CHAPTER III

RESULTS

1. Separation of quaternary ammonium compounds

Thin layer chromatography was performed on 0.25 mm thick silica gel plate. The running solvent was chloroform/ methanol/ 0.1 M HCl (65:30:4, v/v) for 1.5 hr. After TLC plate dried, it was sprayed with Dragendorff' s reagent (Appendix 6). This procedure gave approximate R_f values and colour spots for standard choline, betaine aldehyde, and glycine betaine (by methods described in section 1) which were 0.19, red ; 0.29, orange; and 0.24, yellow respectively (Figure 7). For quaternary ammonium compounds, after appropriate elution through a cation-exchange column (Dowex 50W, 50x4-200, H^+ form) choline, betaine aldehyde, and glycine betaine gave R_f values of 0.21, 0.29, and 0.24 with red, orange, and yellow color spots, respectively (Table1).

2. Efficiency of cation-exchange column

The efficiency of a cation-exchange column to separate quaternary ammonium compounds was tested by adding 1 μCi [^{14}C] choline to *A. halophytica* in 50 mM - NaOH, pH 7.5 containing 0.5 M NaCl and incubating for 60 min. Cells were extracted and passed through a cation-exchange column. From methods described in section 2, table 2 shows the recovery of radioactivity after passing through a cation-exchange column. The radioactivity recovered in the eluted choline, betaine aldehyde, and glycine betaine represented nearly 100% of that before passing through the column.

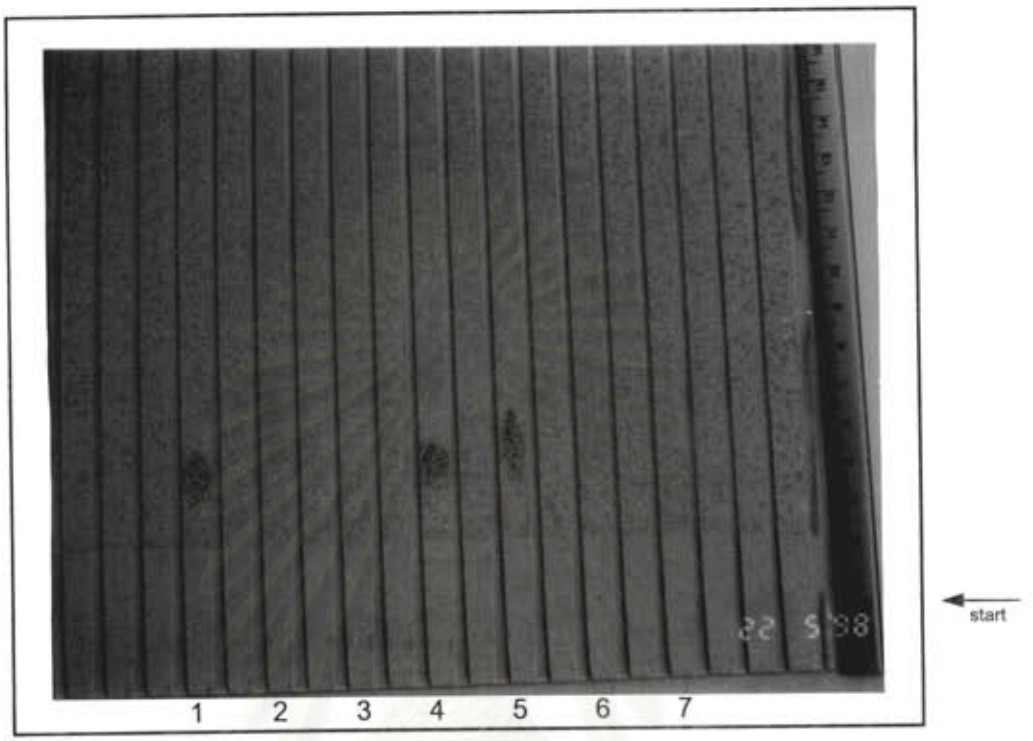

Figure 7 Thin layer chromatography separation of commercial choline, betaine aldehyde, and glycine betaine on silica gel thin layer plate with chloroform/methanol/0.1 M HCl (65:30:4, v/v) for 1.5 h

Lane 1: choline

2: betaine aldehyde

3: glycine betaine

4: mixture of choline, betaine aldehyde, and glycine betaine

5: choline after passing through cation-exchange column

6: betaine aldehyde after passing through cation-exchange column

7: glycine betaine after passing through cation-exchange column

Table1 Separation of standard quaternary ammonium compounds, before and after passing through Dowex 50w,50x4-200 column by thin layer chromatography for 1.5 hr, using chloroform/ methanol/ 0.1 M HCl (65:30:4, v/v)

Quaternary ammonium compounds	Color	Distance from origin (cm)	Approximate R_f
choline	red	3.5	0.19
betaine aldehyde	orange	5.2	0.29
glycine betaine	yellow	4.3	0.24
mixture of choline, betaine - aldehyde and glycine betaine	red, orange, and yellow respectively	3.6, 5.8, 4.2 respectively	0.20, 0.32, 0.23 respectively
choline*	red	3.7	0.21
betaine aldehyde*	orange	5.2	0.29
glycine betaine*	yellow	4.3	0.24

* : After passing through column (Dowex 50x4-200, H^+ form)

