การนำไนเตรตเข้าเซลล์ไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*

นางสาว จุฑาแข วังสุภา

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NITRATE UPTAKE BY HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*

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นางสาวจุฑาแข วังสุภา : การนำไนเตรตเข้าเซลล์ไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*. (NITRATE UPTAKE BY HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*) อ. ที่ปรึกษา : รศ. ดร. อรัญ อินเจริญศักดิ์, 79 หน้า.
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การนำไนเตรตเข้าเซลล์ได้มีการศึกษาในแบคทีเรียทนเค็ม *Aphanothece halophytica* โดยการวัดปริมาณไนเตรตที่เหลืออยู่ในสารละลาย การศึกษากลไกทางจลนพลศาสตร์ของการนำไนเตรตเข้าเซลล์พบว่าในภาวะปกติและภาวะที่มีความเครียดจากแรงดันออสโมติกมีค่า K_s = 416 และ 450 μM และอัตราเร็วสูงสุด = 9.1 และ 5.3 μmol/min/mgChl ตามลำดับ ความสามารถการนำไนเตรตเข้าเซลล์ลดลงอย่างมากโดยการเติมแอมโมเนียมและตัวยับยั้งกระบวนการตรึงคาร์บอนไดออกไซด์คือ DL-glyceraldehyde ตัวยับยั้งการนำแอมโมเนียมไปใช้ เช่น L-methionine sulfoximine ปลดปล่อยการถูกยับยั้งด้วยแอมโมเนียม จากผลการทดลองสามารถสรุปได้ว่าการนำ ไนเตรตไปใช้ประโยชน์ถูกควบคุมโดยแอมโมเนียมและกระบวนการตรึงคาร์บอนไดออกไซด์ ไนไตรต์เป็นตัวยับยั้งแบบแข่งขันที่มีประสิทธิภาพของการนำไนเตรตเข้าเซลล์ โดยมีค่า K_i = 84 μM ไนไตรต์และไนเตรตอาจจะถูกขนส่งด้วยตัวพาชนิดเดียวกัน monensin ลดการนำไนเตรตเข้าเซลล์ซึ่งชี้ให้เห็นว่าการนำไนเตรตเข้าเซลล์ถูกควบคุมโดยการรักษาสภาพของ Na^+ electrochemical gradient ให้คงไว้ amiloride ซึ่งเป็นตัวยับยั้งของกระบวนการ Na^+/H^+ antiport และ Na^+ channels พบว่าลดอัตราการนำไนเตรตเข้าเซลล์ทำให้เห็นว่าการนำไนเตรตเข้าเซลล์ของ *Aphanothece halophytica* อาจมีผลมาจากกระบวนการ Na^+/H^+ antiport *N,N*-dicyclohexylcarbodiimide และ carbonylcyanidetrifluoromethoxyphenylhydrazone เป็นตัวยับยั้งที่ดีของการนำไนเตรตเข้าเซลล์ใน *Aphanothece halophytica* จากผลการทดลองชี้ให้เห็นว่า pH gradients ที่มาจากกระบวนการ H^+/ ATPase น่าจะเป็นตัวขับดันให้เกิดการนำไนเตรตเข้าเซลล์ใน *Aphanothece halophytica*

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต .....
สาขาวิชา .....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา .....
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Nitrate uptake has been studied in the halotolerant cyanobacterium *Aphanothece halophytica* by monitoring nitrate remaining in the solution after contacting with the cells. Study of kinetics of nitrate uptake by cells grown under normal (0.5 M NaCl) and salt stress (2 M NaCl) conditions revealed K_s values of 416 and 450 μM respectively, the maximum velocities (V_{max}) were 9.1 and 5.3 μmol/min/mgChl respectively. The ability of the cells to take up nitrate was effectively reduced by either ammonium addition or selective inhibition of CO_2 fixation by DL-glyceraldehyde. Ammonium assimilation inhibitor, L-methionine sulfoximine was able to release nitrate uptake from ammonium inhibition. Overall results support the contention that nitrate utilization in cyanobacteria is regulated at the level of nitrate uptake through the concerted action of ammonium and CO_2 fixation. Nitrite behaved as an effective competitive inhibitor of nitrate uptake, with a K_i (NO_2^-) of 84 μM. Nitrate and nitrite might be transported by the same carrier. Monensin depressed nitrate uptake in a concentration-dependent manner indicating that the nitrate uptake relies on the maintenance of a Na^+ electrochemical gradient. Amiloride, a potent inhibitor of Na^+/H^+ antiport and Na^+ channels, caused a decreased rate of nitrate uptake suggesting that nitrate uptake in *A. halophytica* was partly due to the Na^+/H^+ antiport.*N*,*N*-dicyclohexylcarbodiimide and carbonylcyanidetrifluoromethoxyphenyl-hydrazone are potent inhibitors of nitrate uptake in *Aphanothece halophytica.* These results might indicate that a pH gradient generated by H^+/ATPase drives nitrate uptake in *Aphanothece halophytica.*

Department…….. Biochemistry……. Student's signature…….

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ABBREVIATION

°C	degree celsius
Hepes	*N*-2-hydroxyehylpiperazine-*N*-ethanesulfonic
l	litre
lux	**Photometric (light density)**
min	minute
ml	millilitre
mM	millimolar
μM	micromolar
nm	nanometre
NR	Nitrate reductase
NRi	Nitrite reductase
MSX	L-Methionine Sulfoximine
DLG	DL-Glyceraldehyde
CCCP	Carbonylcyanidetrifluomethoxyphenylhrazone
DCCD	*N*,*N*-dicyclohexycarbodiimide

CHAPTER I

INTRODUCTION

Nitrogen Assimilation

After carbon, oxygen and hydrogen, nitrogen is the most abundant element in all living cells. Nitrogen is a constituent of a large number of important compounds found in all living cells. Particular notable examples are amino acids, protein (enzymes) and nucleic acid (RNA and DNA), while in other examples polyamines and chlorophyll, may play a major role in some organisms. Most animals do not have the capacity to assimilate inorganic nitrogen, nor to synthesize half the amino acids found in protein, unless assisted by bacteria (e.g. in the rumen of sheep and cattle).

Nitrogen is available to all living cells in the three basic forms: free nitrogen gas, and as combined inorganic or organic compounds. The ability to reduce or fix nitrogen gas is found only among the prokaryotes and in the algae exclusively in the Cyanophyceae. Nitrogen fixation is the conversion of gaseous nitrogen (N_2) to ammonia. Nitrogen fixation requires nitrogenase, an oxygen-sensitive irons-, sulphur-, molybdenum-containing enzyme complex that also brings about the reduction of other substrates containing triple covalent bounds (nitrous oxide, cyanides, isocyanides, cyclopropene, and acetylene).

Nitrate is a major source of nitrogen for photosynthetic organisms including cyanobacteria, algae and plants (Guerrero et al., 1981). Nitrate assimilation takes

place by three sequential steps: (1) nitrate transport into the cell by a specific nitrate permease; (2) reduction to nitrite by assimilatory nitrate reductase; and (3) further reduction to ammonium by assimilatory nitrite reductase. The resulting ammonium is then incorporated into central metabolism through the action of glutamine synthetase and glutamate synthase.

Two families of membrane proteins have been identified that can mediate the active transport of nitrate, presumably by symport with protons, across the plasma membrane: the nitrate-nitrite porters (NNP) and the peptide transporter (PTR) family. Both families have prokaryotic and eukaryotic members and have been assigned to the Major Facilitating Superfamily. Both families share the usual features of the superfamily in having two sets of six transmembrane domains linked by a longer cytoplasmic loop. The molecular identity of these two transport systems is given below.

1. Peptide Transporter (PTR) Family

The PTR family includes members that transport a wide range of nitrogen-containing substrates, including amino acids, peptides, and nitrate (Pao et al., 1998). This family has also been called the proton-dependent oligopeptide transporter or POT (Steiner et al.,1995). A phylogenetic analysis indicates that there are four clearly distinguishable clusters with bacterial proteins in cluster I, animal proteins in cluster II, yeast and one plant, proteinAtPtr2, in cluster III, and the remaining plant protein in cluster IV (Schroeder et al., 1994). All members of this family have two characteristic conserved motifs, one between transmembrane domains 2 and 3, and the other within

the fifth (Paulsen et al., 1994). Many members also have a protein kinase C recognition motif at the beginning of the central cytoplasmic loop. The first plant nitrate transporter gene to be cloned, *AtNRTl.l* (formerly called *CHLl*, belongs to the PTR family (Tasay et al., 1993). The *Arabidopsis* gene was isolated by T-DNA tagging in 1993, but the mutant phenotype was described much earlier and has been selected by resistance to the toxic nitrate homologue, chlorate (Doddem et al., 1979). Assigning substrates to the PTR family members is difficult because the family can transport such a diverse range of nitrogen-containing solutes. Two members that exhibit less than 40% identity to *AtNRTl.l* have been identified as oligopeptide transpoters, and one family member transports both nitrate and basic amino acids (Zhou et al., 1998).

2. Nitrate-Nitrite Porter (NNP) Family

The first members of this family were identified in *Escherichia coli*, and one shown to mediate nitrite efflux (Rowe et al., 1994). Like the PTR family, the NNPs also have a conserved signature sequence (Forde BG, 2000). In *Aspergillus nidulans*, the *crnA* gene was isolated from a mutant obtained by selection using chlorate. This gene encodes a functional nitrate/nitrite transporter that has been characterized by expressing the protein in *Xenopus* oocytes (Zhou et al., 2000). In green algae, *Chlamydomonas reinhardtii*, a group of genes related to *crnA* have been identified that are involved in the high-affinity uptake of nitrate and nitrite (Quesada et al., 1998). Sequence homology to *crnA* was used to clone genes from higher plants, and now a large family has been identified and classified as the *NRT2*s. In *Arabidopsis*, there are at least seven *NRT2* genes, of which several have been cloned, as have four

in barley (Tureman et al., 1996), Differences in the predicted two-dimensional structure within the NNP family was the basis for subdivision into three groups: prokaryotes, fungal and algae/plants (Forde BG, 2000). The fungal members have a long central loop of around 90 amino acids between transmembrane domains 6 and 7. The *Chlamydomonas* and plant family members all have long C-terminal domains of around 70 amino acids that extend into the cytoplasm, and these members can be further subdivided based on the presence or absence of a conserved N-terminal sequence (Forde BG, 2000).

There are two types of nitrate reductase known in algae. The first, and better-known enzyme, is found in eukaryotic algae. This nitrate reductase complex is similar to that found in fungi and higher plants, consisting of haem (cytochrome *b-557*), flavin adenine dinucleotide (FAD), and molybdenum (Mo) as a prosthetic group. The best caracterized algae enzyme is from *Chlorella*; this enzyme has a molecular mass of about 350,000 and a complex structure (Solomonson, 1979). The enzyme catalyzes the reduction of nitrate to nitrite by reduced pyridine nucleotides in accordance with equation:

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD(P)^+ + H_2O$$

With the higher plant enzyme there is evidence that Mo is contained in a small complex (molecular mass less than 30,000) that can be fairly easily separated from the bulk of the enzyme (Hewitt et al., 1980). Haem is present as a cytochrome *b-557* which is reduced by NADH and reoxidized by NO_3^-; cyanide stops its reoxidization by NO_3^- but not its reduction NADH (Hewitt et al., 1980). The pathway of electrons from NAD(P)H to nitrate through nitrate redutase from eukaryotes may be depicted as:

$$NAD(P)H \rightarrow (FAD \rightarrow \text{ cyt b-557} \rightarrow Mo) \rightarrow NO_3^-$$

The pyridine nucleotide specificity of the enzyme differs in different algae. Nitrate reductase is always active with NADH as the electron donor and, in many algae, only with this. Some algae, however, are not able to utilize NADPH (Hattori, 1970).

The second type of nitrate reductase is found in prokaryotic cells and, in the algae, in the blue-green algae. The prokaryotic nitrate reductase is simpler and smaller (molecular mass of about 75,000); it also contains molybdenum but not flavin or cytochrome (Manzano et al., 1976). The important difference from the enzyme of eukaryotes is that it does not use pyridine nucleotide as electron donor but reduced ferredoxin. It therefore catalyzes this reaction:

$$NO_3^- + 2Fd \ +2H^+ \rightarrow NO_2^- + 2Fd \ + H_2O$$

Activation of cytosolic nitrate reductase has been studied in depth over the last 20 years. In higher plants, there are a number of examples of nitrate reductase activation by ferricyanide, light, and higher levels of CO_2, but it seems that redox potential ($NADH/NAD^+$) could be an important regulator of proportion of nitrate reductase found in active form (Solomonson et al., 1979). According to Kaiser et al, there is a light/dark modulation of nitrate reductase, via nitrate reductase phosphorytion / dephosphorytion reaction, dependent on Mg^+ and ATP and requiring regulator proteins (Kaiser et al, 1992). These proteins are a protein kinase (PK) which phosphorylates nitrate reductase but without inactivating it, an inhibitor protein (IP) which binds to phospho-nitrate reductase and inactivates it, and a protein phosphatase (PP) which reactivates nitrate reductase (Glaab and Kaiser, 1995). Recently, a low

molecular weight polypeptide was found in the Lip laboratory, which they called NAP (nitrate reductase activating peptide) capable of stabilizing the activity of nitrate or restoring some types of lost activity (Alikulov et al., 1995). They also found that NAP molecules are identical in both higher plants and fungi.

The reduction of NO_2^- to NH_4^+ is catalyzed by ferredoxin nitrite reductase, characteristic of photosynthetic organisms (Vega et al., 1980) (the second type of nitrite reductase, NAD(P)H-nitrite reductase is found in nonphotosynthetic organisms) This enzyme appears to be much the same in algae and in leaves of higher plants. It is a small molecule with a molecule mass of 60,000-70,000 . This enzyme contains sirohaem which is an iron tetrahydroporphyrin and where NO_2^- probably attaches, it also contains an iron-sulpur center which participates in electron transport .The reaction catalyzed is

$$NO_2^- + 6\ Fe + 8\ H^+ \rightarrow NH_4^+ + 6\ Fe + 2H_2O$$

In the leaves of higher plants, nitrite reductase has been localized definitely in the chloroplast; information about its localization in algae cells is lacking, but some results suggest a close linking between nitrite reduction and the photochemical reaction in the chloroplast.

Regulation of nitrate assimilation

As the assimilation of nitrogen into protein requires both energy and organic skeletons, it is not surprising that there are major interactions between N-assimilation

and photosynthetic metabolism (Beever et al, 1972). The assimilation of both NH_4^+ and NO_3^- is dependent on photosynthesis, that is assimilation requires light and CO_2; removal of either of these prevents assimilation (Tisher et al, 1978). *Chlamydomonas reinhardtii* did not assimilate nitrate and ammonium unless a suitable source of carbon was provided. Suitable sources were CO_2 in light or, in darkness, acetate or the carbon reserves accumulated in nitrogen-starved cells. Nitrate metabolism is strongly light dependent in green algae. Nitrate uptake can be stimulated by light, leading to indirect activation of nitrate reductase (Tisher et al., 1978). The enzyme nitrate reductase can also be directly activated by light; light can furthermore induce nitrate reductase synthesis (Tisher et al., 1978). Nitrate reductase activity was increased both by red and blue light pulses and the effect was partially reversed by far red light in the green marine algae *Ulva lactuca*. Continuous blue light generally is more effective than continuous red light at stimulating nitrate uptake and metabolism (Aparicio et al., 1976). Light also controls nitrite reductase activity in *Chlorella* sp (Tisher et al., 1978). Cole and Toetz reported that light limited nitrate uptake by freshwater reservoir phytoplankton below 10-20% of incident light and also at higher light intensities. Most authors have not observed inhibition of high light intensities. Curtis and Megard reported that nitrite uptake by the green algae *Chlamydomonas* as a function of irradiance did not follow Michaelis-Menten kinetics because of inhibition at high irradiances. The Haldane equation described nitrite uptake better. However, the nitrite uptake as a function of oxygenic photosynthesis followed Michaelis-Menten kinetics. Solomonson and Shepar proposed a model for the regulation of nitrate assimilation in which CO_2 fixation and nitrate assimilation are coordinately controlled by the intracellular ratio of $[O_2]/[CO_2]$ (Solomonson et al., 1977). This model accounts for the observed effects of O_2, CO_2, and light on nitrate assimilation

and provides a rationale for the *in vivo* regulation of nitrate reductase by cyanide. Losada and Vega et al. have emphasized that reduction of N-atom in NO_3^- in an amino acid requires 10 electrons. Reduction of one CO_2 molecule to (CH_2O) requires 4 electrons. It is therefore not surprising that it has been repeatedly shown that assimilation of both NH_4^+ and NO_3^- in normally grown algae is dependent on photosynthesis, with little or no uptake occurring in the dark (Losada et al., 1981). However, this is not an obligate process, and nitrogen-starved *Chlamydomonas* with accumulated carbohydrate reserves assimilates both NO_3^- and NH_4^+ in the dark, but does so at even higher rates in the light.

