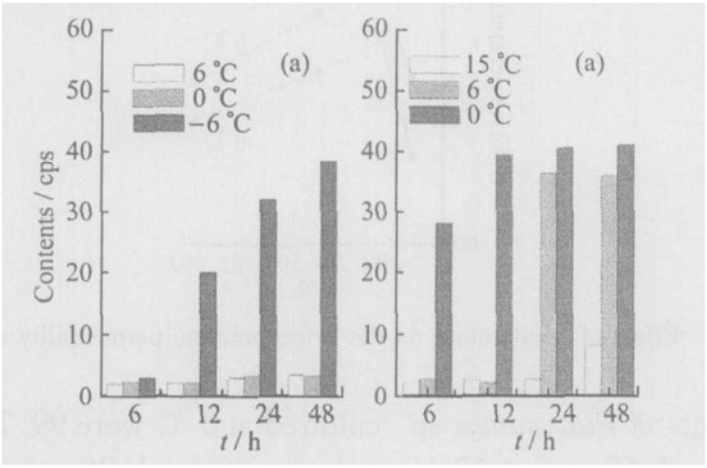

Fig. 3 Effect of temperature on Na^+ content of ICE-L (a) and Platymonas sp. (b)

2.4 *Effect of temperature on K^+ contents of ICE-L*

The effect of temperature on K^+ contents of ICE-L is shown in Figure 4a. The K^+ contents of ICE-L maintained high levels (90–105 cps) at three temperature grades, which showed temperature affected K^+ content of ICE-L slightly. The K^+ content of ICE-L cultured at −6 ℃ reached maximum 112.13 cps at 24 h, which was highest in all tested groups. Then the K^+ content decreased to minimum 86.05 cps at 48 h, which was the lowest in all tested groups.

The effect of temperature on K^+ contents of *Platymonas* sp. is shown in Figure 4b. The K^+ contents of *Platymonas* sp. at 0 ℃ maintained very low levels (2.08–5.99 cps) and were the lowest in three temperature grades. The K^+ contents of *Platymonas* sp. cultured at 6 ℃ fluctuated largely. It decreased from 91.34 cps (6 h) to 20.6 cps (12 h), then increased to 95.86 cps at 24 h, and decreased to 27.63 cps at 48 h. The K^+ contents of *Platymonas* sp. cultured at 15 ℃ changed slightly, ranged from 96.5 cps to 98.92 cps.

The K^+ contents of ICE-L cultured at −6 ℃ fluctuated slightly, which decreased at 12 h, then increased at 24 h and then decreased at 48 h. The K^+ contents of *Platymonas* sp. at 6 ℃ showed similar trend, but fluctuated more largely. The K^+ contents of ICE-L cultured at 0 ℃ and 6 ℃ maintained stable and were similar to those of *Platymonas* sp. cultured at 15 ℃.

2.5 *Effect of temperature on Cl^- contents of ICE-L*

The variation trend of Cl^- contents is similar to that of Na^+ content in ICE-L. The Cl^- content of ICE-L cultured at −6 ℃ decreased from 4.74 cps (6 h) to 1.96 cps (12 h), and then increased sharply to maximum 64.9 cps at 48 h. Both Cl^- contents of ICE-L at 0 ℃ and 6 ℃ were low and maintained relative stable in 48 h (Figure 5a).

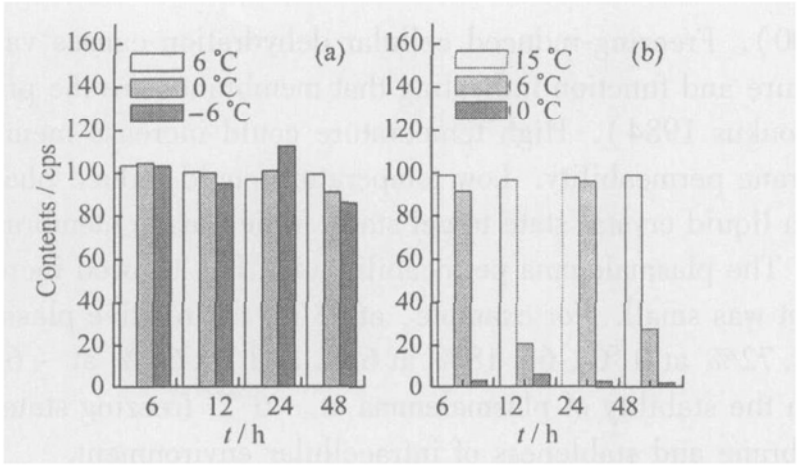

Fig 4 Effect of temperature on K^+ content of ICE-L (a) and Platymonas sp (b)

