CHAPTER I

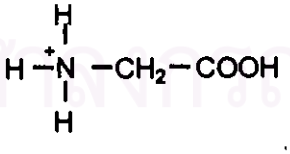
INTRODUCTION

General background

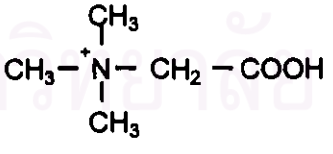
Cellular volume reduction due to osmotic water loss occurs rapidly following exposure to hyperosmotic conditions. Most cells are able to partially regulate the amount of water loss and some are capable of regaining at least a portion of their initial volume (MacKnight 1988). To counter the continued efflux of water, cells actively synthesize or take up solutes(osmolytes) from the extracellular medium. Although inorganic ions are important components of this osmotic response, the level to which their intracellular concentration can rise is limited because of the destabilizing effect on protein structure and function (Yancey et al. 1982). Therefore, hyperosmotically stressed cells also accumulate low molecular weight organic compounds which are nonperturbing to cellular metabolism and may counter the deleterious effects of elevated salt concentrations. The organic osmolytes accumulated differ among cell types, but are restricted to free amino acids, polyols, urea, and quaternary ammonium compounds. A very important quaternary ammonium compound commonly used in cellular osmoregulation is glycine betaine (*N,N,N* - trimethylglycine), a derivative of amino acid glycine (Figure 1). Although this organic osmolyte occurs in an expanding list of diverse organisms the regulatory mechanisms of accumulation have been extensively described for only certain bacteria, a few plants, and cultured renal cells (Garcia-Perez and Burg 1991; Weretilnyk and Hanson 1989; Smith et al. 1988; Lanfald and Strom 1986; Strom et al. 1986; Le Rudulier et al. 1984). Intracellular levels of glycine betaine can be increased either *de novo* synthesis or transport-mediated uptake.

Under osmotic stress, bacteria commonly accumulate potassium ions and organic solutes in their cytoplasm to build up internal osmotic strength and prevent diffusion of water out of the cells. Stewart (1983) pointed out that the organic solutes used by bacteria comprise only a few types of compounds, namely, amino acid, sugar, polyols, and glycine betaines(i.e., fully N-methylated amino acid derivatives). The term osmoprotectant has been coined for molecules which can be accumulated in large amount in the cell and protect against osmotic stress and salt inactivation (Le Rudulier et al. 1984; Strom et al. 1983). *Escherichia coli* displays high versatility in synthesis and uptake of osmoprotectants, and depending on the composition of the growth medium, it accumulated most classes of the compounds listed above (Britten et al. 1962; Le Rudulier et al. 1983; Measures 1975; Perroud and Le Rudulier 1985; Roller and Anagnostopoulos 1982).

Choline is a precursor of glycine betaine in osmotically stressed cells of *Escherichia coli* (Strom et al. 1983). The choline-glycine betaine pathway of *Escherichia coli* is osmotically regulated, i.e., only cells grown in choline-containing medium with an increased osmotic strength display full enzyme activities of the pathway.



Glycine



Glycine betaine

Figure 1 Structure of glycine and glycine betaine

Glycine betaine also stimulates the respiration of the moderately halophilic, heterotrophic bacterium, Ba_1, at a high NaCl concentration (Shkedy-Vinkler and Avi-Dor 1975), and it accumulated in a halophilic, photosynthetic bacterium *Ectothiorhodospira halochloris* (Galinski and Truper 1982) and in several hypersaline strains of cyanobacteria (Mackay et al. 1984; Reed et al. 1984). Thus, glycine betaine appears to play an important role in the osmoregulation of various bacteria with different ranges of osmotic tolerance, as they do in many marine animals and halophytic plants (Burton 1983; Wyn Jones and Storey 1981).

Betaine (glycine betaine) has previously been demonstrated to be a major osmoticum in a member of photosynthetic organisms including both procaryotes and eucaryotes (Gorham et al. 1985; Reed et al. 1986; Storey and Wyn Jones 1977). Glycine betaine is the final oxidation product of choline metabolism (Zhang et al. 1992; Ziesel and Blusztajn 1994). In liver cells, glycine betaine is and important donor of methyl groups for the regeneration of homocysteine (Finkelstein et al.1982; Barak et al. 1993). In kidney cells (Garcia-Perez and Burg 1991; Nakanishi and Burg 1989), bacteria (Landfald and Strom 1986; Smith et al. 1988; Abee et al. 1990; Cayley et al. 1992),and in cells of many plants (McCue and Hanson 1992; Eichenberger et al. 1993) betaine serves as an osmolyte.