Table 2 Recovery of radioactive quaternary ammonium compounds after passing through cation-exchange column

Total radioactivity before passing ion-exchange column ($nCi/10^9$ cells)	[^{14}C]-betaine aldehyde ($nCi/10^9$ cells)	[^{14}C]-glycine betaine ($nCi/10^9$ cells)	[^{14}C]-choline ($nCi/10^9$ cells)	Total count after passing through ion-exchange column ($nCi/10^9$ cells)	Recovery (%)
10.9	9.20	1.16	0.39	10.75	98.6

3. Time course and product of choline oxidation

When 1 μCi of [^{14}C] choline was added to *A. halophytica* (control and stressed) during 0 to 180 min incubation, betaine aldehyde and glycine betaine could be labeled. Figure 8 shows the uptake of 1 μCi [^{14}C] choline by control and stressed cells (methods described in section 3). The rapid rate of [^{14}C] choline uptake occurred during the first 30 min of incubation but the uptake rate increased slowly after 60 min. The pattern of [^{14}C] choline uptake was similar for control and stressed cells.

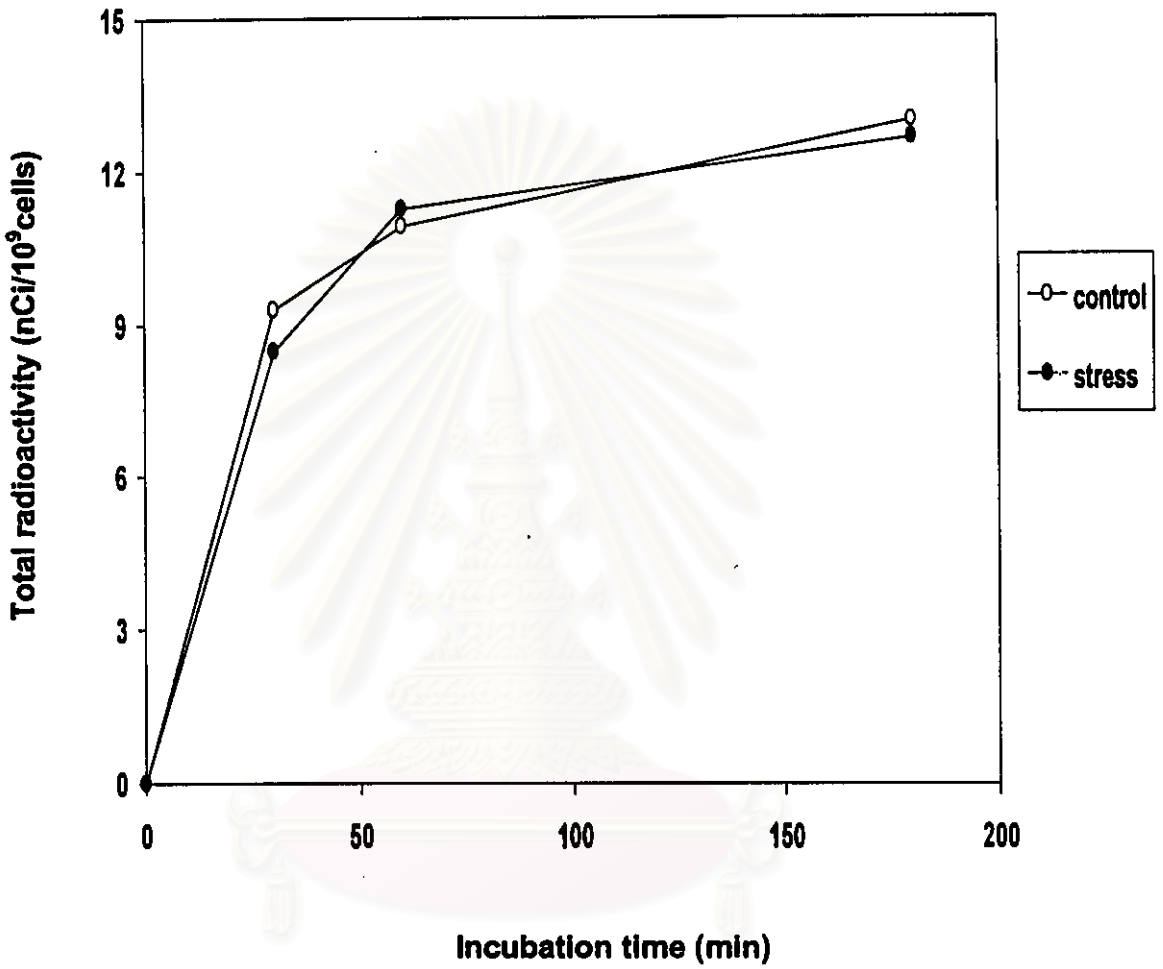

Figure 8 Uptake of [^{14}C] choline by *A. halophytica* grown under control (0.5 M NaCl) and salt - stress (2.0 M NaCl) conditions

Table 3 Conversion of [^{14}C] choline to [^{14}C] betaine aldehyde and [^{14}C] glycine betaine in *A. halophytica*.