Nitrate uptake is generally believed to be the rate-limiting step in nitrate assimilation, and the overall rate of nitrate assimilation is tightly regulated at the nitrate uptake step by the metabolic carbon/nitrogen status of cells (Flores and Herrero, 1994). It has been known for a long time that ammonium suppresses nitrate uptake in many algae species and also in various higher plants (Ohmori et al., 1977). It was demonstrated many times that in the presence of both ammonium and nitrate in the medium, the NH_4^+ is assimilated first, and only when it has gone is NO_3^- utilized (Shen, 1969). Preferential uptake of NH_4^+ has been shown often for both marine and freshwater algae (Hewitt et al., 1980). However, there have been few reports showing preferential assimilation of nitrate; this phenomenon has been reported e.g. for *Pandorina* and *Haematococus*. There are several reasons for this preferential assimilation of ammonium. Active nitrate reductase is not formed in the presence of NH_4^+ nor is the NO_3^- uptake system. And even if active nitrate reductase and an NO_3^- uptake system are present, the addition of NH_4^+ can lead to a rapid cessation of NO_3^- utilization (Losada et al., 1970). There are at least three mechanisms by which nitrate

reductase activity can disappear from cells. These include two sorts of reversible inactivation phenomena and an irreversible loss of enzyme due, presumably, to degradation (Losada et al., 1970). The effect of addition of NH_4^+ to cells assimilating NO_3^- is complex. The first effect appears to be an inhibition of NO_3^- uptake but this is followed by loss of nitrate reductase (and nitrite reductase) activities. The loss of nitrate reductase activity will be partly due to reversible inactivation, and partly due to irreversible loss of enzyme with the rate of proteolytic breakdown of nitrate reductase possibly being greater in the presence of NH_4^+ (Morris, 1974). At the same time addition of NH_4^+ stops the synthesis of nitrate reductase. The regulation of the formation of nitrite reductase has received much less attention. Like nitrate reductase, its formation is repressed in the presence of NH_4^+. Nitrate does not act either as an inducer or as repressor of nitrite reductase. Hattori found that under light-aerobic conditions, nitrite reducing system was induced by nitrite but not by gaseous nitrogen, ammonium and glutamine (Hattari, 1970). No formation was also observed under the anaerobic conditions in the dark. Suppression of NO_3^- uptake by phytoplankon typically occurs in the range 0.5-1.0 μM NH_4^+. This inhibition has been observed to some extent in species of *Gracilaria, Hypnea musciformis*, *Enteromorpha* but not in *Fucus*. It appears that some seaweeds have the ability to take up NO_3^- and NH_4^+ simultaneously, although NH_4^+ is usually taken up at higher rate (). Although NH_4^+ inhibition of NO_3^- assimilation by algae has often been demonstrated both with laboratory cultures and in field experiments it is clear that algae that are highly nitrogen deficient or growing with a limiting nitrogen supply assimilate both NH_4^+ and NO_3^- simultaneously (Hipkin et al., 1977). With highly carbon-defient cells, NH_4^+ does not inhibit NO_3^-; indeed NO_3^- is reduced to NH_4^+ which accumulates. These

findings suggest that it is not NH_4^+ that is the inhibitor but an organic product of NH_4^+ assimilation.

Nitrate uptake in plants

Of all the mineral elements required by plants, nitrogen is needed in the greatest amount and most often limits growth. It is a constituent of proteins, nucleic acids, and many other important cellular components including chlorophyll and several plant hormones. Deficiency results in reduced plant growth, a gradual chlorosis of older leaves, follow by abscission. Often anthocyanins are synthesized in the stems, petioles, and leaf veins from excess carbohydrates that have accumulated because of reduced nitrogen metabolism. Nitrogen acquisition by plants appears to be regulated by negative feedback control from cellular nitrogen pools, which serve as indicators of plant nitrogen status. Studies carried out in carob and rygrass suggest that both influx and efflux are strongly influenced by nitrogen status and that cellular nitrate and ammonium may they participate in regulation of nitrogen acquisition. Nitrogen is available to plants in the soil in a variety of forms including ammonium (as both NH_3 and NH_4^+), nitrate, amino acids, soluble peptides, and complex insoluble nitrogen-containing compounds. Plant species differ in their preferred nitrogen source, absorbing it via the roots primarily in an inorganic form as nitrate or ammonium. Amino acids (including amides and ureides), that also occur in abundance in certain soils, can also be an important source of nitrogen. Preece emphasized the necessity of optimizing both nutrient requirements and plant growth regulators for morphogenic responses (Preece, 1995). Various forms of nitrogen in the culture medium modulate the endogenous levels of cell metabolites including proteins, nucleic acids, plant growth regulators (Preece, 1995). This nitrogen

requirement may or may not be met by either inorganic pr organic forms of nitrogen depending on the species and culture condition. The initial events of cells division, cell size increase and entry into S-phase of DNA synthesis were increased and the rate of cell mortality decreased when *Asparagus officinalis* mesophyll cells were cultured on MS medium with 30 mM L-glutamine as the sole nitrogen source (Jullien *et al*., 1979). In this culture system an organic form of nitrogen is most appropriate since asparagus cells lack NR activity and thus are unable to use nitrate ions.

Following uptake, inorganic nitrogen is first reduced to ammonium before it is incorporated into the amino acids glutamine and glutamate. Nitrate can be either converted to nitrite and subsequently to ammonium by the action of enzymes nitrate reductase (NR, E.C.1.6.6.1) and nitrite reductase (NiR, E.C. 1.6.6.4) (Crawford, 1995; Srivastava) respectively, or the nitrate may be stored in the vacuoles. Ammonium, produced by these reactions, is further utilized in combination with carbon skeletons to produce glutamine and glutamic acid. Glutamine synthetase (GS, E.C. 6.3.1.2) and glutamate synthase (GOGAT, E.C. 1.4.1.14) catalyze this reaction (Bhadula and Shargool, 1995). A small fraction may be utilized via glutamate dehydrogenase (GDH, E.C. 1.4.1.2-4) (Lam et al., 1995). Once it has entered into the organic cycle nitrogen can be incorporated into other amino acids, amides, proteins, nucleic acids, chlorophylls, alkaloids, polyamines, vitamins and plant growth regulators. Recent studies also suggest that nitrate may function as a signal molecule of plant growth via increased gene expression for enzymes responsible for the uptake and utilization of nitrate (i.e., NR, NiR, GS, and GOGAT). Seelye *et al*. have recently shown that exogenously supplied ammonium enhanced GS activity, ammonium content and growth of asparagus callus as compared to those with no ammonium supplementation.

However, high ammonium supplementation reduced GS activity and growth of the calli. Ammonium assimilation usually occurs in the roots whereas nitrate assimilation can occur both in roots and in leaves, depending on species and environmental conditions. Transport to the leaves occurs via the xylem whereas redistribution from the leaves to nitrogen-requiring organs occurs predominantly in the form amino acids via the phloem. This redistribution is essential for supplying tissues that do not participate in nitrogen assimilation. When nitrogen assimilation occurs in the root, amino acids are transported to the mature leaves in the transpiration stream through the xylem. A high proportion of amino acids arriving in the mature leaves is cycled from the xylem into the phloem for redistribution to nitrogen sinks. When assimilation occurs predominantly in the shoot, it is essential that cycling of amino acids take place to cover the demand of the roots for these nutrient. In the root, excess amino acids can move from the phloem to the xylem for recycling (Tillard *et al*., 1998). Nitrogen can also be transported across the plasma membrane of certain cells in other forms such as small peptides and purine and pyrimidine bases and their derivatives (Liu *et al*., 1999).

Nitrate and ammonium are the most common inorganic nitrogen compounds used as nutrient salts in tissue culture media for *in vitro* plant cultures. Ammonium and nitrate can have different effects on plant development. Bioenergenic considerations reveal that the assimilation of nitrate nitrogen by plants requires more energy than the assimilation of ammonium (Raven, 1985; Salsac *et al*., 1987). According to Pate, Smirnoff et al., Pearson, and Stewart, plants can be roughly divided into three groups: i) those that prefer or utilize ammonium; ii) those that preferentially utilize nitrate; iii) those that use both ammonium and nitrate. However,

numerous experiments have reveals that, as a rule, plants perform best on mixtures of nitrate and ammonium. The pathways for the assimilation and utilization of nitrogen from the environment are well defined for *in vivo* growth of plants. However, very limited information is available for plant tissues grown in culture. The most common inorganic nitrogen form acquired by plants grown *in vivo* is nitrate. Intact plants are usually equipped to acquire optimal quantities of nitrate when the exogenously available nitrate concentrations vary from 10 μM to 100 μM.

Nitrate uptake from the rhizosphere to the cytoplasm of root cells is thermodynamically disfavoured in the terms of both the electric potential gradient (negative inside the cell) and the chemical potential gradient. The uptake is eletrogenic and has been shown to be conducted as an H^+/NO_3^- symport system with a flux stoichiometry of $2H^+/1\ NO_3^-$ (Glass *et al*., 1992; Mistriki and Ullrich, 1995), producing transient plasmalemma depolarization upon addition of nitrate. This depolarization is counteracted by the plasmalemma H^+-ATPase and modulated by induction of nitrate uptake system and nitrogen status of root cells (Ullrich, 1992). These findings may thus suggest an adaptation of root membranes to exposures of different nitrate concentration, providing the expression of depolarization responses.

Most nitrate uptake studies to date have used almost exclusively depletion methods and experiments with the stable isotopic tracer ^{15}N. Recently, however, the short-lived radiotracer ^{13}N was used to provide estimates for nitrate net flux and flux partitioning within the plant as well as cytoplasmic and apparent free space nitrate concentrations (Krozucher *et al*., 1995). These experiments enable discussion of real meaning of the compartment known as apparent free space, in which the aforesaid

authors found a high amount of nitrate relative to the external nitrate concentrations. The recognition of this space, using depletion methods, is generally done through observation of the increase in net uptake, not affected by root temperature, respiratory inhibitors, anaerobiosis or inhibitors of proteins synthesis (Kronzucker *et al*., 1995). After this net uptake the rate decreases. The rapid exchange of nitrogen initially taken up with KCl together with the insensitivity to the inhibitors favours the identification of this space as the apparent free space. The time course of the adsorption varies from minutes to hours, depending on the species studied. Herbaceous crop species require only minutes for the filling of apparent free space (Lee, 1989) while woody species may take hours or even days. Following a lag phase of minutes or hours (depending on species) after first exposure to exogenous nitrate, nitrate uptake rates gradually increase from low to high rates until a plateau is reached. This pattern of uptake is consistent with the existence of two transport systems: a constitutive one, always present in plasmalemma (cHATS), and an inducible one (iHATS), only present when nitrate is available. The cHATS, which serves the function of NO_3^- -sensing (Behl *et al*., 1988); is associated with a plasmamembrane-bound nitrate reductase (PM-NR), found in the roots of barley, maize and *Chlorella* cells (Tishner *et al*., 1993; Tishner *et al*., 1995). This protein is anchored in cell membranes; its function is stimulation of nitrate uptake. In starved carob plants, the cHATS seems to be responsible for net uptake rates of 0.5 μmol g^{-1} h^{-1} (Cruz et al., 1993). This rate is similar to those observed in crop species (Lee and Drew, 1986; King et al., 1993), but high in comparison to those of spruce roots (Kronzucher *et al*., 1995). These responses may be due to genetic adaptation of the species too particular abiotic conditions, mainly nitrate availability in soil solution, or to differences in growth conditions and experimental procedures. The iHATS, described by King et al., is a saturable system

activated at low NO_3^- concentration (<1mM). Investigation of nitrate transport mutants and respective protein suggest that a carrier protein perform this transport mechanism (Tishner *et al*., 1993). The low affinity system (LATS) is likely to be a passive transport system representing uniport by a carrier protein or, more likely, by anions channels (Glass and Siddiqui, 1992). It starts to take up nitrate only after the medium concentration of nitrate is above 1.0 mM and seems to be responsible for the transport of nitrate from the apoplast into the symplast of root parenchyma cells. No saturation value is known even when external concentration reaches 40 mM nitrate. This means that once nitrate is present in the root medium at high concentrations the rate of uptake is proportional to its concentration. Another mechanism proposed for the uptake of nitrate is the K-shuttle, an exchange NO_3^-bicarbonate (Lips *et al*., 1970). This exchange allows uptake of nitrate from the rhizosphere, directly into the stele of root through the endodermis. The affinity of this system may be between that of HATS and of LATS. It will be functional at high concentrations of nitrate in plants in which nitrate reduction occurs mainly in the shoots. Since it depends on the rate of nitrate reduction in the shoot and the consequent synthesis of malate, it is never responsible for the accumulation of nitrate in the shoots.

Nitrate uptake in cyanobacteria

Nitrate is a major source of nitrogen for cyanobacteria. Nitrate assimilation in cyanobacteria involves two steps: (i) the uptake process and (ii) enzymic reduction (). The enzyme involved in the reduction of nitrate to nitrite in cyanobacteria is ferredoxin-dependent nitrate reductase (E.C. 1.7.7.2). It consists of a single polypeptide of 58-85 kDa, a molybdenum cofactor, four non-heme iron and four labile sulfide (two Fe_2S_2 clusters) per molecule (Mikami and Ida, 1984). The enzyme

nitrite reductase (E.C. 1.7.2.1), which mediates the reduction of nitrite to ammonium, is a polypeptide of 52-68 kDa, having Fe4S4 cluster and a siroheme as prosthetic group (Arizmendi and Serra, 1990). Ammonium resulting from nitrate and nitrite is assimilated mostly through the GS/GOGAT pathway rendering glutamate, the principal nitrogen donor in the biosynthesis of other organic nitrogen compounds. The transport of nitrate has been the least understood step of nitrate assimilation, although its importance has been recognized for a long time (Beevers and Hageman, 1983). The first unequivocal demonstration of a gene responsible for nitrate transport and of its essential role in nitrate assimilation was done in the cyanobacterium *Synechococus* sp. PCC7942 (Omata et al., 1989), and the subsequent works have shown the structure of the nitrate transporter of the cyanobacterium (Omata et al., 1993). Clusters of genes involved in cyanobacteria *Synechococus* sp. PCC7942 nitrate assimilation has been mapped. They are in an operon that is regulated by nitrogen availability and are cotranscirbed. The operon consists of two nitrogen regulated genes nirB and ntcB, one structural gene nirA for nitrite reductase, four genes for nitrate transport (nrtA, nrtB, nrtC, and nrtD), and one structural gene NarB for nitrate reductase. Nitrate transport is essential for the growth of the cyanobacterium at physiological concentrations of nitrate and has been shown to be involved in the active transport of nitrite as well. The deduced amino acid sequences of the NrtB, NrtC, NrtD proteins indicate that the transport is a member of the ABC (ATP-binding cassette) superfamily of active transporters. A 4 kb fragment of the marine diazotrophic, filamentous cyanobacterium Trichodesmium sp. strain WH9601 contained gene sequences edcoding the nitrate reduction enzymes, nirA and narB. A third gene positioned between nirA and narB encodes a putative membrane protein with similarity to the nitrate permeases of *Bacillus subtilis* (NasA) and *Emericella nidulans* (CrnA). The gene was shown to

functionally complement a Delta nasA mutant of *B.subtilis* and was assigned the name napA (nitrate permease). NapA was involved in both nitrate and nitrite uptake by the complemented *B.subtilis* cells.Naps is distinct from the nrt genes that encode the nitrate transporter of freshwater cyanobacteria.

Comparison of the structures of nitrate transporters from eukaryotic and prokaryotic, photosynthetic and non- photosynthetic organisms indicate that the nrt nitrate/nitrite transporter represent a prokaryotic nitrate transporter distinct from the nitrate transporter of eukaryotes. The activity of the nitrate-assimilation system of cyanobacteriua is regulated transcriptionally in response to changes in nitrogen availability ; biosynthesis of the NrtA protein, expression of nitrate-transporting ability, and expression of NR and NiR activities are all subject to nutritional repression by ammonium (Herrero et al., 1981). In *Synechococus* sp. PCC7942, the concerted regulation of the activities related to nitrate assimilation is ascribed to co-transcription of gene nirA-nrtABCD-narB as a single operon (Suzuki *et al*. 1992, 1993). Ammonium, either added to the ammonium or generated internally by reduction of nitrate, negatively regulates the transcription of the nirA operon (Suzuki, 1993). Since L-methionine sulfoximine (MSX),an inhibitor of ammonium fixation by glutamine synthetase, induces development of NR and NiR activities in *Synechococus* (Herrero et al., 1981), it has been assumed that a nitrogenous compound(s), resulting from the assimilation of ammonium, inhibits the expression of the nitrate assimilation system (Gurrero and Lara, 1987). Nitrate assimilation by cyanobacteria is subjected not only to transcriptional regulation but also to post-translational regulation (Gurrero and Lara, 1987). Addition of ammonium to medium causes prompt cessation of nitrate uptake in nitrate-utilizing cells of *Synechococus* sp. PCC6301 (Anacystis

nidulans), a strain closely related to the strain PCC7942 . By determining the intracellular concentration of nitrate, Lara *et al.* showed that ammonium inhibits active transport of nitrate (Lara *et al*., 1987). As in the regulation of the nirA operon, fixation of ammonium to Glu is required for the negative effects of ammonium to prevail on the activity of nitrate transport (Lara *et al*., 1987). It is supposed that a metabolite(s) of Glu acts as a negative effector of nitrate transport. Nitrate transport by *Synechococus* sp. PCC 7942 cells was inhibited by ammonium and inhibitors of CO_2 fixation, Ammonium assimilation inhibitors, such as L-methionine D,L-sulfoximine, were know to prevent the negative effects of ammonium and of inhibitors of CO_2 fixation on nitrate uptake, leading to propose the CO_2 fixation was required to counteract the feed back inhibition of nitrate assimilation. In NR-less mutants, L-methionine D,L-sulfoximine prevented the negative effects of ammonium on nitrate transport, but nit always prevented those of inhibiting CO_2 fixation. The mutant strain NC2, constructed by deleting 3 portion of nrtC, showed high nitrate transport activity insensitive to ammonium but sensitive to inhibitors of CO_2 fixation. These finding indicate that the C-control and N-control of nitrate transport are independent at both the physiological and the molecular level (Rodriquez *et al*., 1998). In the unicellular cyanobacteria *Synechococus* PCC 7942 that do not fix molecular nitrogen, interactions between N assimilation and C assimilation occur through the signal transducer P-II and the global nitrogen regulator NtcA. Under high CO_2 concentration, P-II liganded to ATP and bound to 2-oxoglutarate becomes phophorylated and negatively controls the high affinity transport for bicarbonate. In contrast, under low CO2, P-II being only liganded to ATP becomes de phophorylated and negatively controls the nitrate/nitrite active transport system.