Organic osmolytes are osmotically active substances that accumulate in cells of many species during hypertonic states and are believed to play an important role in cell volume homeostasis. The role of trimethylamines as osmolytes appears to be highly conserved, as they have been demonstrated to function in this capacity in *Escherichia coli* (Landfald and Strom 1986) and cartilagenous fish (Yancey et al. 1982).

Glycine betaine and its precursors choline and glycine betaine aldehyde have been found to confer a high level of osmotic tolerance when added exogenously to cultures of *Escherichia coli* at an inhibitory osmotic strength. Choline works as an osmoprotectant only under aerobic conditions, whereas glycine betaine aldehyde and glycine betaine function both aerobically and anaerobically. No endogenous glycine betaine accumulation was detectable in osmotically stressed cells grown in the absence of the osmoprotectant itself or the precursors. A membrane-bound, O_2-dependent, and electron tranfer-linked dehydrogenase was found which oxidized choline to glycine betaine aldehyde and aldehyde to glycine betaine at nearly the same rate (Landfald and Strom 1986).

It was also demonstrated that glycine betaine accumulated in *Vibrio costicola*, a moderately halophilic eubacterium that can grow in media containing 0.4-3.5 M NaCl, with its optimum growth occurring at around 1.0 M NaCl (Flannery et al. 1952; Forsyth and Kushner 1970; Kamekura et al. 1985). This ability to grow over a wide range of salt concentration may be partly due to the accumulation of the compatible solute glycine betaine (Brown 1976). The moderate halophile *Vibrio costicola*, growing on a chemically-defined medium, tranformed choline into glycine betaine by the membrane-bound enzyme choline dehydrogenase and the cytoplasmic enzyme betaine aldehyde dehydrogenase. Choline dehydrogenase was strongly induced and betaine aldehyde dehydrogenase less strongly induced by choline. The formation of these enzymes was also regulated by the NaCl concentration of the growth medium, increasing with increasing NaCl concentrations. Intracellular glycine betaine concentration also increased with increasing choline and NaCl concentration in the medium (Choquet et al. 1991).

In higher plants, some plant species in families such as *Chenopodiaceae, Amaranthaceae,and Gramineae* accumulate glycine betaine in response to water or salt

stress (Gorham et al. 1985). Salinity is one of the important elements to limit crop productivity. Since the synthesis and accumulation of low molecular weight organic "compatible" solutes such as glycerol, sugars, and quaternary ammonium compounds are known to be essential for adaptibility of plant cells to high salinity (Gorham et al. 1985; Hanson and Hitz 1982; Storey and Wyn Jones 1977; Wyn Jones et al. 1977; Yancey et al. 1982), it can readily be surmised that changes in carbon, nitrogen, and/or energy metabolisms are tightly connected to the overall process. Hanson and co-worker (Hanson et al. 1985; Weigel et al. 1986) have reported that glycine betaine synthesis occurs in chloroplasts from spinach leaves. Subsequently, Robinson and Jones (1986) have reported the accumulation of glycine betaine up to 0.3 M in spinach chloroplasts to provide osmotic adjustment during salt stress.

Cyanobacteria accumulate glycine betaine

It is also known that highly halotolerant cyanobacteria accumulate glycine betaine as a major osmoticum, a quaternary ammonium compound, whereas less tolerant cyanobacteria accumulate either sucrose or glucosylglycerol (Reed et al. 1986). In cyanobacteria, osmoregulation involves the accumulation of both organic and inorganic solutes. However, a key factor in the adjustment of many cyanobacteria to hypersaline environment is the increase in the intracellular level of a species-specific low molecular weight organic solute. The role of low molecular weight carbohydrates in the maintenance of osmotic balance has been shown for several cyanobacteria as well as in many higher plants. Osmoregulation in cyanobacteria grown at high external NaCl concentrations involves the accumulation of organic and inorganic solutes. For several fresh water cyanobacteria the role of low molecular weight carbohydrate as osmoregulators has been shown; for example, glucosylglycerol in *Synechocystis* 6714 (Mackay et al. 1983.) and sucrose in *Synechococcus* 6311 (Blumwald et al. 1983).