Labeling time (min)	[^{14}C] choline oxidation (nCi/10^{9}cells)			
	betaine aldehyde		glycine betaine	
	control	stress	control	stress
0	0	0	0	0
30	8.42	6.68	0.66	0.80
60	9.20	7.50	1.16	2.55
180	8.84	3.69	2.26	5.88

Choline was oxidized to betaine aldehyde in control and stressed cells (Figure 9, Table 3). [^{14}C] Betaine aldehyde was synthesized at high rate during the first 30 min and reached maximum at 60 min (about 9.2 nCi/10^{9}cells for control and 7.5 nCi/10^{9}cells for stressed cells). After 60 min, in control cells the rate of [^{14}C] betaine aldehyde biosynthesis fell slightly but in stressed cells fell rapidly. Figure10 shows that glycine betaine could also be synthesized from choline. In control cells, the rate of [^{14}C] glycine betaine biosynthesis appeared to increase linearly during the first 60 min and the rate was slightly lower afterwards. For salt-stressed cells, the rate during the first 30 min was similar to the control cells but rose sharply during 30 to 180 min.

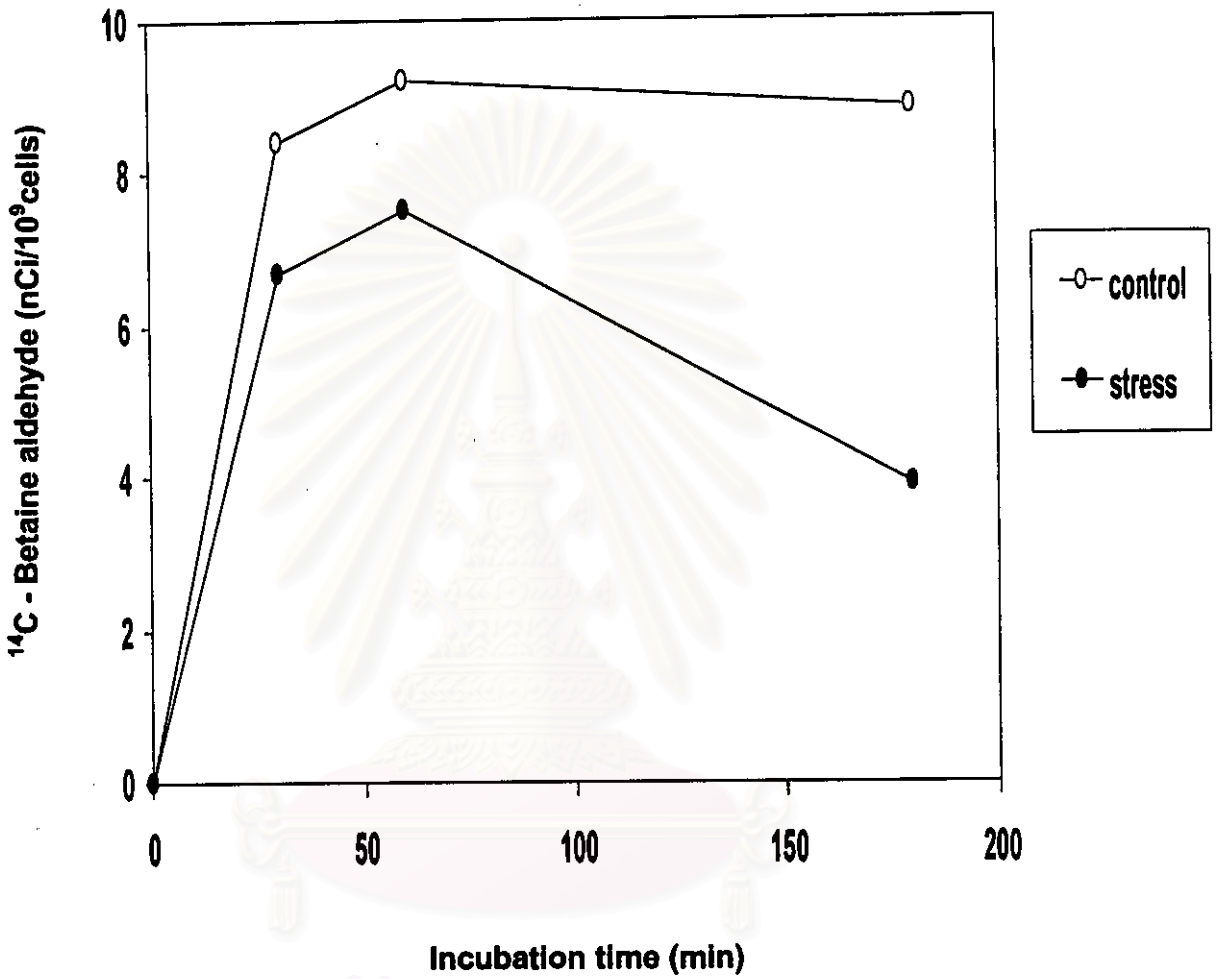

Figure 9 Formation of [^{14}C] betaine aldehyde from [^{14}C]- choline

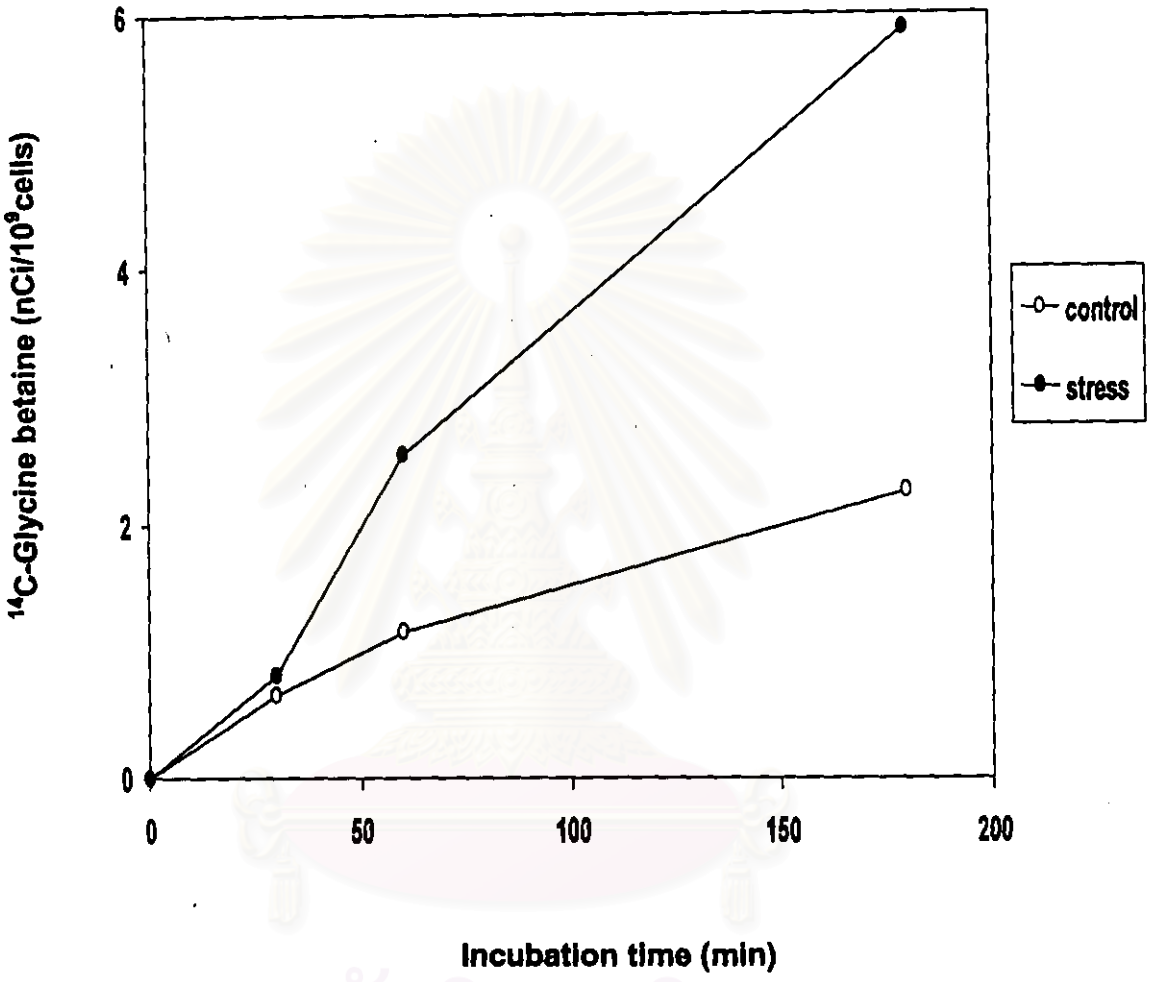

Figure 10 Formation of [^{14}C] glycine betaine from [^{14}C]- choline