By monitoring intracellular accumulation of nitrate in *Synechococus* sp. PCC7942, Rodriguez *et al*. showed a selective and strong dependence of nitrate transport on millimolar concentrations of Na^+. Monensin, an ionophore that collapses the eletrochemical gradient of Na^+, was shown to depress nitrate transport (Rodriquez *et al*., 1992). Based on detailed kinetic studied on the sodium-dependent nitrate transport, Rodriguez et al. proposed a sodium/nitrate symport driven by the energy of eletrochemical gradient of Na^+ as the most likely mechanism of nitrate transport.

High light stress (40 W/M^2) induced alterations in the nitrogen assimilatory enzymes in *Spilulina platensis* were studied under the Ca^{2+} and phosphate (Pi) supplemented as well as starved conditions. Results revealed that activities of nitrate reductase, amino acid transferases (AST/GOT and ALT/GPT), and protease enzymes in the high light incubated cells were relatively higher under the Ca^{2+} and Pi starved conditions, on the contrary, relative rates of glutamine synthetase (GS) and ATPase activities were lower in the Ca^{2+} and Pi-starved. But the *Spilulina* cells under the Ca^{2+} and Pi-added conditions showed enhanced activity of both GS and ATPase enzymes.

The presence of NaCl in the nutrient solution promoted nitrate uptake in parent Anabaena sp. PCC 7210, mutants SP7 (defective in nitrate reductase activity) and SP17 (partially defective in nitrate reductase activity), but not in the mutant SP9 (defective in nitrate transport and reduction). Nitrate reductase activity of the parent and mutant SP17 increased with increasing concentration of nitrate in saline medium, while mutants SP7 and SP9 did not respond to the altered salinity (Ashwani and Tiwari, 1999).

The effect of low temperature on nitrate assimilation was examined in the cyanobacterium *Synechococus* sp. PCC6301 to determine the factor that limits growth. The decrease in the rate of nitrate consumption by cells as a function of temperature decreased with decreasing temperature. Cells could not actively take up nitrate at 15 C, although nitrate reductase and nitrite reductase were still active .

Enhancement of cyanobacteria salt tolerance by combined nitrogen

Salt overloading in soils is by far the major hindrance for plant growth, and a crucial problem for agriculture (Serrano and Gaxiola, 1994). Each organism displays a salinity tolerance range, which includes its optimal growth conditions, though larger ranges of salt concentrations (resistance range) may still be compatible with cell survival. Crop plants are particularly limited in their tolerance range, whereas other photosynthetic organisms, including microalgae and cyanobacteria, may display wider acclimation capacities. Exposure of cells to salt concentrations that are physiologically above those present intracellularly threatens them via two deleterious effects, namely, increase of both the osmotic pressure and the ion concentration. The water potential decreases, leading to loss of water by the cells, and simultaneously to influx of ions into the cytoplasm. Stress from NaCl, in particular, causes a dramatic increase in the concentration of inorganic ions.

Cyanobacteria, the only prokaryotes performing oxygenic photosynthesis and probable ancestors of chloroplasts, constitute valuable models for the study of the molecular mechanisms involved in tolerance to high salinity, or to its corollary, drought, a major agricultural problem. The critical demands of cyanobacteria exposed to high salinity, i.e., accumulation of osmoprotectors and extrusion of sodium ions, are met through immediate activation and/or long term (protein synthesis-dependent) adaptation of various processes: (i) uptake and endogenous biosynthesis of osmotica, the nature and amount of which are strain and salt concentration dependent (ii) probable modifications of membrane lipid composition (iii) increased energetic capacity, at the level of cyclic electron flow around photosystem I (through routes induced under these conditions) and cytochrome c oxidase, and (iv) enhancemeant of H^+-ATPase activity and active extrusion of sodium ions.

Extrusion of sodium ions phenomena have an important influence on the halotolerance of various organisms. Cyanobacteria do not accumulate Na^+ although a transient net Na^+ uptake may occur in response to hypersaline upshock (Reed et al., 1985). It was shown further that the ability to curtail Na^+ influx can also be induced by certain environmental factor like alkaline pH or presence of combined nitrogen in the form of nitrate or ammonium in the growth medium (Apte *et al*., 1985). Presence of certain nitrogenous compounds in the growth medium significantly enhanced the salt tolerance of freshwater cyanobacterium *Anabaena* sp. strain L-31 as well as the brackish water cyanobacterium *Anabaena torulosa.* Amoung these, nitrate, ammonium, and glutamine were most effective followed by glutamate and aspartate. These nitrogenous compounds also inhibited Na^+ influx in both *Anabaena* sp. with the same order of effectiveness as that observed for protection against salt stress.

The inhibition of Na^+ influx on addition of the nitrogenous substances was rapid; nitrate and ammonium inhibited Na^+ influx competitively. Uptake of nitrate and ammonium increased during salt stress but was not correlated with growth. Intracellular levels of nitrate and ammonium were found to be inadequate to constitute a major component of the internal osmoticum. The results suggest that inhibition of Na^+ influx by combined nitrogen is a major mechanism for protection of cyanobacteria against salt stress.

In the previous works, not only the effect of salinity on nitrate assimilation is not well understood but also mechanism with respect to carbon assimilation is still unknown. Moreover, sources of driving force for transport is still controversial. Consequently, in this work, the unicellular cyanobacterium, *Aphanothece halophytica* (this organism is classified into *Chroococcales* order, *chroococcacean* cyanobacteria subgroup, Geitler 1932; Stanier et al. 1971) was chosen as a source for the study of (a) nitrate uptake by *A. halophytica* grown under normal and salt stressed condition (b) the effect of other ions on nitrate uptake by *A. halophytica* grown under normal and salt stressed condition, and (c) the effect of various inhibitors on nitrate uptake by *A. halophytica* grown under normal and salt stressed condition.

CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Instruments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model J-21C, Beckman Instrument Inc, USA

Digital Lux Meter FT710; Taiwan

Glass Microanalysis Filter Holder: Millipore, USA

HPLC Model Hewlett Packard series 1050, Japan

Illuminated/Refrigerated Orbital: Sanyo, England

Laminar Flow BVT-124: International Scientific Supply, Thailand

PH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Vortex: Model K-550-Ge: Scientific Industries, USA

Water Bath: Charles Hearson, England

Water Bath Shaking: Heto lab Equipment, Denmark

2.2 Chemicals

Amiloride: Sigma, USA

Ammonium chloride: Merck Ag Darmatadt, Germany

Boric acid: Merck Ag Darmatadt, Germany

Calcium chloride: Merck Ag Darmatadt, Germany

CCCP (Carbonylcyanidetrifluoromethoxyphenylhydrazone): Sigma, USA

Citric acid: Fluka, Switzerland

Cobalt chloride: Merck Ag Darmatadt, Germany

EDTA (Ethylenediaminetetraactic acid): Sigma, USA

Ferric sulfate: Mallinckrodt Chemical, USA

Hepes: Sigma, USA

MSX (L-methionone sulfoximine): Sigma, USA

Magnesium chloride: Merck Ag Darmatadt, Germany

Magnesium sulfate:Merck Ag Darmatadt

Monensin: Sigma, USA

DCCD (N,N-dicyclohexylcarbodiimide): Sigma, USA

Potassium dihydrogen phosphate: BDH, England

Sodium chloride; Merck Ag Darmatadt, Germany

Sodium tungstate: BDH, England

Sodium nitrate: Merck Ag Darmatadt, Germany

Sodium nitrite: Merck Ag Darmatadt, Germany

Sorbitol: BDH, England

2.3 Bacterial Strains

Aphanothece halophytica was initially isolated from Solar Lake in Israel. The organism was kindly provided by Dr. T. Takabe of Research Institute of Meijo University, Japan.

Methods

2.4 Culture of *A. halophytica*.

Ten percents inoculum of *Aphanothece halophytica* was inoculated into a 250 ml. Flask containing 100 ml of Turk Island Salt solution + modified BG_{11} medium (in which molybdenum in the composition of trace element A_5 solution was replaced with tungsten) (see Appendix 1, Thongekkaw 1995) with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on rotary sharker with 160 rpm at 30 C, 2000 lux of continuous illumination. After 10 days, 10 ml culture was inoculated into the same medium containing either 0.5 M NaCl or 2.0 M NaCl and shaken on a rotary shaker (160 rpm) at 30 C with 2000 lux of continuous illumination.

2.5 Determination of nitrate uptake system.

2.5.1 Determination of nitrate content by HPLC.

The nitrate content was determined by anion-exchange HPLC method using analytical Hypersil-10 sax column (10 μm porous silica gel packing 250 mm × 4.6 mm i.d.) recommended by Romero et al. (1989). HPLC analyses were performed with HP series 1050, 4 pumps and UV detector, which was set at 210 nm. 30 mM potassium dihydrogen phosphate buffer pH 3 was used as mobile phase and operated at the flow rate of 1.0 ml/min.

2.5.1.1 Standard curve of nitrate.

The nitrate standard curve was established. The standard nitrate solutions were prepared at 50, 100, 200, 400 and 600 μM in 30 mM potassium dihydrogen phosphate buffer pH 3. Twenty μl of each concentration was injected onto sax column and run with above condition described in 2.5.1.

The nitrate standard curve was plotted between standard nitrate concentrations and peak areas, represented by axis X and Y respectively. Below is equation derived from the nitrate standard curve shown below was used to estimate the nitrate content in *A. halophytica*.

$$Y = 7.3226X + 118.91$$

Where $R^2 = 0.9994$

2.5.1.2 Preparation of nitrate extract.

The nitrate extract was filtered through 0.45 μm nylon filter membrane before determining by HPLC.

2.5.1.3 Limitation of nitrate determination using analytical Hypersil-10 sax column and HPLC.

To estimate the limitation of the method, the standard nitrate was prepared at the concentration of 0.05, 0.1, 1, 2 and 5 μM. The nitrate content was determined as described previously in the section 2.5.1. For assessment of the reliability of the method, at least three replications were needed.

2.5.2 Time courses of nitrate uptake.

Ten percents of *Aphanothece halophytica* were inoculated into a 250 ml flask. Containing 100 ml of Turk Island Salt Solution + Modified BG_{11} medium (see Appendix 1, Thongekkaw, 1995) with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on a rotary shaker with 160rpm at 30 C, 2000 lux of continuous illumination. After 10 days, 10 ml culture was inoculated into the same medium containing either 0.5 M NaCl (normal) or 2.0 M NaCl (salt stress) and shaken on the rotary shaker (160 rpm) at 30 C with 2000 lux of continuous illumination. After 10 days, the culture was centrifuged at 2000 g for 10 min and the cell pellet was suspended in 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3, 12 mM $NaHCO_3$ containing either 0.5 M NaCl or 2.0 M NaCl followed by the addition of 100 μM $NaNO_3$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by rapid filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1.

2.6 Characterization of nitrate uptake system.

2.6.1 Kinetics of nitrate uptake.

A. *halophytica* was grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. To determine the kinetic constants of the nitrate uptake system, initial rates of uptake were determined over a wide range of nitrate concentrations (100 to 1000 μM $NaNO_3$) using cells grown under normal and salt stress conditions (0.5 M and 2.0 M NaCl). The Half saturation value (K_s) and maximum velocity (V_{max}) were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot, i.e., 1/[s] vs 1/[v].

2.6.2 Na^+ requirement of the uptake system.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. Initial rates of uptake were determined in the presence of various Na^+ concentrations using NaCl as a source.

2.7 Effect of various ions on nitrate uptake.

2.7.1 Effect of NH_4^+ on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of NO_3^-+ NH_4^+ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1. To study effect of ammonium concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various NH_4^+ concentrations using NH_4Cl as a source. To study effect of MSX (inhibitor of ammonium assimilation) to remove negative effect of NH_4^+, preincubation the cells with 1 mM MSX for 30 min in the dark was done before assay. The assay was started by simultaneous addition of NO_3^-+ NH_4^+ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined foe nitrate content by HPLC as described in section 2.5.1.

2.7.2 Effect of NO_2^- on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of NO_3^-+ NO_2^- and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1. To study effect of nitrite concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various NO_2^- concentrations using $NaNO_2$ as a source.

2.7.3 Effect of various anions on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of NO_3^-+ Cl^- or NO_3^- + PO_4^{3-} and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1.

2.7.4 Effect of DLG on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation the cells with 30 mM DLG for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1 To study effect of DLG concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various DLG concentrations.

2.8 Effect of various inhibitors on nitrate uptake.

2.8.1 Effect of monensin on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contains 0.5 M sorbitol in 25 μM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation the cells with 20 mM monensin for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as

described in section 2.5.1 To study effect of monensin concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various monensin concentrations.

2.8.2 Effect of amiloride on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation the cells with 0.1 mM amiloride for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1. To study effect of amiloride concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various amiloride concentrations.

2.8.3 Effect of DCCD on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contains 0.5 M sorbitol in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation the cells with 0.05 mM DCCD for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM

$NaNO_3$ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1 To study effect of DCCD concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various DCCD concentrations.

2.8.4 Effect of CCCP on nitrate uptake.

Cells were grown in the medium, harvested and osmotic stress experiment was done as described in 2.5.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbital in 25 mM Hepes buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation the cells with 10 μM CCCP for 30 min in the dark were done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ and illumination. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through 0.45 μm nylon filter membrane. The total supernatant was determined for nitrate content by HPLC as described in section 2.5.1 To study effect of CCCP concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various CCCP concentrations.

CHAPTER III

RESULTS

3.1 Characterization of nitrate uptake system.

3.1.1 Kinetics of nitrate uptake.

We also determined the concentration needed to saturate the uptake system of control and salt stress conditions (0.5 M and 2.0 M NaCl) on the rate of nitrate uptake by *A. halophytica*. Figure 3.1 shows that nitrate uptake in control condition is higher than in stress condition. The low level of nitrate uptake observed at higher concentrations of external nitrate was probably caused by passive diffusion. Rate of nitrate uptake in stress condition got saturated at a concentration of 600 μM $NaNO_3$.

When the cells were assayed with 100 μM $NaNO_3$ the linear rapid of nitrate uptake occurred during the first 2 min and the uptake was rather constant after that. Initial uptake rates were determined over a wide range of nitrate concentration (100 to 1000 μM). The nitrate uptake system was saturable and displayed typical Michaelis-Menten type kinetics (Figure 3.2 A and Figure 3.2 B). The Lineweaver-Burk transformation of the data under these conditions was performed, the line of best fit was performed by using a least squares linear regression, the apparent K_s values for control and stress condition were 416 and 450 μM respectively, the maximum velocity (V_{max}) was 9.1 and 5.3 μmol/min/mgChl respectively.

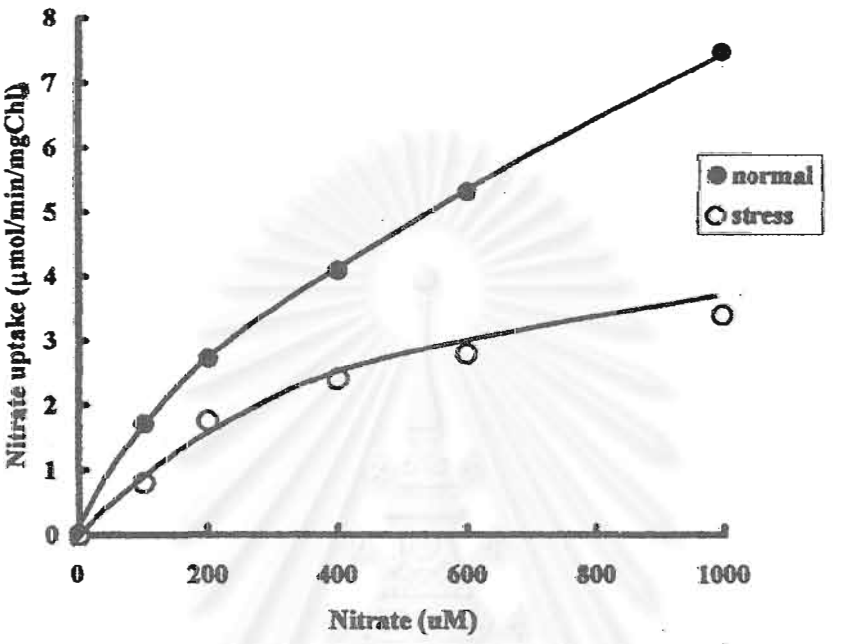


Figure 3.1 Kinetics of nitrate uptake by A. halophytica under normal and stress condition.