A combination of inorganic and organic solutes (sucrose and glycerol) is involved in osmotic adjustment in the euryhaline cyanobacterium, *Synechocystis* PCC 6714, which can grow in both nonsaline and saline media (Reed et al. 1984). In early studies, Miller et al.(1976) suggested that the halophilic unicellular cyanobacterium *Aphanothece halophytica* (*Synechococcus* sp.) responded to changes in external water status by adjusting its intracellular K^+ concentration (see also Yopp et al. 1978), as in *Halobacterium* spp. (Brown 1976, 1978). However, more recent studies of osmotic adjustment in cyanobacteria have demonstrated that organic osmotica , rather than inorganic ions, may play a major role in the maintenance of positive turgor under conditions of salt-stress. This was first shown by Borowitzka et al.(1980) for the marine unicellular strain *Synechococcus* RRIMP-N-100, which accumulated the heteroside glucosylglycerol in response to salt stress. Subsequent studies have also implicated the disaccharides sucrose and trehalose in the osmotic adjustment processes of several marine and freshwater cyanobacteria (Blumwald and Tel-Or 1982; Reed and Stewart 1983; Reed et al. 1984). Furthermore they have shown that the halophilic cyanobacterium *Synechocystis* DUN 52 accumulated the quaternary ammonium compound glycine betaine when grown in the presence of high salt concentrations (Mohammad et al. 1983). Glycine betaine also serves as an internal osmoticum in the halophilic bacterium *Ectothiorhodospira halochloris* (Galinski and Truper 1982) and in several halophytes (Wyn Jones and Gorham 1983).

The intracellular concentrations of the monovalent inorganic cations K^+ and Na^+, low molecular weight carbohydrates (identified as glucosylglycerol, sucrose, and trehalose) and quaternary ammonium compounds have been determined for 4 strains of cyanobacteria (*Aphanothece halophytica, Coccochloris elabens, Dactylococcopsis salina* and *Synechocystis* DUN 52) originally isolated from hypersaline habitats (i.e. habitats with a salinity greater than that of seawater) over a range of external salt concentration

(from 50% to 400% seawater). Intracellular cation levels (Na^+ and K^+) were determined and shown to be only minor changes in response to salinity. Intracellular carbohydrates were found to comprise a negligible component of the intracellular organic solutes. Quaternary ammonium compounds, however ,were recorded in osmotically significant quantities in these strains, showing major variation in response to salinity. Examination of intact cells and cell extracts using ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy confirmed the presence of the quaternary ammonium compound glycine betaine as the major osmoticum in the 4 strains ; no other compounds were detected during NMR assays. These results suggest a common mechanism of osmotic adjustment, involving quaternary ammonium compounds, in cyanobacteria from hypersaline environment (Reed et al. 1984).

Accumulation of glycine betaine in cyanobacterium, *Aphanothece halophytica*

Glycine betaine was first shown to be the major osmoticum in a halotolerant cyanobacterium *Synechocystis* DUN52 (Mohammad et al. 1983). The unicellular cyanobacterium, *Aphanothece halophytica* is a highly halotolerant organism that can grow at high external NaCl concentration up to 3 M (Garlick et al. 1977; Reed et al. 1984). It was demonstrated that glycine betaine is accumulated as the major osmoticum inside *A. halophytica* cells in response to changes in external salinity (Reed et al. 1984). Furthermore, Incharoensakdi et al.(1986) reported that the presence of glycine betaine at 0.5 M, slightly promoted ribulose-1,5-bisphosphate (RuBP) carboxylase activity in *A. halophytica*. KCl at 0.25 M inhibited RuBP carboxylase about 55%. Glycine betaine relieved the inhibition by 0.25 M KCl and original uninhibited activity was restored at 1 M glycine betaine. Other osmoregulatory solutes such as sucrose and glycerol also reduced KCl inhibition, though to a lesser extent than glycine betaine.

High concentration of salts have been reported to inhibit the activity of many enzymes of both eucaryotic and procaryotic origin (von Hippel and Schleic 1969). It has also previously been reported that salt inhibits enzyme activity of RuBisCO from *A. halophytica*, whereas glycine betaine protects the enzyme against salt inhibition (Incharoensakdi et al. 1986) and is not harmful to the metabolic activities of the cell even at high concentration.