4. Formation of [^{14}C] glycine betaine from various precursors

When [^{14}C] choline, [^{14}C] ethanolamine, and [^{14}C] glycine precursors were added to *A. halophytica* (control and stressed cells), glycine betaine was found to be labeled by methods described in section 4 (Table4). In control cells, the rate of [^{14}C] glycine betaine biosynthesis was low throughout the entire incubation period as compared to stressed cells (Figures 10,11, 12). For stressed cells, [^{14}C] glycine betaine biosynthesis from [^{14}C] choline was dependent on incubation time and no saturation was observed during 180 min. On the other hand, when either [^{14}C] ethanolamine or [^{14}C] glycine was a precursor [^{14}C] glycine betaine biosynthesis seemed to be saturated after 60 min.

Table 4 Time course for the conversion of various [^{14}C] precursors to [^{14}C]- glycine betaine in *A. halophytica*.

Labeling time (min)	[^{14}C] glycine betaine (nCi/10^{9}cells)					
	[^{14}C] choline precursor		[^{14}C] ethanolamine precursor		[^{14}C] glycine precursor	
	control	stress	control	stress	control	stress
0	0	0	0	0	0	0
30	0.66	0.80	0.13	1.18	1.35	6.41
60	1.16	2.55	0.28	1.25	0.85	6.54
180	2.26	5.88	0.25	1.32	1.57	7.39