The effect of temperature on Cl^- content of *Platymonas* sp is shown in Figure 5b. The Cl^- contents of *Platymonas* sp cultured at 0 ℃ increased from 2.44 cps (6 h) to 12.08 cps (24 h), and then decreased slightly to 11.42 cps at 48 h. The Cl^- content of *Platymonas* sp cultured at 6 ℃ decreased from 4.2 cps (6 h) to 1.77 cps (12 h), and then increased sharply to maximum 65.8 cps at 48 h. The Cl^- contents of *Platymonas* sp cultured at 15 ℃ kept relative stable in 24 h, ranged from 2.07 cps to 2.54 cps, then increased to 10.0 cps at 48 h.

The Cl^- contents of ICE-L cultured at − 6 ℃ and *Platymonas* sp at 6 ℃ had similar variation trend, namely Cl^- content decreased to minimum at 12 h and then increased to maximum at 48 h, and their Cl^- contents at 48 h were basically same.

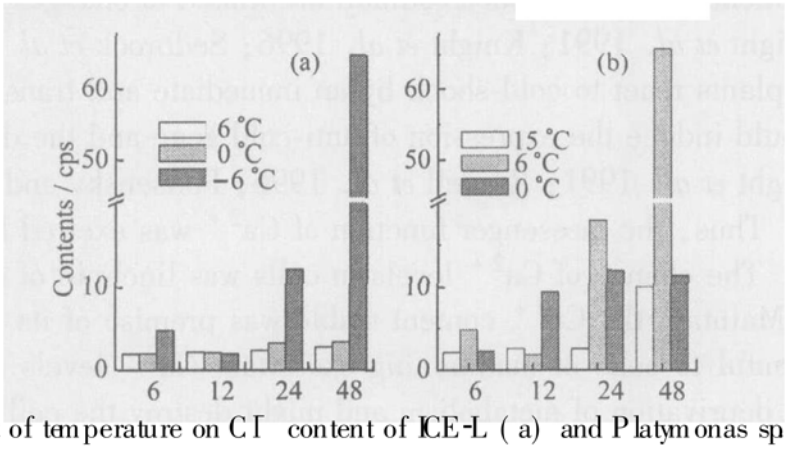

Fig 5 Effect of temperature on Cl^- content of ICE-L (a) and Platymonas sp (b)

3 Discussion

3.1 The role of the relative permeability of plasmalemma in ICE-L survival

All the plasmalemma permeability of ICE-L cultured at three temperature grades was increased in short time and then decreased. This suggested that ICE-L could maintain the stability of plasmalemma at low temperature. The plasmalemma permeability at 0 ℃ was the lowest and increased whether the temperature increased or decreased, and that indicated that the plasmalemma of ICE-L was most stable at 0 ℃. Temperature induced change in membrane fluidity is one of the immediate consequences in plants during temperature stresses and might represent a potential site of perception and/or injury (Horvath *et al.* 1998;

Orvar *et al.* 2000). Freezing-induced cellular dehydration causes various perturbations to membrane structure and function indicating that membranes are the primary targets of freezing injury (Steponkus 1984). High temperature could increase membrane fluidity, which increased membrane permeability. Low temperature could induce phase transition of membrane lipid from liquid crystal state to gel state, which made membrane crack and permeability increased. The plasmalemma permeability of ICE-L showed increase trend at −6 ℃, but the increment was small. For example, at 48 h, the relative plasmalemma permeability of ICE-L was 63.72% at 0 ℃, 66.18% at 6 ℃ and 70.60% at −6 ℃. Therefore, ICE-L could maintain the stability of plasmalemma at −6 ℃ freezing state and maintain normal function of membrane and stableness of intracellular environment.