Biosynthesis pathways

There is evidence for several biosynthetic routes to choline among high plants with different routes predominating in different species (and perhaps tissues). These routes interconnected series of decarboxylation and *N*-methylation reactions at the free base, phospho-base or phosphatidyl-base levels (Figure 2).

In wilted barley leaves, glycine betaine accumulated at about 200 nanomoles per 10 cm leaf per day. Results with ^{14}C-labeled precursors were qualitatively and quantitatively consistent with *de novo* synthesis of this glycine betaine from serine via ethanolamine, choline and betaine aldehyde (Figure 3) and indicated that water stress may increase the activities of all steps in this pathway except the last (Hanson,and Scott 1980).

The choline - glycine betaine pathway

Biosynthesis of glycine betaine results from oxidation of choline via a two-step reaction with betaine aldehyde as the intermediate (Figure 4). This series of the reactions may be catalyzed by different enzymatic systems. In microorganisms and mammals a membrane-bound choline dehydrogenase (EC 1.1.99.1) is employed in conjunction with a soluble betaine aldehyde dehydrogenase (EC 1.2.1.8) (Haubrich and

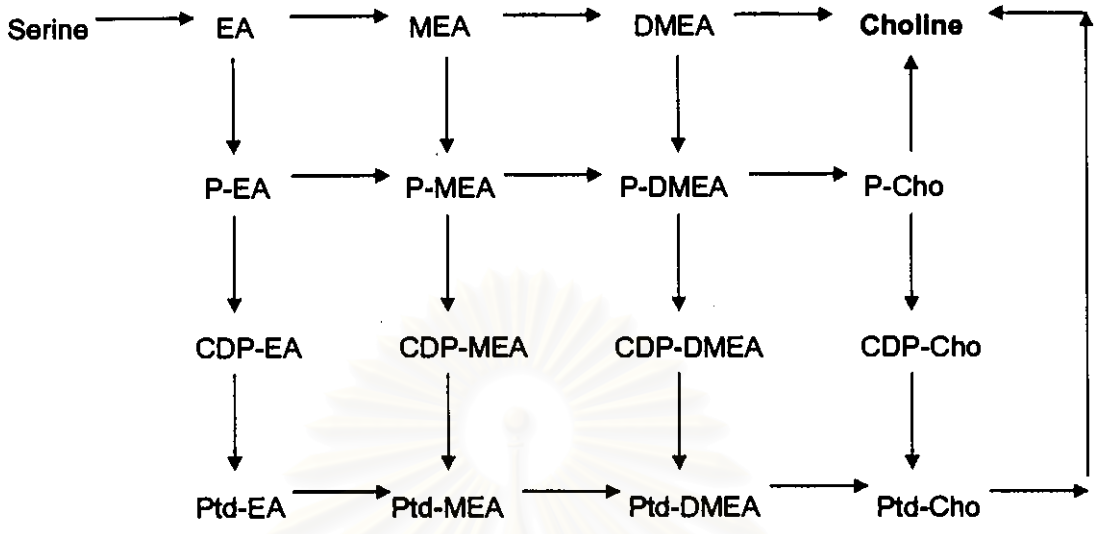


Figure 2 Pathways of choline synthesis in higher plants

Abbreviations: Serine and its metabolytes: Ser, serine; EA, ethanolamine; MEA, *N*-methylethanolamine; DMEA, *N,N*-dimetyhlethanolamine; the phosphate esters of these compounds are designated by the prefix P-, and the corresponding cytidine diphosphate and phosphatidyl derivatives by the prefixes CDP-or Ptd-, respectively