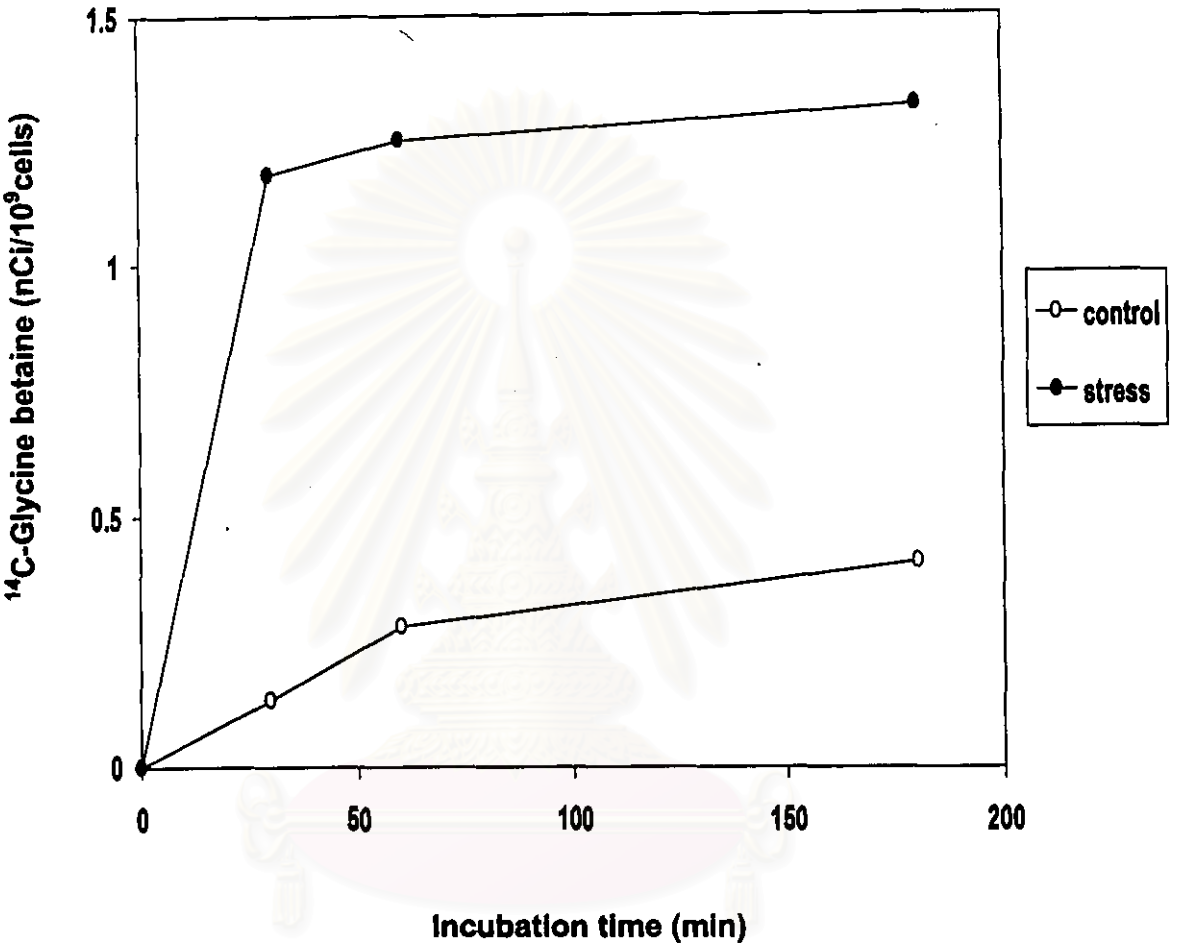

Figure 11 Formation of [^{14}C] glycine betaine from [^{14}C]- ethanolamine

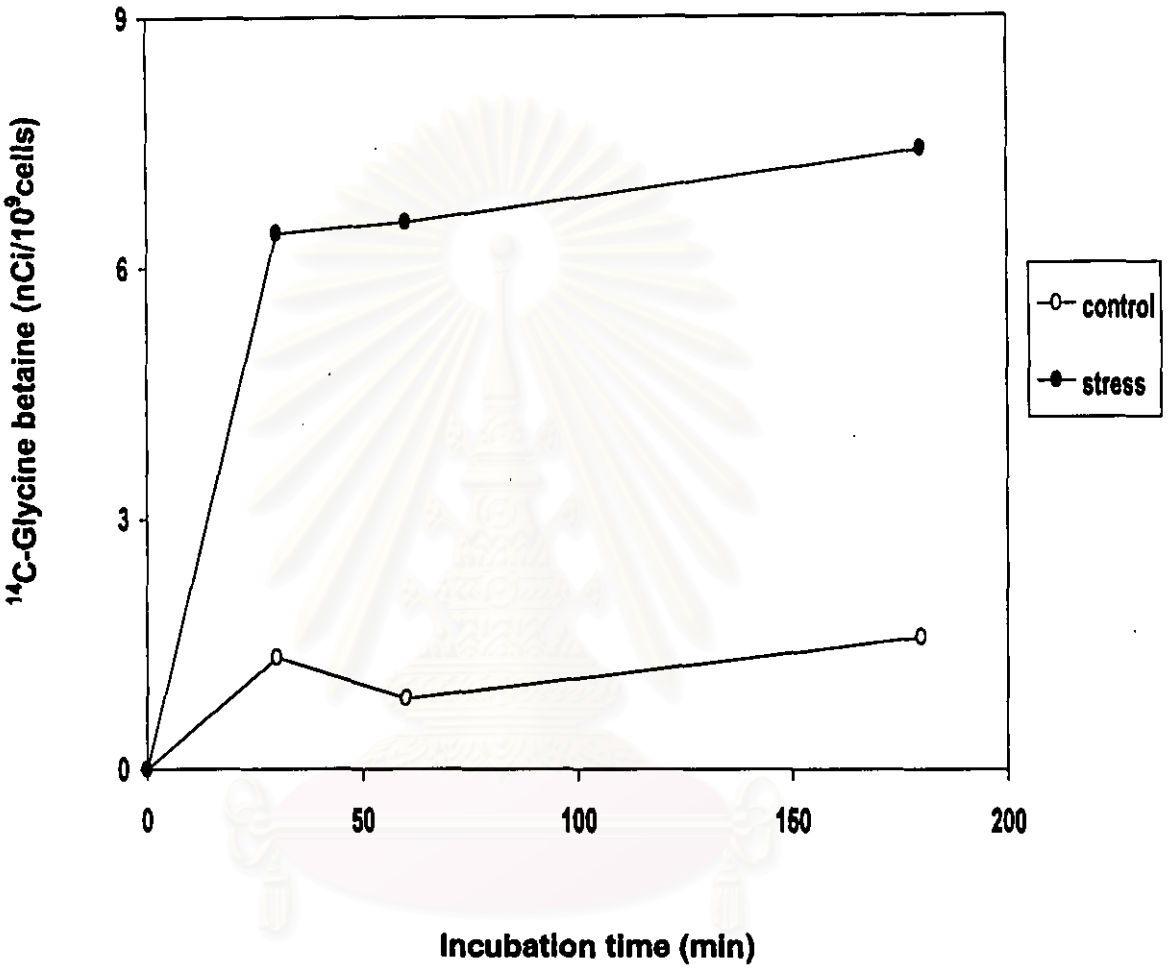

Figure 12 Formation of [^{14}C] glycine betaine from [^{14}C]- glycine

5. Effect of salt stress on [^{14}C] glycine betaine biosynthesis and glycine betaine accumulation

The growth of *A. halophytica* was studied by measuring the changes in cell number after transfer of the cells from 0.5 M NaCl to 2.0 M NaCl (Figure 13). The growth rate of *A. halophytica* in medium containing 2.0 M NaCl was similar to that in 0.5 M NaCl. However, a lag period of 2 days occurred in salt-stressed cells. Figure 14 shows the accumulation of glycine betaine in *A. halophytica* under control and stress conditions from methods in section 5. Under salt stress, intracellular glycine betaine increased with increasing time of stress, whereas the levels of glycine betaine increased very little in the control medium (0.5 M NaCl). It was noticeable that a slight decrease of glycine betaine appeared during day 4 to day 6 period.

Table 5 Effect of salt stress on the accumulation of glycine betaine and [^{14}C] glycine betaine biosynthesis in *A. halophytica*.

Culture time (days)	Cell density ($x10^9$ cell.ml^{-1})		Accumulation of glycine betaine (nmol/10^6 cells)		Biosynthesis of [^{14}C]-glycine betaine in 3 h (nCi/10^9cells)	
	control	stress	control	stress	control	stress
0	0.46	0.46	3.42	3.42	0.32	0.32
2	0.67	0.40	9.06	29.41	0.28	0.64
4	1.07	0.91	20.95	48.05	0.34	2.65
6	1.72	1.41	15.65	60.36	0.52	3.11