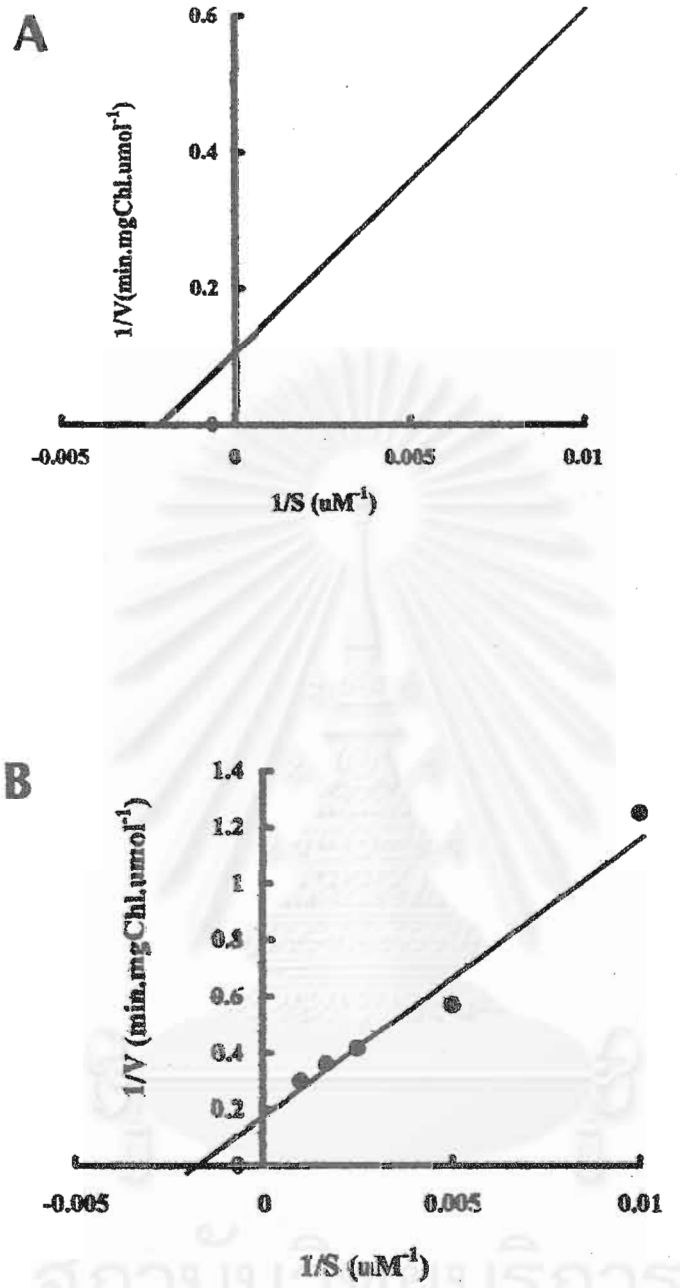


Figure 3.2 Lineweaver-Burk transformation of the data grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

3.1.2 Effect of Na^+ on nitrate uptake.

It has been reported that sodium is required for Nrt-dependent nitrate uptake in *Synechococcus* sp. strain PCC7942 (Rodriguez at el., 1994). We therefore determined whether nitrate uptake by *A. halophytica* is influenced by external sodium. The rate of nitrate uptake was determined in reaction mixtures containing 0 to 20 mM NaCl. Figures 3.3 A and 3.3 B show that Na^+ has no effect on nitrate uptake rate for cells grown under normal and salt stress conditions when NaCl concentration was increased up to 20 mM. Nitrate uptake rates were constant. These results indicate that nitrate uptake in *A. halophytica* in both conditions are indifferent to the extracelluar sodium concentrations. These results support the conclusion of Qito and Valley, who inferred that nitrate transport in *Klebsiella oxytoca* is indifferent to the extracellular sodium concentrations.

3.2 Effect of other ions on nitrate uptake.

3.2.1 Effect of NH_4^+ on nitrate uptake.

Figure 3.4 A and Figure 3.4 B show the effects on nitrate uptake of exposure to 100 μM ammonium chloride in the presence and absence of L-Methionine sulfoximine. In figure 6 the data shows that when L-Methionine sulfoximine, a potent inhibitor of Glu synthetase, was not added to the assay medium, cells of *A. halophytica* consumed nitrate from the medium, but the rate of nitrate uptake was slower than that in the presence of L-Methionine sulfoximine. These results also showed that the addition of L-Methionine sulfoximine is essential to obtain the

maximal rate of nitrate uptake in normal condition however in salt stress condition L-Methionine sulfoximine did not release feedback inhibition of ammonium. When 25 to 200 μM ammonium chloride were added to the assay medium (without L-Methionine sulfoximine) nitrate uptake rates declined with ammonium chloride higher than 100 μM. Addition of L-Methionine sulfoximine to the assay medium containing cells whose nitrate consumption had been arrested by addition of ammonium chloride did not affect resumption of nitrate consumption in both normal and stress conditions (Figure 3.5 A and Figure 3.5 B).

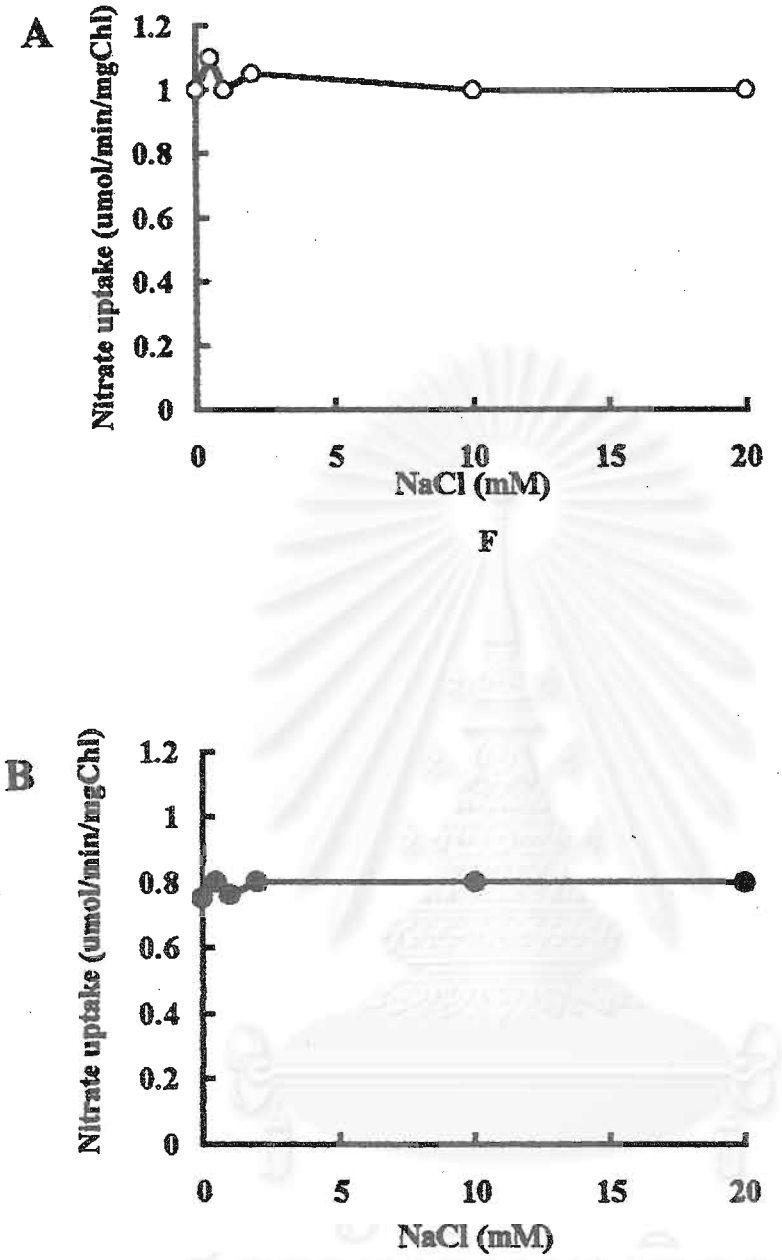
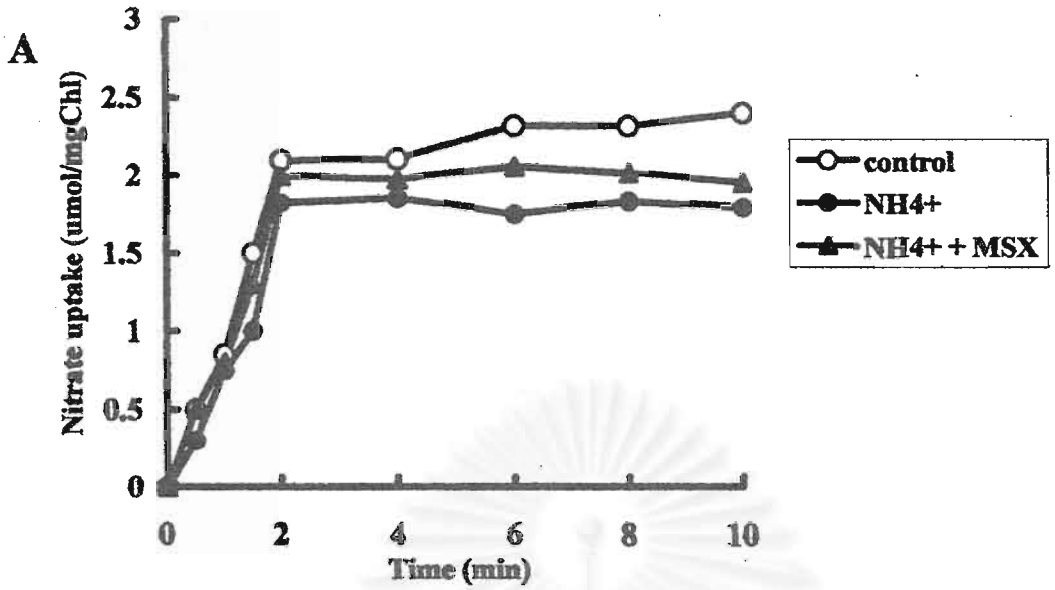


Figure 3.3 Effect of Na+ concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)



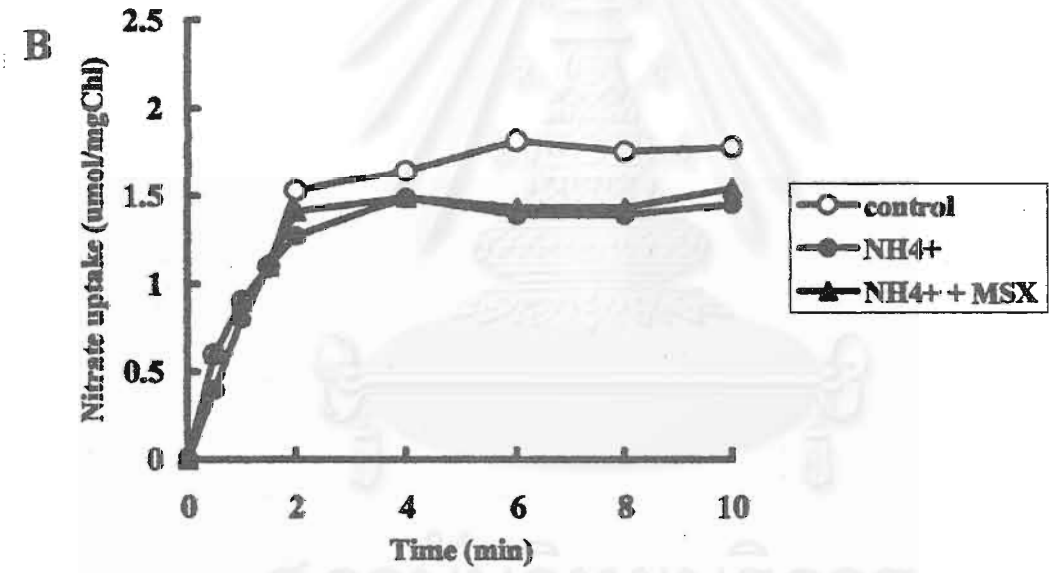


Figure 3.4 Effect of ammonium and MSX on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress 2 M NaCl (B)

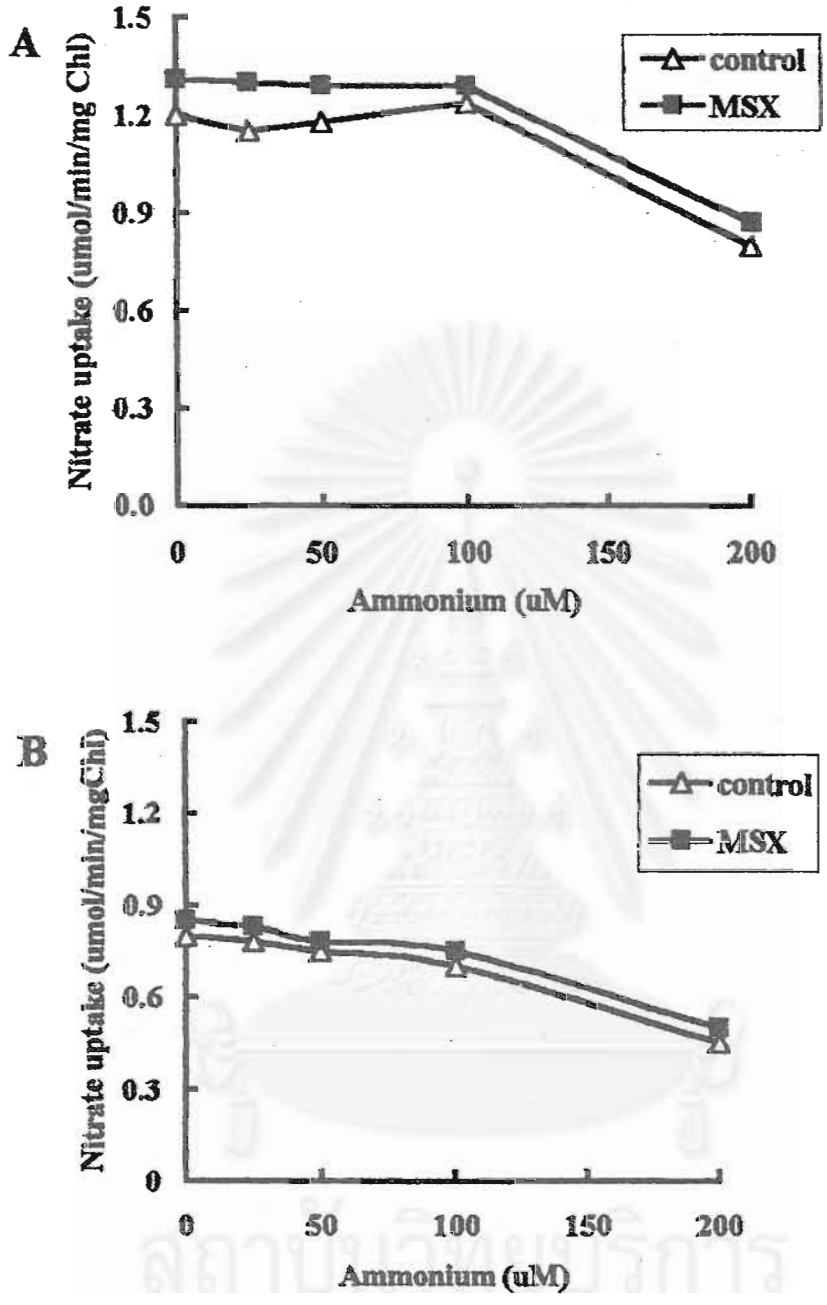


Figure 3.5 Effect of MSX concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

3.2.2 Effect of NO_2^- on nitrate uptake

Nitrite, an alternative nitrogen source, behaved as an effective competitive inhibitor of nitrate uptake. Figure 3.6 A and Figure 3.6 B showed the effect on nitrate uptake of exposure to 100 μM $NaNO_2$. In both cases (normal and stress condition) the addition of NO_2^- to the uptake media inhibited the net uptake rate of NO_3^-. The inhibition appeared to be competitive because the regression lines of the double reciprocal plots approached a common intercept. K_i value by plotting the concentration of nitrite versus slope was 84 μM. This indicates that nitrite binds to the nitrate transporter with very high affinity. Figure 3.7 A and Figure 3.7 B illustrate the response of nitrate uptake rate to four concentrations of nitrite (50, 100, 200 ,and 400 μM). In comparison to the maximum rate (no nitrite) increasing the concentration of NO_2^- in the uptake solution gradually inhibited the uptake of NO_3^-.