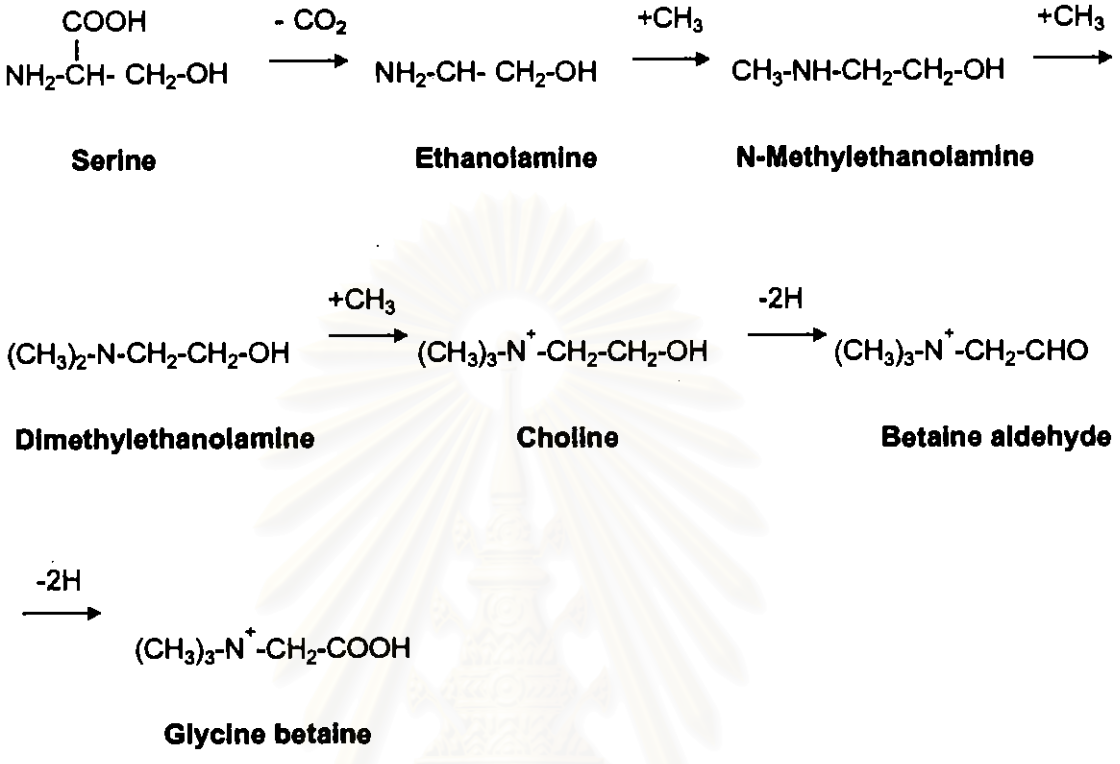
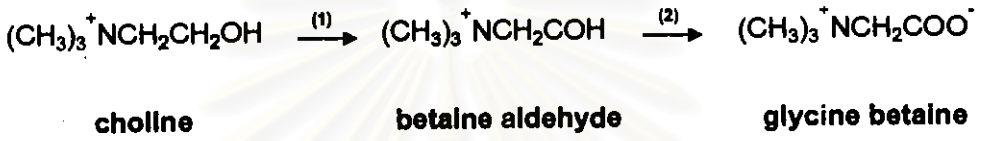


Figure 3 Pathway of glycine betaine biosynthesis in water-stressed barley leaves

Gerber 1981; Lanfald and Strom 1986; Nagasawa et al 1976; Rotchild and Barron 1954; Lin and Wu 1986; Wilken 1970; Wilken et al. 1970). Plants utilize a soluble choline monooxygenase in combination with betaine aldehyde dehydrogenase (Brouquisse et al. 1989; Pan 1988)



1: choline dehydrogenase or choline monooxygenase

2: betaine aldehyde dehydrogenase

Figure 4. Biosynthetic pathway of glycine betaine from choline via betaine aldehyde.

Choline dehydrogenase was discovered in 1937.It has been most extensively studied in mammalian liver where it is found exclusively in mitochondria (Kimura and Singer 1962; Williams and Screenivasan 1953). The enzyme was located on the matrix side of the inner mitochondrial membrane (Streumer-Svobodova and Drahota 1977). Several groups have attempted its isolation and purification (Chi-Shui and Ru-Dan 1986; Haubrich and Gerber 1981; Redina and Singer 1959; Tsuge et al. 1980; Russell and Scopes 1994) and it is generally believed to be a single-chain polypeptide in *Pseudomonas* strain with a molecular mass of approximately 60,000-70,000 Da (Russell and Scopes 1994). It is thought to be linked to ubiquinone (coenzyme Q) in the electron

transport chain to which it transfers the electrons from the oxidation reaction (Barrett and Dawson 1975).