3.2 *The function of Ca^{2+} in ICE-L survival at low temperature*

The cytosolic Ca^{2+} seems to be linked to the acquisition of tolerance to low temperature stresses. Results in this study showed that the Ca^{2+} content increment was induced by low temperature 0− −6 ℃ in ICE-L cytoplasm, and the more temperature decreased, the more Ca^{2+} content increased. The Ca^{2+} content of ICE-L reached maximum at 12 h, which was 40 times of normal value of cells (2.5−2.6 cps). Then the Ca^{2+} content of ICE-L decreased to normal level at 48 h. This trend occurred in *Platymonas* sp. too at 6 ℃. Elevated cytosolic Ca^{2+} levels in response to low temperature are mainly due to Ca^{2+} influx from extracellular stores (Monroy and Dhindsa 1995).

Many environmental and endogenous stimuli are linked to changes in Ca^{2+} of cytoplasm in in plants (Knight *et al.* 1991; Knight *et al.* 1996; Sedbrook *et al.* 1996). It has been demonstrated that plants react to cold-shock by an immediate and transient rise in cytosolic calcium, which could induce the expression of anti-cold gene and the development of freezing tolerance (Knight *et al.* 1991; Russell *et al.* 1996; Polisensky and Braam 1996; Sangwan *et al.* 2001). Thus, the messenger function of Ca^{2+} was exerted by regulate dissociative Ca^{2+} in cells. The change of Ca^{2+} levels in cells was linchpin of cells response to external stimulator. Maintain the Ca^{2+} content stable was premise of its messenger function. However, it is harmful to cells of maintaining exorbitant Ca^{2+} levels in long time, which could result in the deprivation of metabolism and might destroy the cell architecture (Wang and Jian 1994). Therefore, in order to maintain the stability of Ca^{2+} contents, it is necessary to active Ca^{2+} translation systems, such as Ca^{2+} − ATPase, which could make high Ca^{2+} levels stimulated revert to normal levels after accomplish signal transduction. The results demonstrate that Ca^{2+}, as a second messenger, is required for cold induced gene expression and development of freezing tolerance.

Ca^{2+} contents of ICE-L sharply increased in short time (6−12 h) at −6 ℃ and induced the expression of anti-cold genes, and then the Ca^{2+} levels decreased. This may be the reason that superfluous Ca^{2+} was transported out of cytoplasm by Ca^{2+} − ATPase and decreased to normal levels, thereby maintained the cell function of metabolism and signal transduction (Knight *et al.* 1996). These indicated that Ca^{2+} plays important roles in the cold acclimation of sea microalgae as second messenger. The optimum growth temperature of ICE-L is −3−6 ℃, Ca^{2+} content of ICE-L maintain low levels at 6 ℃ while its increased largely at −6 ℃ as second messenger and could revert normal levels quickly. The

optimum growth temperature of *Platymonas* sp. is about 20 ℃, Ca^{2+} play the same function as it in ICE-L at 6 ℃. But at 0 ℃, Ca^{2+} content of *Platymonas* sp. increased to maximum at 6 h and decreased to minimum at 12 h, which indicated *Platymonas* sp. could be destroyed at low temperature 0 ℃, the ability of Ca^{2+} regulation in cells was deprived after 6 h, and Ca^{2+} was leaked from cytoplasm.

3.3 *The function of Na^+, K^+ and Cl^- in ICE-L survival at low temperature*

At normal growth temperature (0– 6 ℃), the Na^+ and Cl^- contents of ICE-L cytoplasm were low and maintained relative stable, and the value of Na^+/Cl^- in cells also kept relative stable. The Na^+ and Cl^- contents of ICE– L cytoplasm were increased quickly at low temperature – 6 ℃, but their increased speeds were different. The Na^+ increased largely while Cl^- contents decreased, which make the value of Na^+/Cl^- ratio increased remarkably at 12 h. Then the value of Na^+/Cl^- ratio in cells decreased with the enhancement the ability of cells absorb Cl^- and reverted to the levels of 6 h after 48 h (Figure 6a). The value of Na^+/Cl^- ratio in *Platymonas* sp. maintained relative stable at 6 ℃ and 15 ℃. Cultured at 0 ℃, the value of Na^+/Cl^- ratio in *Platymonas* sp. was 12.56 at 6 h, which much more than those at two other temperature, and decreased to 4.1 at 12 h, and then maintained relative stable (Figure 6b).