3.2.3 Effect of various anions on nitrate uptake

Previous studies on several genotypes of the genus *Hordeum* compared net uptake of NO_3^- from the solution containing equimolar concentration of Cl^-. The kinetics of net NO_3^- suggested that the presence of Cl^- might inhibit NO_3^- uptake (Smith et al., 1971). We test the hypothesis by adding nitrate with various anions to the assay medium. Figure 3.10 A and Figure 3.10 B show effects of anions (Cl^- and PO_4^{3---}) on nitrate uptake. Both 100 μM Cl^- and 100 μM PO_4^{3-} did not inhibit nitrate uptake compared with the control treatment in both conditions. The results suggest that Cl^- and PO_4^{3-} have no effect on nitrate uptake in *A. halophytica*

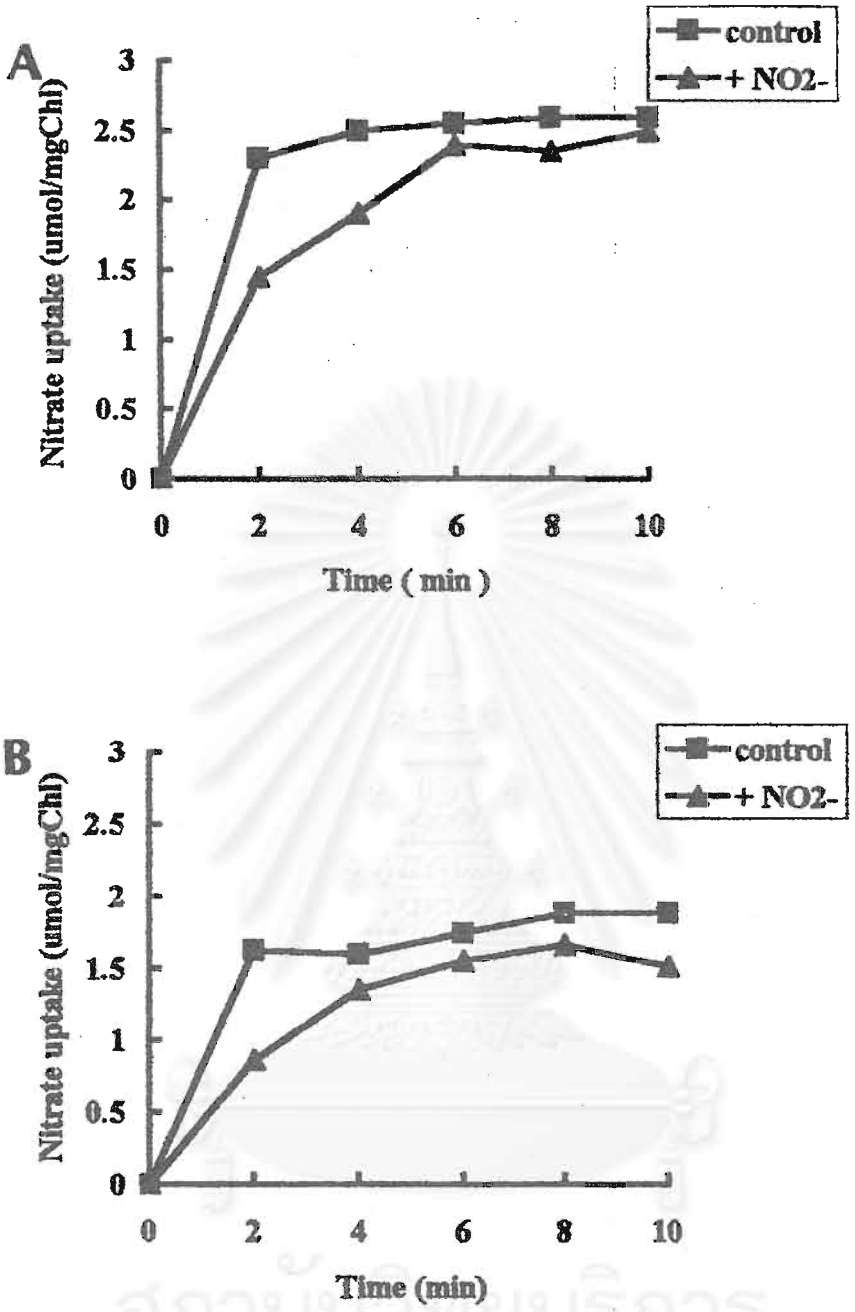


Figure 3.6 Effect of nitrite on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

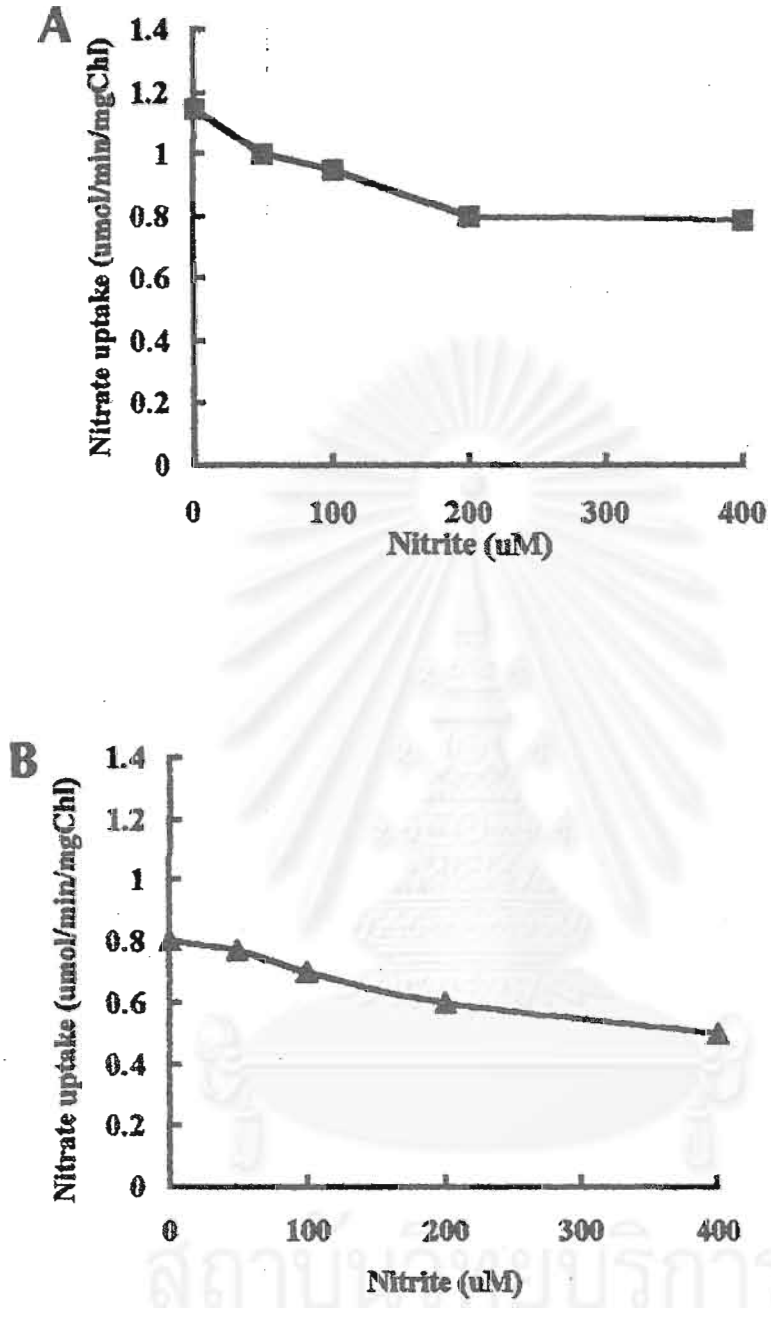


Figure 3.7 Effect of nitrite concentration on nitrate uptake with cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

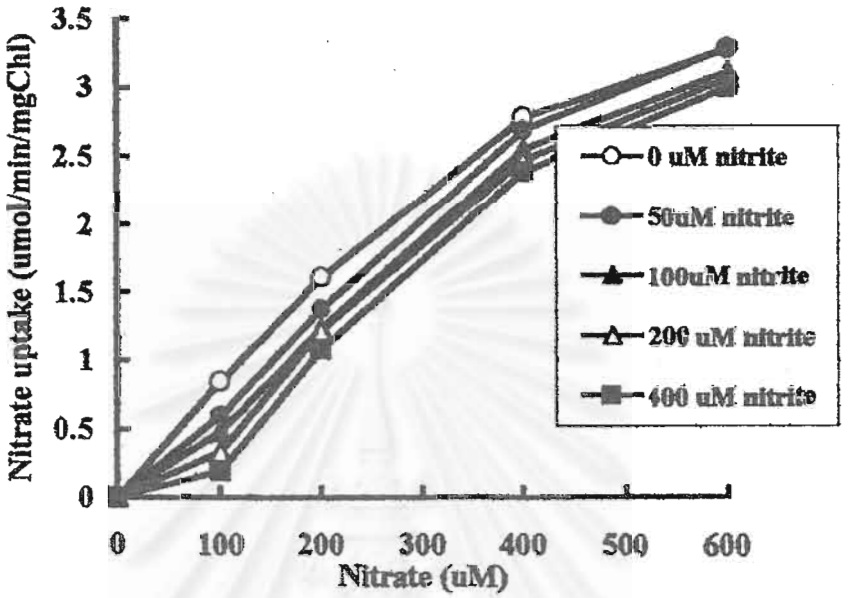


Figure 3.8 Effect of nitrite on nitrate uptake (0.5 M)

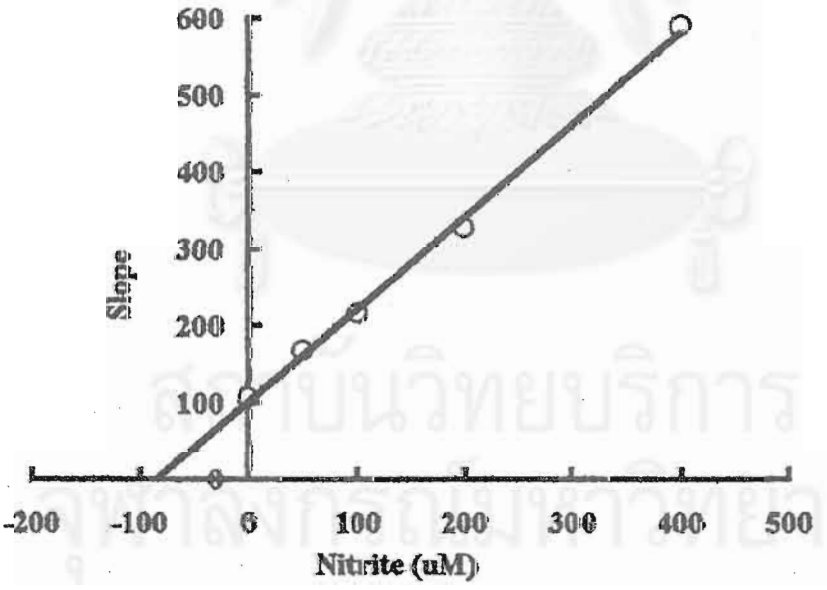


Figure 3.9

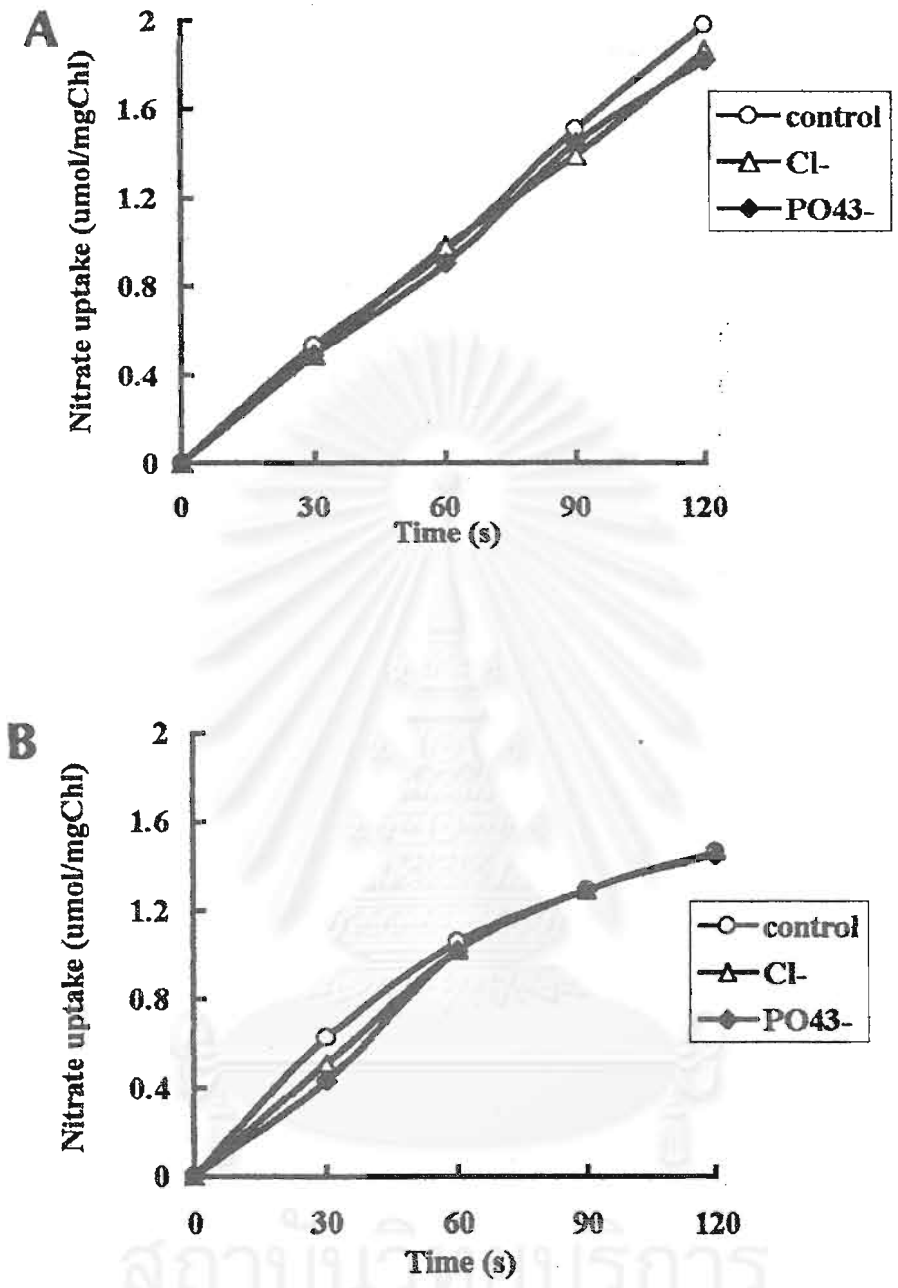


Figure 3.10 Effect of various anions on nitrate uptake by cell grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

3.2.4 Effect of DLG on nitrate uptake

As previously reported in *Anacystis nidulans* (Lara et al.,1985), nitrate uptake was severely inhibited by preincubation of *Anacystis nidulans* cells with DLG(a selective inhibitor of CO_2 fixation). We have tested this hypothesis in tungstate-treated A. halophytica cells by preincubation cells for 30 min in the dark prior to the assay with DLG before assay. In both control and salt stress cells the data show that when DLG, a selective inhibitor of CO_2 fixation, was present in the assay medium, cells of *A. halophytica* consumed nitrate from the medium, but the rate of nitrate uptake was slower than that with in the absence of DLG (Figure 3.11 A and Figure 3.11 B). In comparison to the maximum rate the rates declined with increasing concentration of DLG (10 to 300 mM) (Figure 3.12 A and Figure 3.12 B). These results strongly indicated the dependence of nitrate uptake upon active carbon assimilation. The results presented here confirm that nitrate uptake in *A. halophytica* is strictly dependent on active CO_2 fixation.

3.3 Effect of various inhibitors on nitrate uptake

The effects of various inhibitors that disrupt Na^+ electrochemical gradients (monensin), inhibited Na^+/H^+ antiportor and/or Na^+ channel (amiloride), proton channel blocker (DCCD) and protonophore (CCCP) on the initial rate of nitrate uptake via osmatic stress condition were studied. Inhibitors were preincubated with the cells in 30 min prior to assay. The results were shown in Figures 3.13 A-3.20 B. All inhibitors inhibited nitrate uptake in both control and stress system. In comparison to the maximum rate, as obtained with out inhibitors, the rates

declined with increasing concentration of all four inhibitors (Figure 3.14 A, 3.14 B, 3.16 A, 3.16 B, 3.18 A, 3.18 B, 3.20 A, and 3.20 B)

The concentrations of monensin, amiloride, DCCD and CCCP producing 50% inhibition of nitrate uptake in control condition were 23, 245, 32, and 22 μM, respectively. The concentrations of monensin, amiloride, DCCD and CCCP producing 50% inhibition of nitrate uptake in stress condition were 25, 160, 75, and 18 uM, respectively. CCCP, and DCCD are suggested to be a more potent inhibitor of nitrate uptake in *A. halophytica*. Inhibition of all two conditions was complete in the presence of 300 μM CCCP and 300 μM DCCD (Figure 3.18 A, 3.18 B, 3.20 A, and 3.20 B). These results might indicated that a pH gradient generated by H^+/ATPase drives nitrate uptake in *A.halophytica.*

The monensin effect indicates that nitrate uptake relies on the maintenance of a sodium electrochemical gradient across the plasmalemma, which might represent the immediate source of energy for active nitrate uptake. Amiloride treatment of *A.halophytica* cells results in a decreased rate of nitrate uptake. These data may suggest that nitrate uptake in *A.halophytica* cells was caused by the Na^+/H^+ antiport.