In general choline dehydrogenase activity is highest in the liver and kidney of the rat and other mammals although in humans its activity in the kidney is some what higher than in the liver (Streumer-Svobodova and Drahota 1977). Some activity is also present in the mammalian brain, but is negligible in other organs. Within the kidney, choline dehydrogenase activity has been localized to the proximal tubule (both convoluted and pars recta segments) and the inner medulla (Wirthensohn and Guder 1982). Choline dehydrogenase was strongly induced and betaine aldehyde dehydrogenase less strongly induced by choline. The formation of these enzymes was also regulated by the NaCl concentration of the growth medium, increasing with increasing NaCl concentrations. Intracellular glycine betaine concentration also increased with increasing choline and NaCl concentrations in the medium. This increase was almost completely blocked by chloramphenicol, which does not block the increase in salt-tolerant active transport on transfer from a low to a high salt concentration (Choquet et al. 1991; Lanfald and Strom 1986). The oxidation of choline to betaine aldehyde in rat liver mitochodrial preparation required no additional cofactors, suggesting that mitochodria contained an endogenous electron acceptor. Oxygen might have been the ultimate electron acceptor because cyanide (an inhibitor of cytocrome oxidase) totally inhibited the formation of [methyl-^{14}C] betaine aldehyde from [methyl-^{14}C]choline. This inhibition could, however, be overcome by the addition of phenazine methosulfate (PMS). This would be expected if the electrons from choline dehydrogenase were shunted to PMS.

Betaine aldehyde was the major product of choline oxidation in rat liver mitochondria in the absence of NAD^+. In the presence of NAD^+, betaine aldehyde was further oxidized to glycine betaine, thus it is likely that choline dehydrogenase and betaine aldehyde dehydrogenase were separate enzymes. However, it is possible that

choline dehydrogenase and betaine aldehyde dehydrogenase were one enzyme requiring NAD^+ only for the catalyzed oxidation of betaine aldehyde to glycine betaine. The results obtained with the use of the partially purified protein support the former hypothesis, as no glycine betaine formation was detected when this preparation was incubated in the presence of [methyl-^{14}C]choline and NAD^+. Thus, it is concluded that choline dehydrogenase is a separate enzyme from betaine aldehyde dehydrogenase, and suggest that betaine aldehyde dehydrogenase requires NAD^+ (or $NADP^+$) for activity (Zhang et al. 1992).

The cytoplasmic enzyme betaine aldehyde dehydrogenase, which appears to require NAD^+ to catalyze the reaction (Wilken et al. 1970), is also regulated by the NaCl concentration of the growth medium, increasing with increasing NaCl concentrations. In the moderate halophile *Vibrio costicola*, betaine aldehyde dehydrogenase was stimulated by 0.5 M salts and could function up to 2.0 M salts (Choquet et al. 1991). As with glycine betaine accumulation, the activity of betaine aldehyde dehydrogenase increases threefold in response to gradual salinization of spinach plants to 300 mM NaCl or to sudden salt shock with 200 mM NaCl (Weigel et al. 1986). The native molecular weight of this enzyme is approximately 111,000 Da (Weretilnyk and Hanson 1989)

In this work, the unicellular cyanobacterium, *Aphanothece helophytica* (this alga is classified into *Chroococcales* order, *chroococcacean* cyanobacteria subgroup, Geitler 1932; Stanier et al. 1971) was chosen as a source for the study of (a) the determination of quaternary ammonium compounds and (b) the elucidation of the pathway of choline oxidation (Figure 4) in salt stress by radiotracer method after ion-exchange chromatography, and (c) the localization of choline dehydrogenase activity between membranous and cytoplasmic fraction. This cyanobacterium is a short cylindrical shape cyanobacterium covered with mucous membrane (as shown in Figure 5) which mutiplies by binary fission.

Figure 5 Microscopic picture of *Aphanothece halophytica* grown in Turk Island Salt Solution + modified BG_{11} medium at day 14 (x2250)

Objectives

The objectives of this thesis are the following :

1. To study the biosynthetic pathway of glycine betaine in a halophilic cyanobacterium, *A. halophytica* by various precursors.
2. To study the accumulation and the biosynthesis of glycine betaine in *A. halophytica* under salt stress.
3. To fractionate choline dehydrogenase from *A. halophytica.*
4. To study the relationship between salt stress and choline dehydrogenase activity in *A. halophytica*