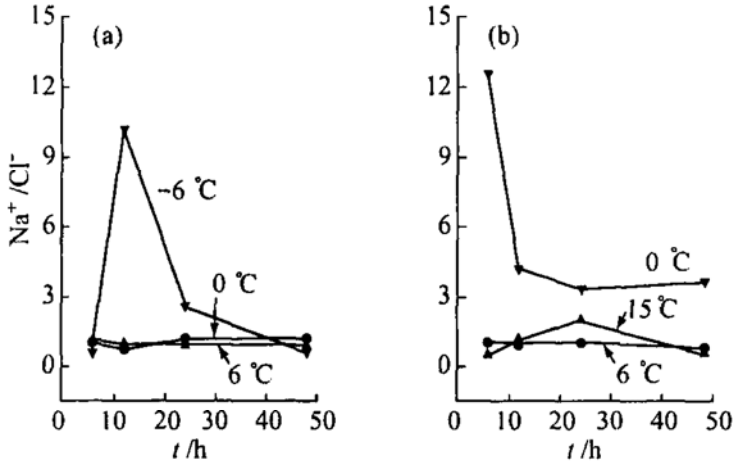

Fig. 6 Effect of temperature on Na^+/Cl^- ratio of ICE-L (a) and *Platymonas* sp. (b)

The K^+ contents of ICE-L maintained relative stable at three temperature grades. The value of Na^+/K^+ ratio in ICE-L ranged 0.02~ 0.03 at 6 ℃ and 0 ℃, but increased largely at – 6 ℃ (Figure 7a). The K^+ content maintained relative stable and the Na^+ contents increased at – 6 ℃ in ICE-L, while the Na^+ contents were still much lower the K^+ content. Therefore the enhancement of Na^+ contents in cytoplasm didn't induce the K^+ exosmosis. The K^+ contents were still much higher than the Na^+ contents in ICE-L indicated the K^+ requirement of cells was much higher than that of Na^+. Potassium is an important nutriment in plant cells. It is significant for membrane integrality and enzymes activity to keep the stability of cytoplasm K^+. The Na^+ contents increased and the K^+ contents decreased at 0 ℃ in *Platymonas* sp., which made the Na^+/K^+ ratio values increase to 19.76 at 48 h (Figure 7b). The reason of the enhancement of the Na^+/K^+ ratio values was the leakage of K^+.

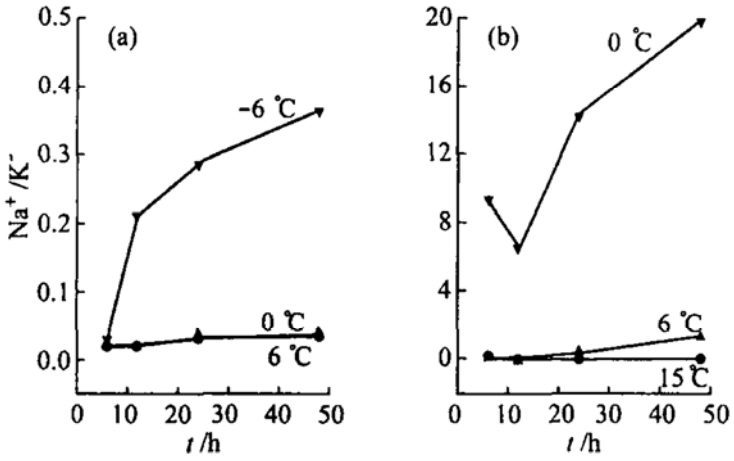

Fig 7 Effect of temperature on Na^+/K^+ ratio of ICE-L (a) and *Platymonas* sp (b)

The culture medium of Antarctic ice algae was freezing at – 6 ℃. Therefore, the environmental condition in which Antarctic ice algae incubated was not only low temperature and but also high salinity (Riaux-Gobin 2000). The increase of salinity could induce Antarctic ice algae to enhance the absorbed dose of Na^+ and Cl^-, which could resist the change of osmotic pressure and cells destruction during ice formation. Though the Na^+ and Cl^- contents of cytoplasm and the Na^+/K^+ ratio values increased, the K^+ of cytoplasm wasn't exosmosis. Therefore, the most likely cause was the channels of Na^+ and Cl^- in membrane were opened and the Na^+ and Cl^- content increased. At the same time, these suggested Na^+ and Cl^- were transported into cytoplasm mainly by passive transport too (Trevena *et al*. 2000).

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