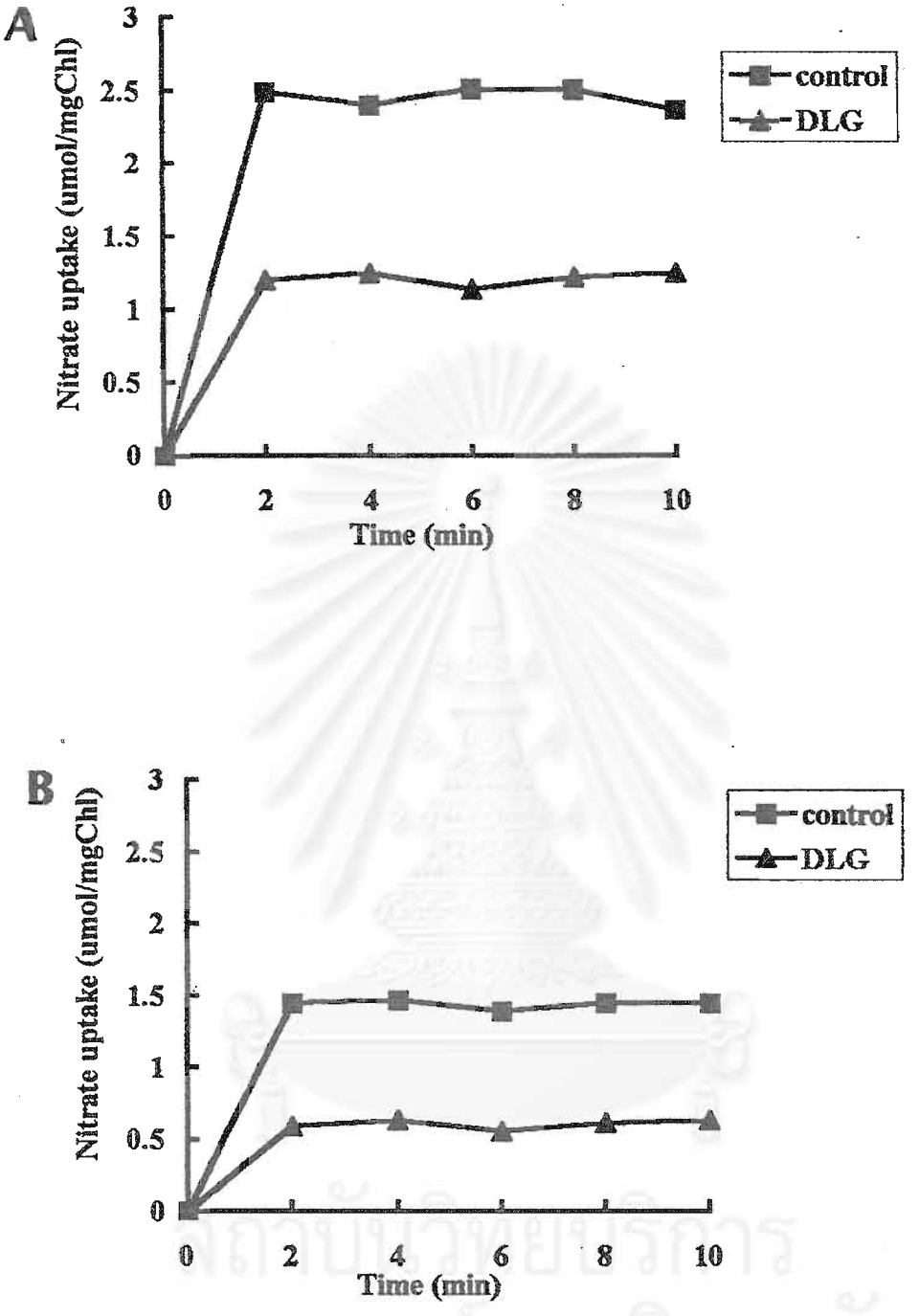


Figure 3.11 Effect of DLG on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

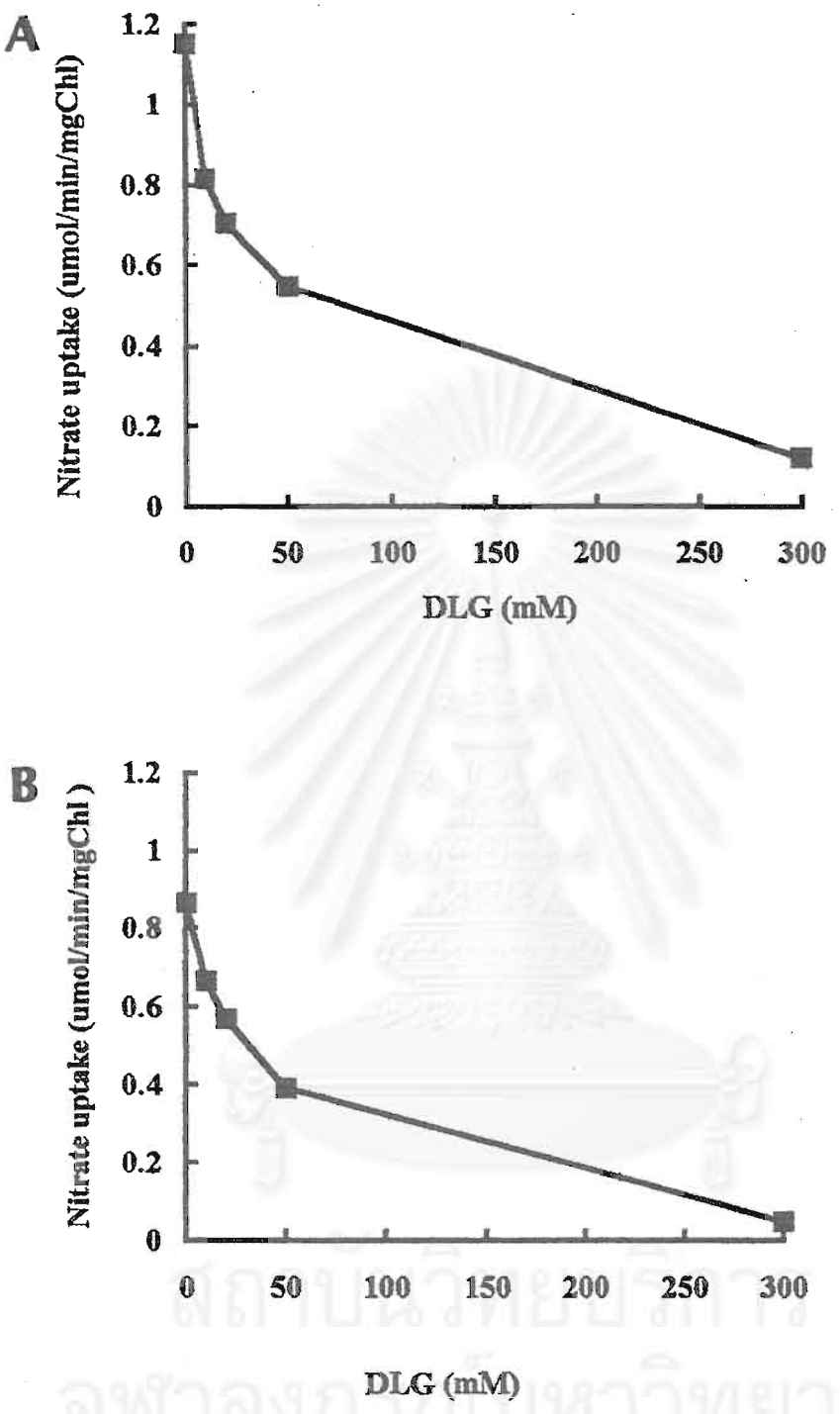


Figure 3.12 Effect of DLG concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

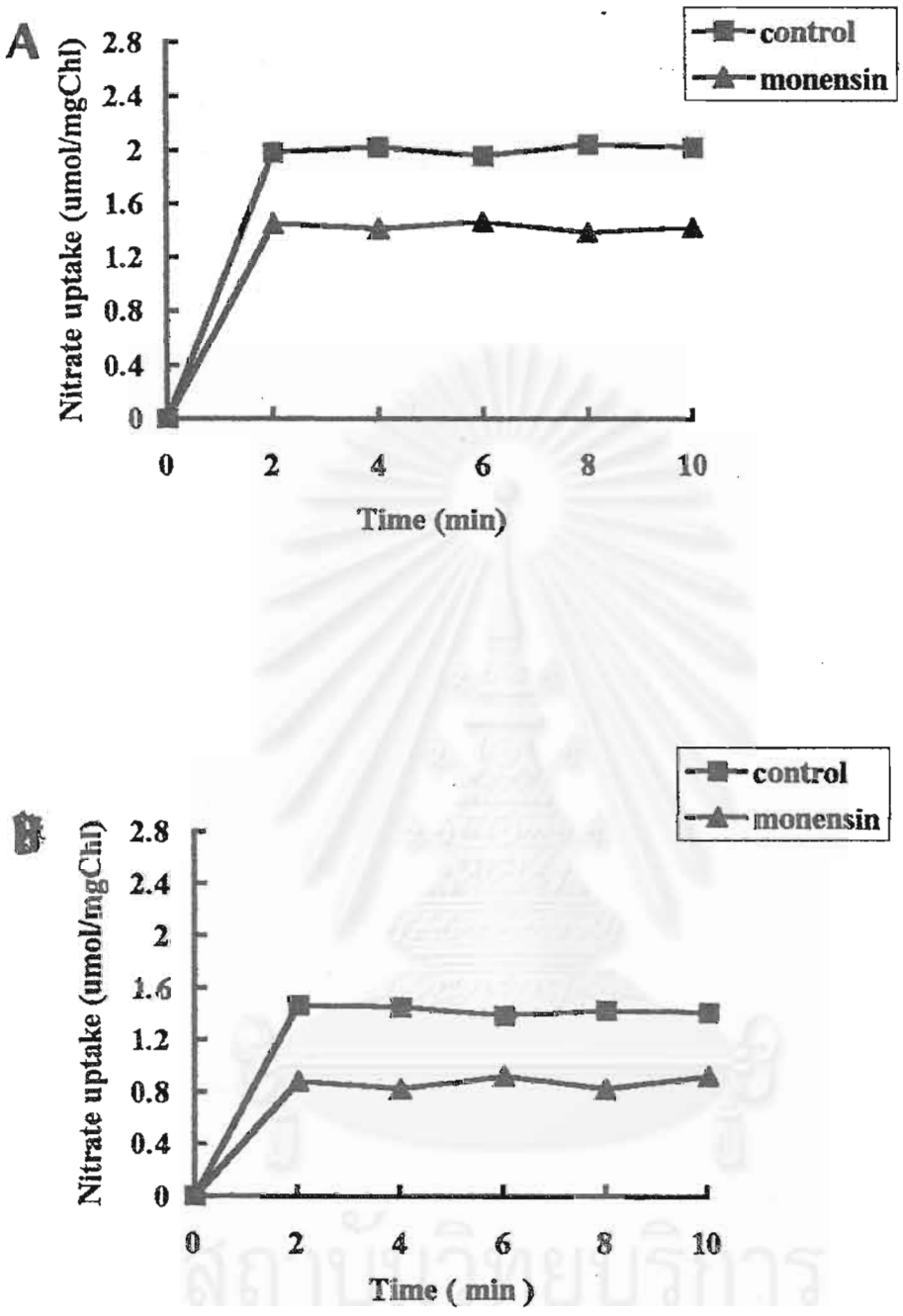


Figure 3.13 Effect of monensin on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

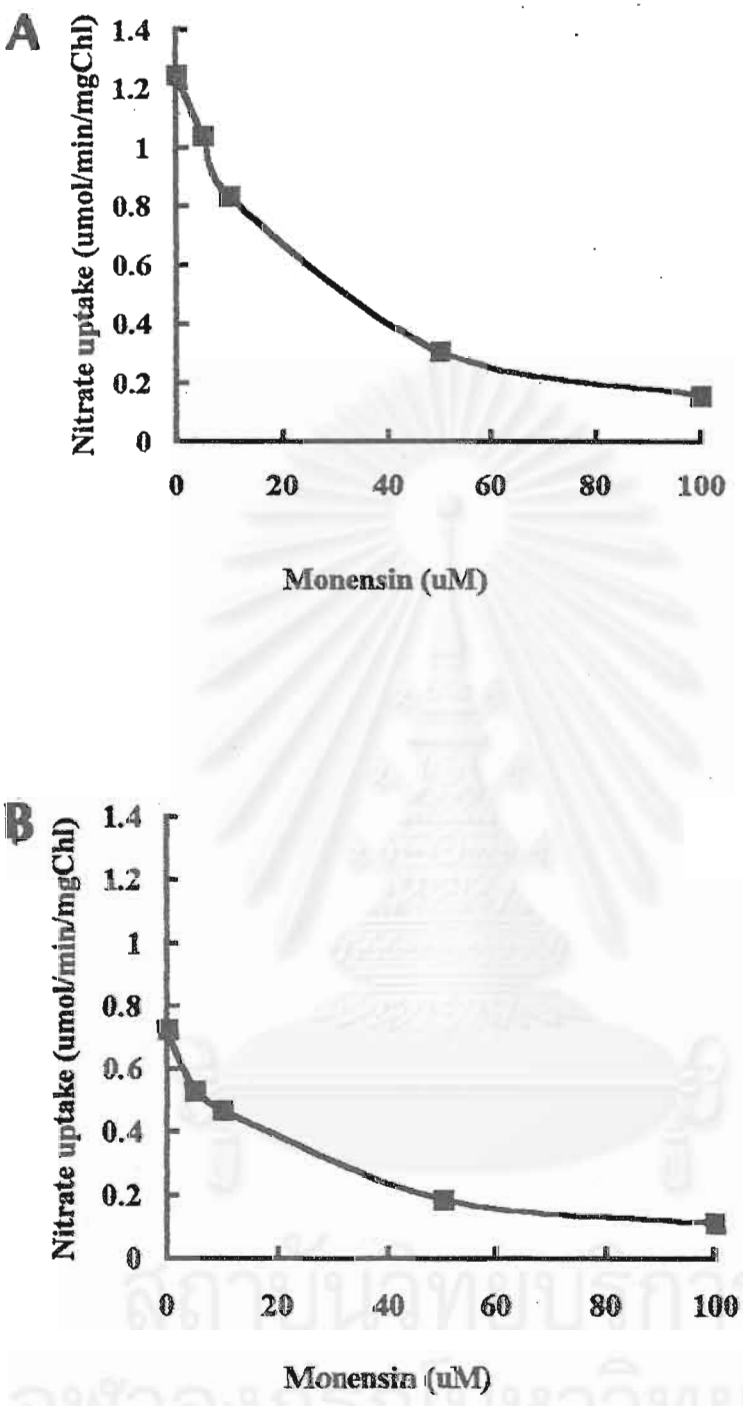


Figure 3.14 Effect of monensin concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

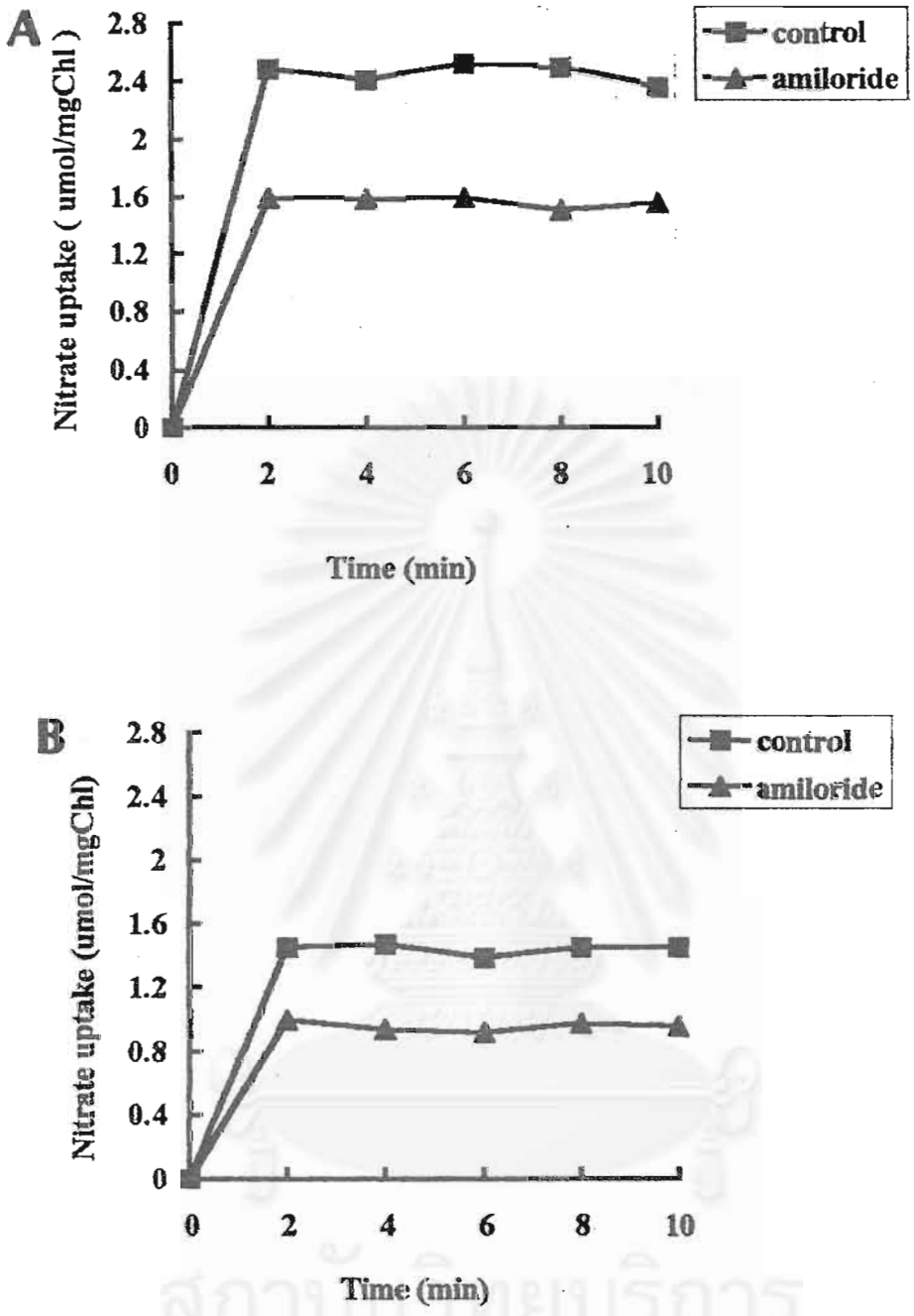


Figure 3.15 Effect of amiloride on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