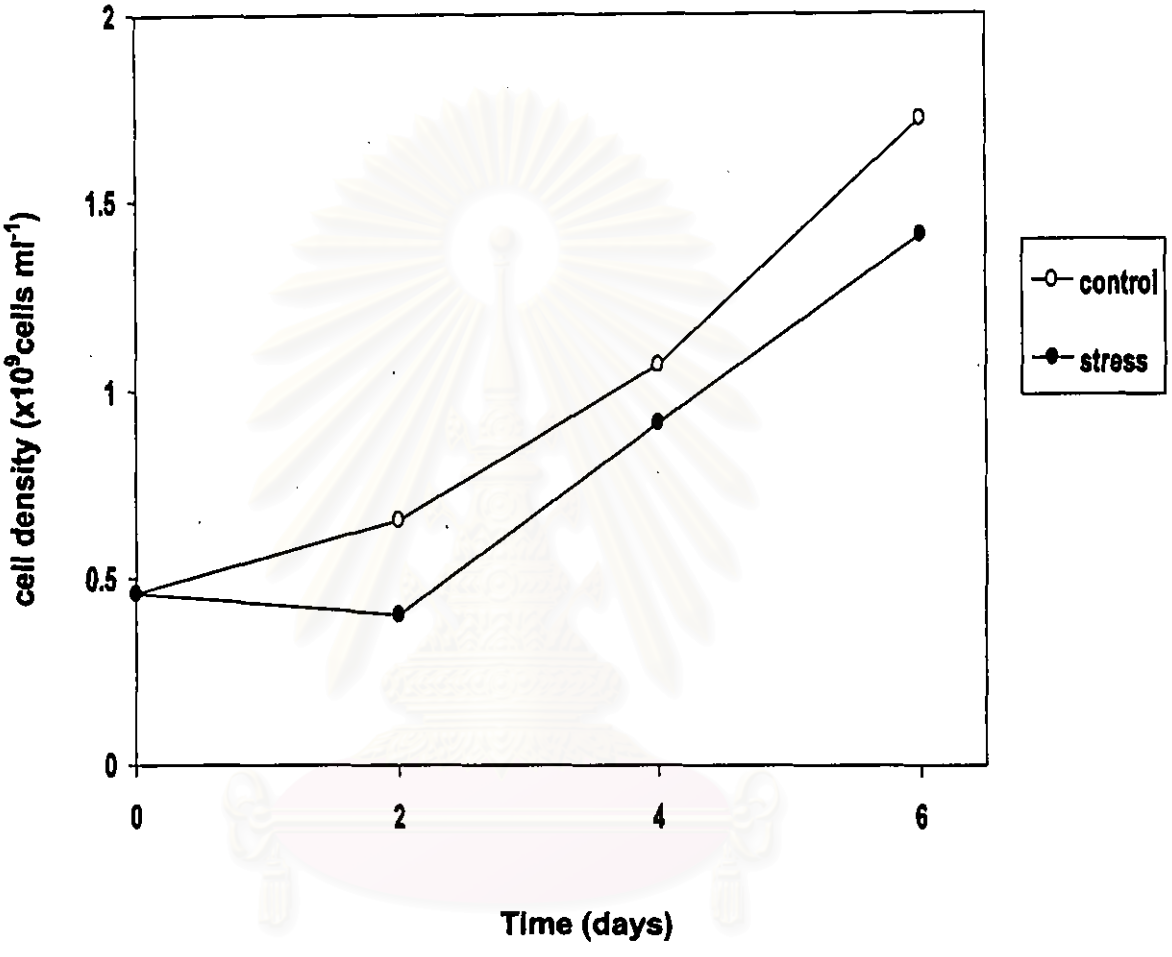

Figure 13 Growth of *A.halophytica* under salt stress

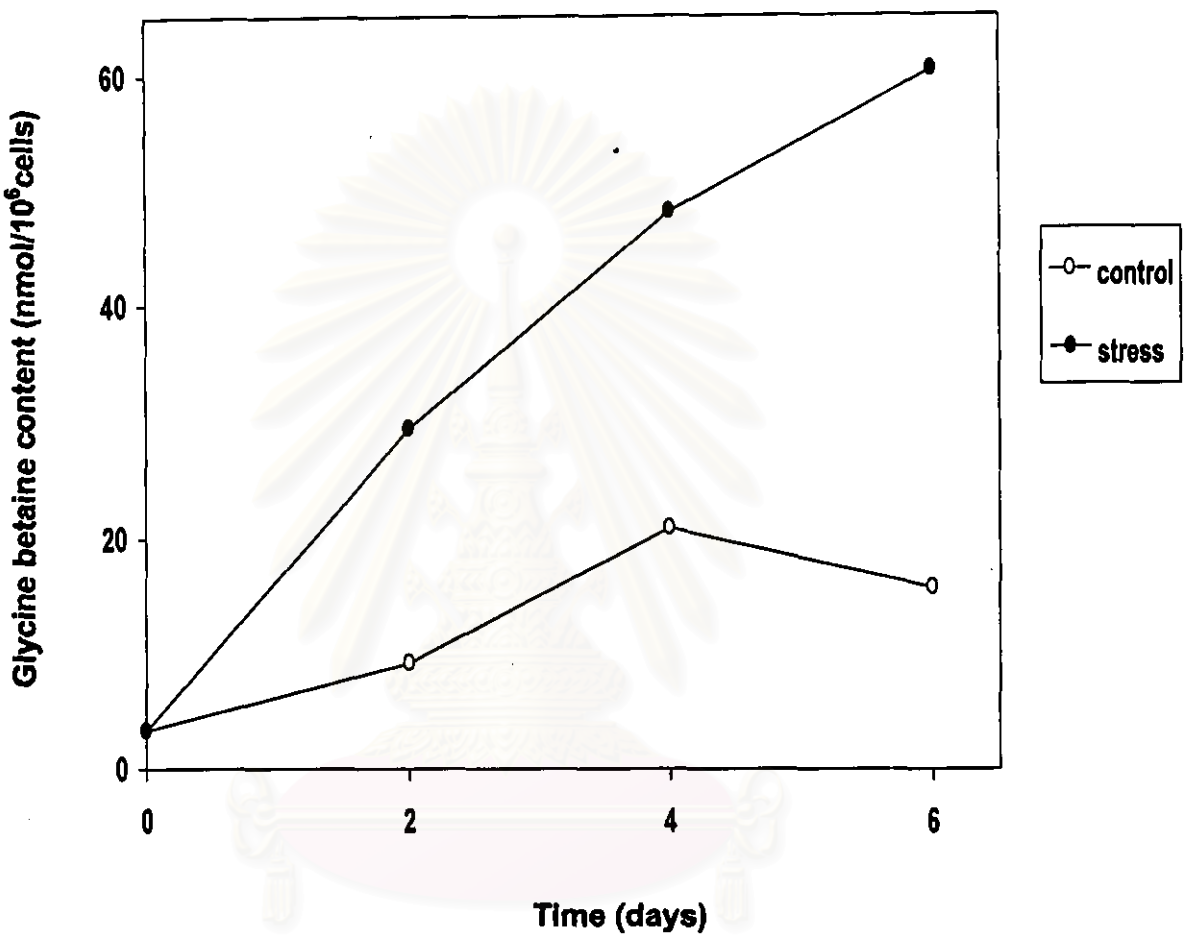

Figure 14 Effect of salt stress on glycine betaine accumulation

Uptake of choline by *A. halophytica* under low and high salinities was studied, using [^{14}C] choline. When the osmolarity of the growth medium was raised by the addition of NaCl (2.0M) an increase in choline uptake was observed in salt stressed cells whereas in control cells very little choline uptake occurred (Figure15).