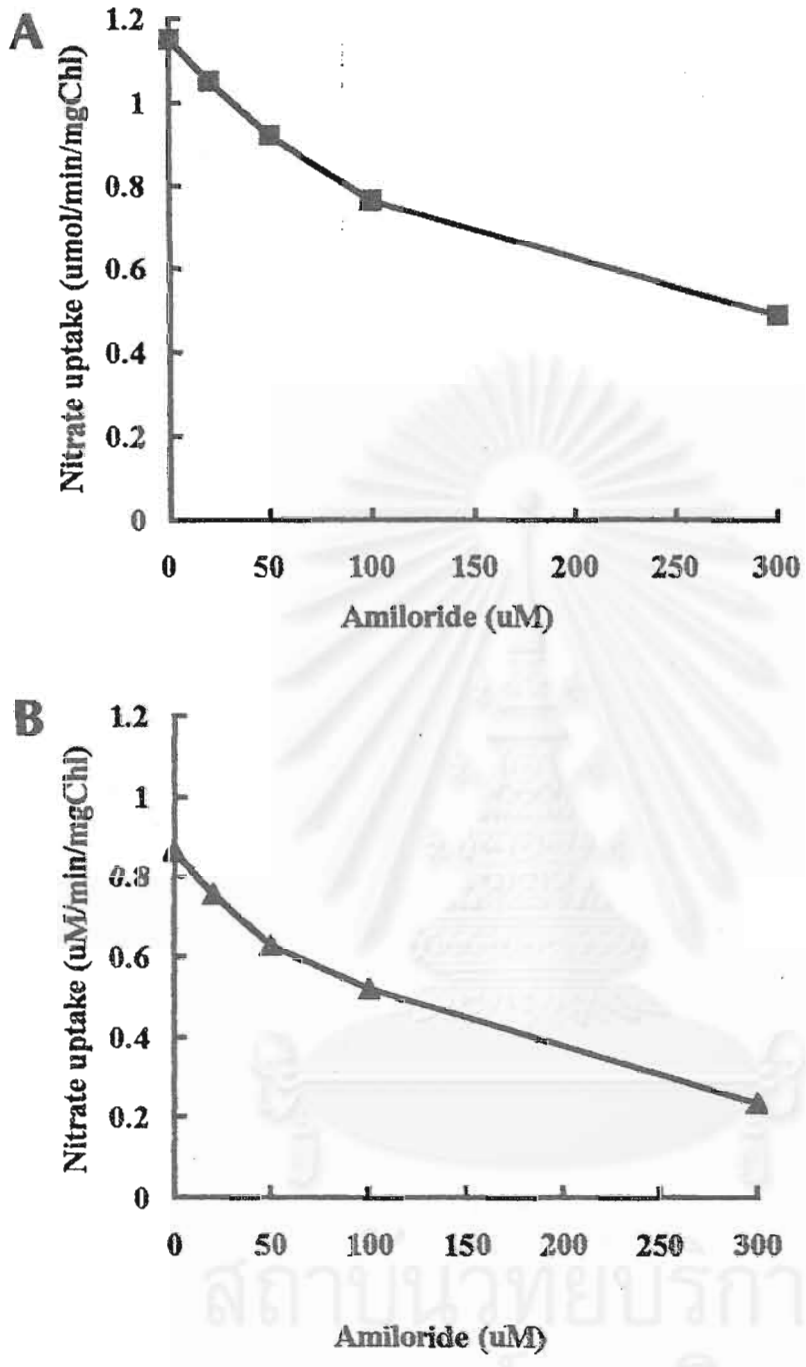


Figure 3.16 Effect of amiloride concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

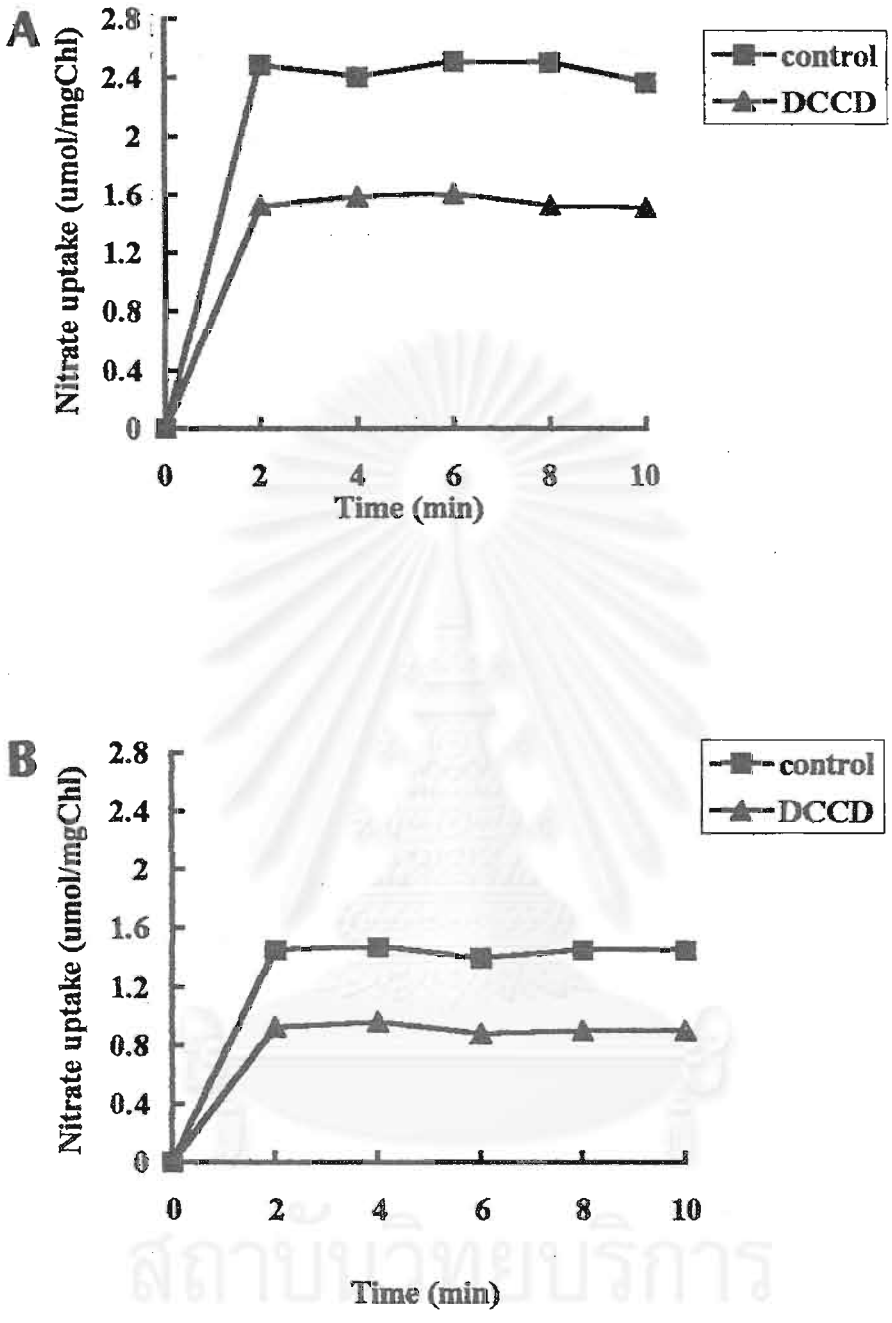


Figure 3.17 Effect of DCCD on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

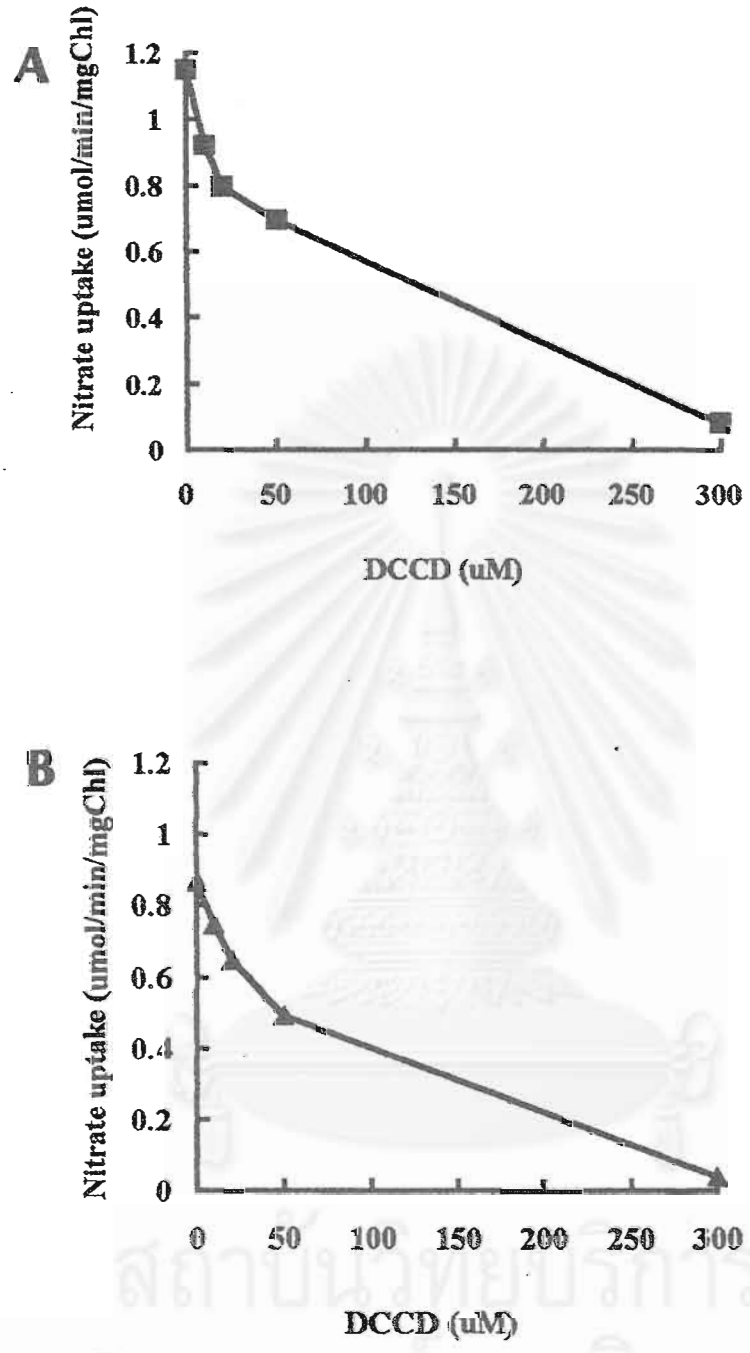


Figure 3.18 Effect of DCCD c concentration on nitrate uptake by cells grown un under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

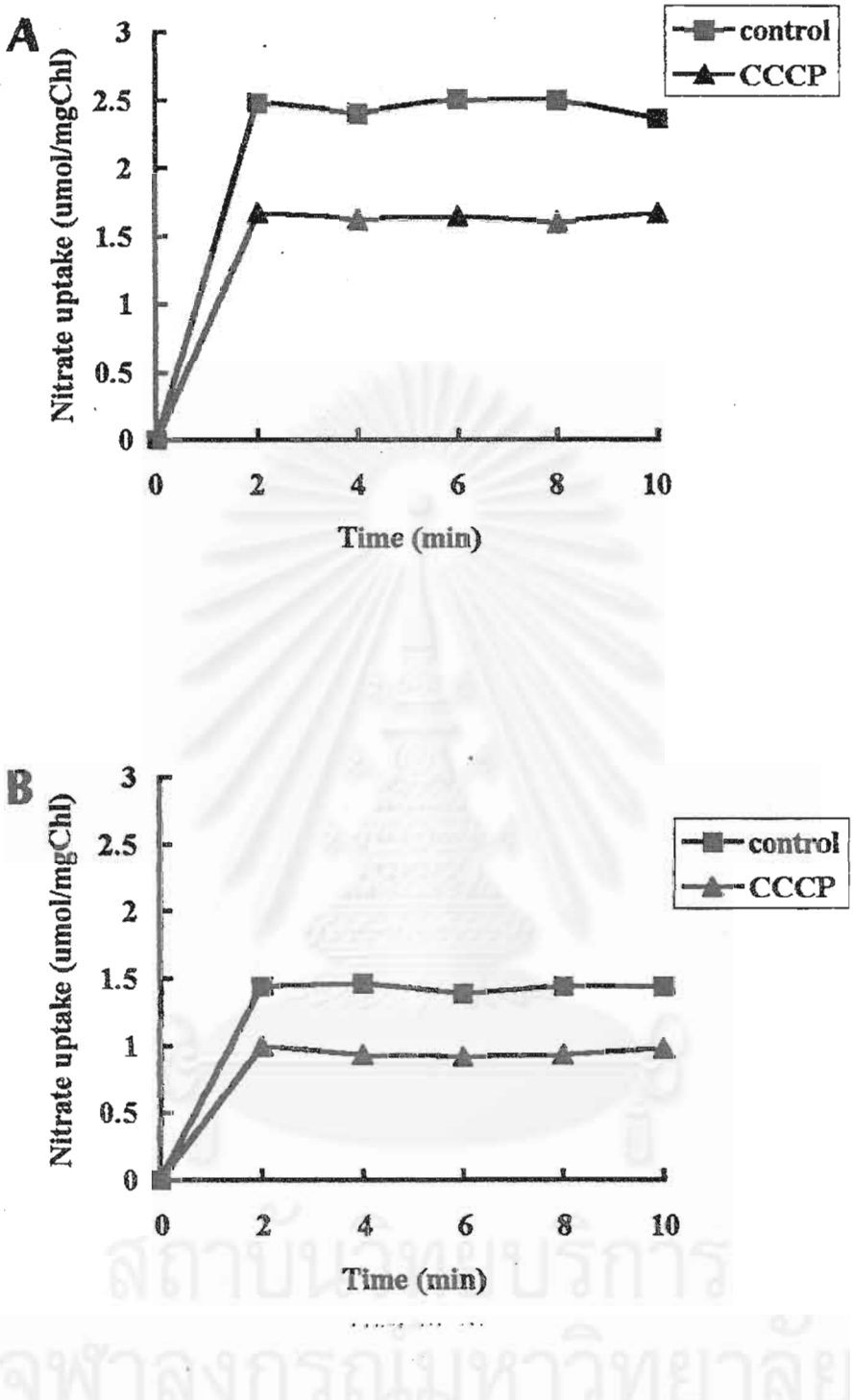


Figure 3.19 Effect of CCCP on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

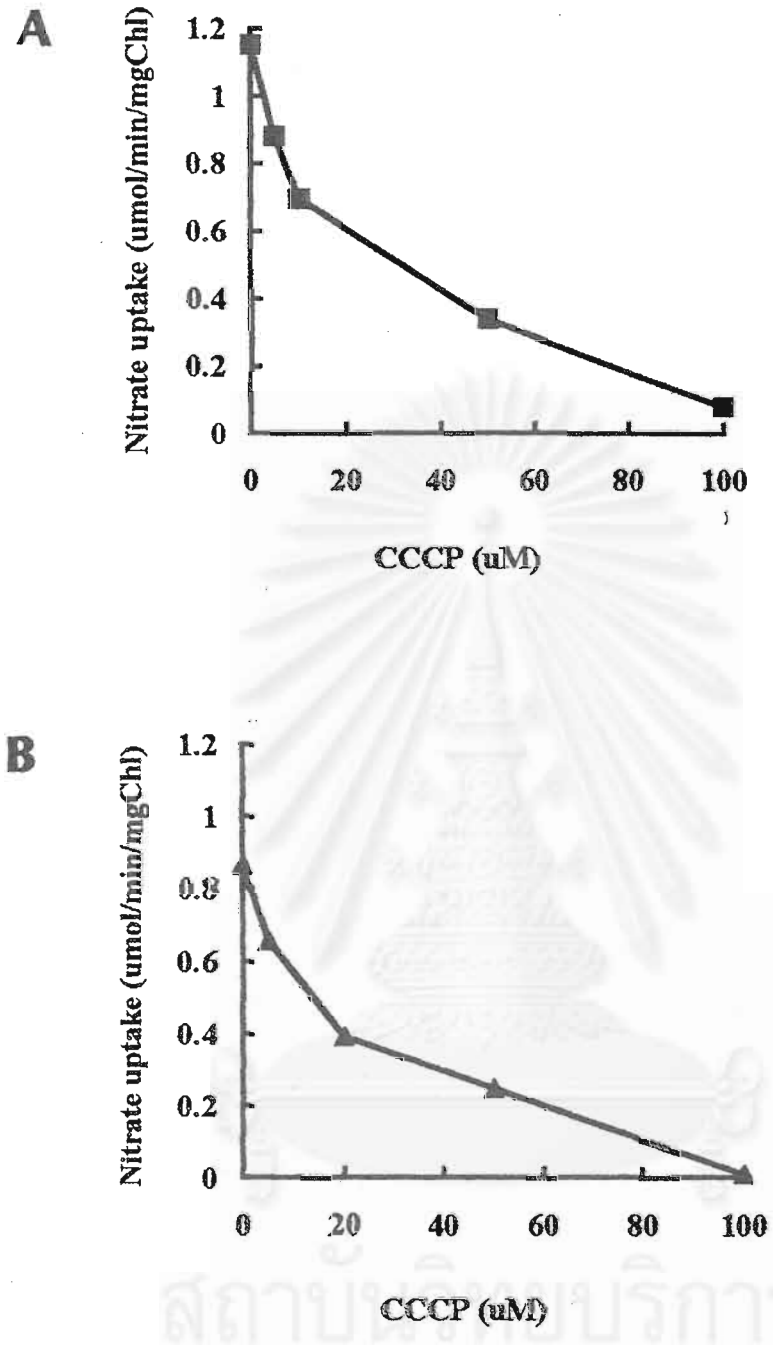


Figure 3.20 Effect of CCCP concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

CHAPTER IV

DISCUSSION

Determination of nitrate uptake system

The effect of salinity on nitrate uptake has been studied in the halotolerant cyanobacterium *Aphanothece halophytica* with reduced nitrate reductase activity and can therefore minimally reduce the transported nitrate. This has been accomplished by tungstate treatment of *A.halophytica* to generate cells with reduced levels of nitrate reductase activity. Molybdenum is a prosthetic group of cyanobacterial nitrate reductase with an essential role in catalysis (Guerrero et al., 1987). Under conditions of molybdenum deprivation, tungsten can be incorporated into newly synthesized apoprotein in place of molybdenum, leading to the formation of an inactive nitrate reductase (Herrero et al., 1986). Figure 3.1 shows that nitrate uptake in normal condition is higher than in stress condition. Furthermore, the low level of nitrate uptake observed at higher concentrations of external nitrate is probably a case of passive diffusion.

When the substrate concentration was varied the nitrate uptake system followed typical Michaelis-Menten kinetics. Using Lineweaver-Burk transformation of the data, *A.halophytica* showed K_s values in control and stress condition of 416 and 450 μM respectively. Lower K_s values of 26.2 μM for *Anabaena* sp. PCC 7210 and 29.75 μM for another diazotrophic cyanobacterium *Anabanae cylindrica* have been reported by Meeks et al.(1983). However, much lower K_s values of 1-4 μM have been reported for unicellur non-nitrogen-fixing *Synechococcus* spp. (Tisher et al., 1984). In non-nitrogen-fixing *Oscillatoria agardhii,* in addition to high-affinity

transport system (K_s, 2 μM), a low-affinity system (K_s, 23-60 μM) has also been reported (Zevenboom et al., 1979). Hanisak and Harlin found that the values of V_{max} and K_s for nitrite uptake in *Codium fragile* generally increased with temperatures between 6 and 24 C. In comparison with macrophytes, nitrogen uptake rate is only rarely expressed per unit of area. Richardson and Schwegler reported uptake rate of 55 mg N M^{-2} d^{-1} for *Cladophora glomerata* in a Michigan marsh. In waters with high nitrogen concentration the uptake rate may reach much higher values, however Davis et al. reported the uptake rate as high as 1,900 mg N m^{-2} d^{-1} for periphyton growing in wastewater treatment plant effluent. Nitrate uptake by illuminated cells of *A. halophytica* increased linearly with time in the first 2 min. Therefore, in most of the experiments, uptake rates were estimated at 2 min. The cessation of nitrate uptake after a certain time suggests that accumulated nitrate (the product of uptake) may induce a decrease in the rate of nitrate influx as the system approaches equilibrium or that thermodynamically favored nitrate efflux counteracts the nitrate transport activity.

Effect of Na^+ on nitrate uptake

In figures 3.3 A and 3.3 B, the results indicate that nitrate uptake in *A. halophytica* in normal and stress condition are indifferent to the extracellar sodium concentrations. These results support the conclusion of Qito and Valley, who inferred that nitrate transport in *Klebsiella oxytoca* is indifferent to the extracellular sodium concentrations.

However, nitrate transport in *Anacystis* exhibits a strong and selective requriment for Na^+ (Rodriguez et al., 1992). Very low transport activity is observed in the absence of univalent cation, or in the presence of either Li^+ or K^+. For Na^+

concentrations in the micromolar range, a rapid efflux of nitrate, following the nitrate eletrochemical gradient, takes place after an initial intracellular accumulation. This efflux suggests a severe limitation in the driving force for active nitrate transport.

Furthermore, nitrate transport activity is sensitive to monensin, an ionophore that relaxes the eletrochemical gradient for sodium (Skulachev et al.,1985). At saturating sodium concentration, nitrate transport exhibits complex saturation kinetics with respect to external nitrate, with a K_s for nitrate of 1 μM and apparent substrate inhibition at nitrate concentrations above 25 μM in *Anacystis nidulans* (Rodriguez et al., 1992). Other authors have reported substrate inhibition of nitrate uptake in *Anacystis nidulans* (Tischner et al., 1984) and *Chlorella sorokiniana* (Tischner et al., 1981), and suggest that binding of two nitrate ions to the nitrate transporter would result in an active complex. The Na^+-requirement exhibited by nitrate transport in *Anacystis nidulans* provides the basis for interpreting the apparent substrate inhibition of nitrate transport in terms of a symport of nitrate and sodium (Rodriguez et al., 1992). A detailed kinetics study has provided evidence that nitrate transport in *Anacystis nidulans* also exhibits complex saturation kinetics with respect to sodium, although it is not inhibited by excess sodium. This asymmetry of the system with respect to substrate inhibition, as well as the complex kinetic patterns exhibited at a varying concentration of both nitrate and sodium, have a best fit to a model for a reaction mechanism (London and Steck 1969, Segel 1975) involving sodium-nitrate as the true substrate of carrier and Na^+ as a non-essential ion activator.

Effect of NH_4^+ on nitrate uptake

The effects of NH_4^+ on nitrate uptake are more complex, due to the possibility of affecting nitrate uptake at a number of levels (transcript abundance, protein level, or direct effects of NH_4^+ on the nitrate transporter). Using nitrite as a tracer of nitrate, Aslem et al. suggested that NH_4^+ increased nitrate efflux rather than diminished influx, while the use of $^{13}NO_3^-$ demonstrated that influx was strongly reduced (Glass et al,1985; Lee and Drew,1989). A recent paper by Kronzucher et al. established that in barley roots, the provision of NH_4^+ in the external medium simultaneously decreased NO_3^- influx and increased efflux, the absolute effect upon influx being more significant. Moreover, this effect occurred within minutes of supplying NH_4^+, suggesting that NH_4^+ itself was acting directly upon inducible high-affinity transport system. This direct effect of NH_4^+ on nitrate uptake does not preclude long-term effects. For example, in longer term experiment Breteler and Siegerist (1984) showed that Methionine sulfoximine (MSO), an inhibitor of Glu synthetase, relieved the inhibitory effect of NH_4^+ on nitrate uptake in dwarf bean. These authors concluded that the NH_4^+ effect arose from products of the assimilation of NH_4^+ rather than from NH_4^+ itself. However, King et al. (1993) observed no relief of NH_4^+ inhibition of NO_3^- uptake by MSO in barley roots. Clearly, part of the confusion in the literature has resulted from the aforementioned multiple levels at which NH_4^+ is capable of inhibiting NO_3^- uptake. There is every reason to expect that NH_4^+ might have direct effect on the transport systems as well as effects at the level of transcription via products of NH_4^+ assimilation. It was demonstrated many times that in the presence of both ammonium and nitrate in the medium, the ammonium is assimilated first, and only when it has gone is NO_3^- utilized. However there have been few reports showing preferential assimilation of nitrate; this phenomenon bas been reported e.g. for *Pandorina* and *Haematococcus.*

Effect of NO_2^- on nitrate uptake

Nitrite is a particularly interesting candidate for regulation because it appears to be taken up by the same transporters as nitrate (Aslam et al., 1992). The kinetics of nitrite inhibition of nitrate uptake in the *A. halophytica* strongly supports the contention that nitrate and nitrite are transported by the same transport system. This is in agreement with the reported competitive inhibition by nitrate and nitrite uptake in *A. nidulans* (Madueno et al., 1987) and in the eukaryotic microalgae *Phaeodactylum tricornutum* (Bibao et al., 1981) and *Cyanidium caldarium* (Fuggi, 1989). However in other microalgae the existence of two different transport systems for nitrate and nitrite was proposed (Cordoba et al., 1986). The kinetics evidence in *Hordeum vulgae* indicated that NO_3^- and NO_2^- may share the same transporter, whereas use of inhibitor and an antibody against nitrate reductase (NR) suggested separated transporters with which the two ions may react competitively (Ward et al., 1988). Likewise, in both barley and *Chlorella*, anti-NR immunoglobulin G fragments purified from the anti-NR serum reversibly inhibited NO_3^- uptake more than 90% relative to the control, but had little effect on NO_2^- uptake. This suggested that the transport proteins may be antigenically different. The reported inhibition of the induction of NO_3^- transport by tungsten but not of NO_2^- transport in sunflower seedlings further suggests that the two transport proteins may be different.

Effect of various anions on nitrate uptake

From the results, it was indicated that the Cl^- and PO_4^{3-} had no effect on the nitrate uptake of the *A.halophytica*. On the other hand, only NO_2^- could affect the nitrate uptake.

Effect of DLG on nitrate uptake

As the assimilation of nitrogen into protein requires both energy and organic skeleton, it is not surprising that there are major interactions between N-assimilation and photosynthetic metabolism. The assimilation of both NH_4^+ and NO_3^- is dependent on photosynthesis, that is assimilation requires light and CO_2; removal of either of these prevents assimilation. The results presented here confirm that nitrate uptake in *A. halophytica* is strictly dependent on active CO_2 fixation. Nitrate assimilation is also subjected to posttranslation regulation by products of N and C assimilation in *Anacystis nidulans* (Lara et al.,1989). Thus, nitrate uptake is completely and reversibly inhibited after addition of ammonium to the medium and its rate is strictly coupled to that of carbon fixation (Lara et al.,1989). In *Synechocooccus sp.* strain PCC 7942 the results suggest that nitrate transport was inhibited by ammonium and by DLG (inhibitors of CO_2 fixation) and ammonium assimilation inhibitors prevented the negative effects of ammonium and DLG but the C-control and N-control of nitrate transport are independent at both the physiological and the molecular level. Di Martino Rigano et al. reported that N-sufficient cells of red algae *Cyanidium caldarium* obtain carbon skeletons for ammonium assimilation exclusively by photosynthetic reactions. Addition of a nitrogen source to N-limited or starved algae cells in the dark increases respiratory CO_2 release, demonstrating the activation of respiratory carbon flow (Syrett, 1953). Carbohydrate formed via photosynthetic CO_2 fixation are either transported from the site of assimilation to other parts of the algae

or used in the chloroplast to supply energy production, biosynthesis or storage. The carbon requirements for amino acids synthesis are independent of the form of inorganic nitrogen assimilated. The major differences between the assimilation of inorganic NO_3^- and NH_4^+ are the energy costs associated with the reduction of NO_3^- to NH_4^+. Compartmentalization of NO_3^- assimilatory pathway enzymes increases the complexity of integrating and controlling nitrogen and carbon metabolism during assimilation.

Effect of various inhibitors on nitrate uptake

In an attempt to resolve the nature of nitrate uptake in *A. halophytica* we examined its response to several metabolic inhibitors. The uncoupler carbonylcyanide-*m*-chlorophenylhydrazone and the ATPase inhibitor N,N-dicyclohexylcarbodiimide each severely inhibited nitrate uptake in *A.halophytica* in both conditions. These results might indicate that a pH gradient generated by H^+/ATPase drives nitrate uptake in *A. halophytica*.

The plasma membranes of various prokaryotes have Na^+/H^+ antiporter activity. We could not ignore the possibility that the uptake of nitrate into the cells was due to a Na^+/H^+ antiport driven by a pH gradient. Amiloride has been used as an inhibitor of the Na^+/H^+ antiport and/or Na^+ channel blocker (at different concentrations), in a wide variety of eukaryotic systems (Krulwich, 1983). Amiloride treatment of *A.halophytica* cells resulted in a decreased rate of nitrate uptake. These data may suggest that nitrate uptake in *A.halophytica* cells was caused by the Na^+/H^+ antiport. However, there has been a report showing that nitrate transport in *Anacystis* is stimulated by amiloride (at pH8) consistent with inhibition of a Na^+-importing/H^+-extruding antiport (Lara et al., 1993 and Kaplan

et al., 1989). Increased Na^+-dependent nitrate transport activity in amiloride-treated cells indicates that the electrochemical gradient of Na^+ required for nitrate transport is not generated by the Na^+/H^+ antiport, but by other means, the sodium circuit may thus be the primary chemiosmotic event in cyanobacteria plasma membranes, at least in cells or species grown at or adapted to alkaline pH (Miller et al., 1984, Brown et al., 1990, Richie 1992).

Nitrate uptake in *A. halophytica* is sensitive to monensin, an ionophore that relaxes the electrochemical gradient for sodium, the inhibition being higher than 50 % for monensin concentration as low as 23 μM. These observations indicate that nitrate uptake in *A. halophytica* relies on the maintenance of an electrochemical gradient of Na^+ across the plasma membrane, which might represent the immediate source for active nitrate transport. Monensin is a carboxylic polyether ionophore that, in artificial system, causes the electroneutral exchange of Na^+ for H^+ and thereby collapses the Na^+ gradient between the cells and the medium (Pressman, 1976). *Synechococcus* cells also actively extrude Na^+ through an Na^+/H^+ antiporter (Blumwald et al., 1984) or a primary Na^+ pump (Brown et al., 1990). The active extrusion of Na^+ creates an electrochemical potential for Na^+ (Ritchie, 1992). The energy conserved in an electrochemical potential for Na^+ may, therefore, serve as an energy source for the secondary active transport of anions. The Na^+ electrochemical potential comprises an electrical component and a chemical component. The inhibitory effect of monensin and amiloride, which dissipate a chemical component, on nitrate uptake in *A. halophytica* suggest that at least this component of the electrochemical potential of Na^+ plays a role in this transport process. Indeed, an Na^+/H^+ antiporter has been reported to play a role in the extrusion of Na^+ which is one of the

adaptive mechanisms for salt tolerance in *A.halophytica* (Waditee,et al., 2001).

However, although monensin is generally considered to mediate Na^+/H^+ exchange, it is also capable of mediating K^+/H^+ exchange (Na^+:K^+ selectivity=16:1) and will bring all cations that can be transported to equilibrium (Pressman and Fahim, 1982). Monensin-mediated fluxes of Na^+, K^+, and H^+ have been observed in red blood cells (Painter and Pressman, 1982) and in several gram positive bacteria (Delort et al., 1989). Depending on the ionic gradients and the rates of exchange, the initial alkalinization of the cytosol, mediated by Na^+ exchange, will with time be countered by K^+/H^+ exchange-mediated acidification, resulting in net Na^+ in/K^+ out exchange and a return of the cytosol to a physiological pH)

CHAPTER V

CONCLUSION

The results of the present investigation can be summerized as follows:

1. Nitrate uptake in normal condition is higher than in salt stress condition.
2. From Lineweaver-Burk transformation of the data, the apparent K_s's of control and salt stress condition were 416 and 450 μM respectively, the V_{max}'s were 9.1 and 5.3 μmol/min/mgChl respectively.
3. Nitrate uptake in both conditions appear to be indifferent to the extracellular sodium concentrations.
4. Ammonium, present together with nitrate, prevented nitrate uptake in *A. halophytica* in both normal and salt stress conditions but inhibition of ammonium assimilation by treatment of the cells with MSX resulted in the slight relief of negative effect of ammonium in nitrate uptake in cells grown under normal condition.
5. The addition of nitrite to the assay mixture inhibited the net uptake rate of nitrate. A $K_{i,}$obtained by plotting the concentration of nitrite versus slope was, 84 μM.
6. Cl^- and PO_4^{3-} did not inhibit nitrate uptake in both normal and salt stress conditions.
7. Nitrate uptake was also inhibited by DLG (a selective inhibitor of CO_2 fixation)
8. The uptake of nitrate by *A. halophytica* was susceptible to a variety of agents that inhibit energy generation process.

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APPENDICES

APPENDIX 1

Turk Island Salt Solution+modified BG_{11} medium contained the following components:

1. Preparation of Turk Island Salt Solution

Stock Solution A : KCI 33.3 g

$MgCI_2.6H_2O$ 275.0 g

$CaCI_2.2H_2O$ 73.3 g

and made up to 5 litres with distilled water

Stock Solution B : $MgSO_4.7H_2O$ 347.0 g

and then made up to 5 liters with distilled water

To make Turk Island Salt Solution, 500ml of Stock Solution A was added to 500 ml of Stock Solution B. To this mixture 140.8 g of NaCI was added and then final volume was made to 5 liters with stilled water.

2. Composition of modified BG_{11} medium (BG_{11} medium + $NaNO_3$ solution)

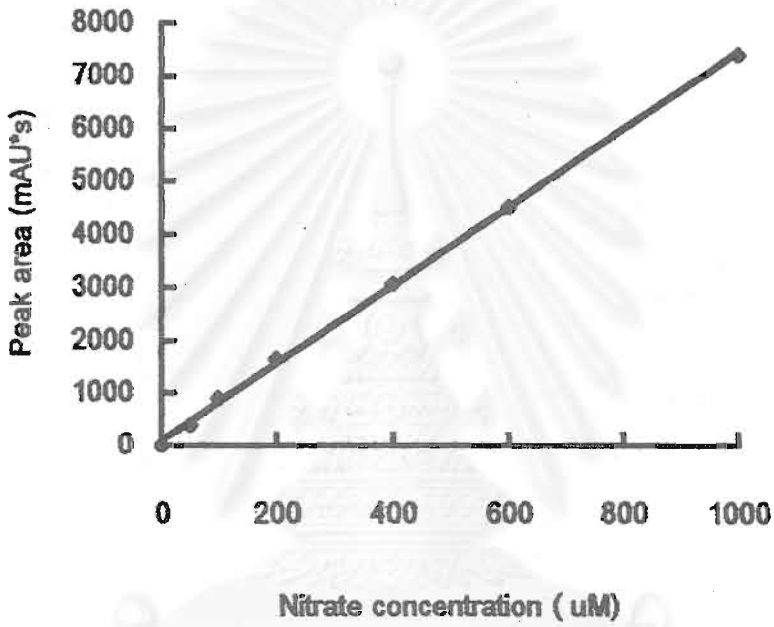
$NaNO_3$	(75 g/500 ml)	50 ml
KH_2PO_4	(8 g/200 ml)	5 ml
$MgSO_4.7H_2O$	(15 g/200 ml)	5 ml
$CaCI_2.2H_2O$	(7.2 g/200 ml)	5 ml
Na_2CO_3	(4 g/200 ml)	5 ml
Citric acid	(1.2 g/200 ml)	5 ml
$EDTA.Na_2$	(0.2 g/200 ml)	5 ml
$FeSO_4.7H_2O$	(1.2 g/200 ml)	5 ml
∗Trace element A_5 Solution + Co		5 ml

∗ Trace element A_5 Solution + Co contained the following component in gram per litre H_3PO_4 : 2.86 ; $ZnSO_4.7H_2O$: 0.2 ; $CuSO_4.5H_2O$: 0.08 ; $MnCI_2.4H_2O$: 1.81 ; $Na_2MoO_4.2H_2O$: 0.39 ; $Co(NO_3)_2.6H_2O$: 0.049

Culture medium of *Aphanothece halophytica* was prepare by adding all solution of item 2 at indicated volume to 5 liters of Turk Island Salt Solution and the pH was adjusted to 7.6 by slowly adding 2 M NaOH. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minutes.

APPENDIX 2

Standard curve of nitrate



BIOGRAPHY

Miss Jutakae Wangsupa was born on June 25, 1976 in Bangkok, Thailand. She graduated with a Bachelor of Science Degree in Food science from Faculty of Applied science, King Mongkut institute of Technology North Bangkok, Thailand in 1992 and studied for a Master Degree in Biochemistry program since 1998.