The effect of salt stress on the biosynthesis of [^{14}C] glycine betaine was studied by incubating control or salt-stressed cells grown at various times with [^{14}C] choline for 3 h. Figure 16 shows that [^{14}C] glycine betaine biosynthesis increased during growth under salt stress with a drastic increase during day 2 and day 4 of growth. Control cells did not seem to increase [^{14}C] glycine betaine biosynthesis although slight increase of the biosynthesis occurred during day 4 and day 6 of growth.

6. Effect of salinity on choline dehydrogenase activity in membrane and cytoplasmic fractions

From methods in sections 6,7, and 8, choline dehydrogenase, which catalyzes the oxidation of choline to betaine aldehyde, was distributed mainly in membrane fraction of *A. halophytica* regardless of salinity in the growth medium (Figure 17). NaCl, up to a concentration of 2.0 M, stimulated choline dehydrogenase in both membrane and cytoplasmic fractions.

The choline dehydrogenase activity staining by methods in section 9 in 7.5% native gel electrophoresis showed detectable bands for choline dehydrogenase of *A. halophytica* in both membranous and cytoplasmic fractions (Figures 18, 19)

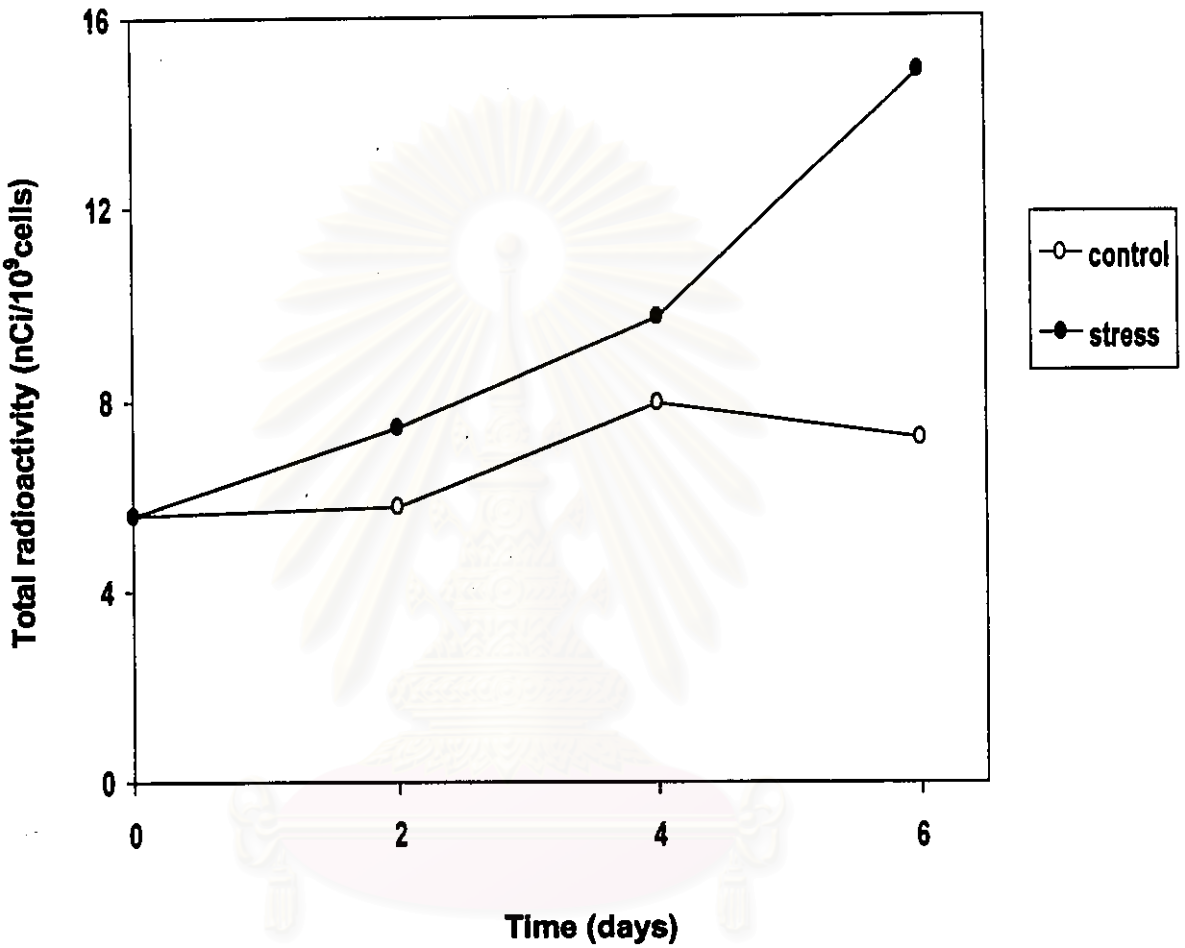

Figure 15 Uptake of [^{14}C] choline by control and salt-stressed *A. halophytica* grown for various times. The reaction time for the uptake was 3 h.

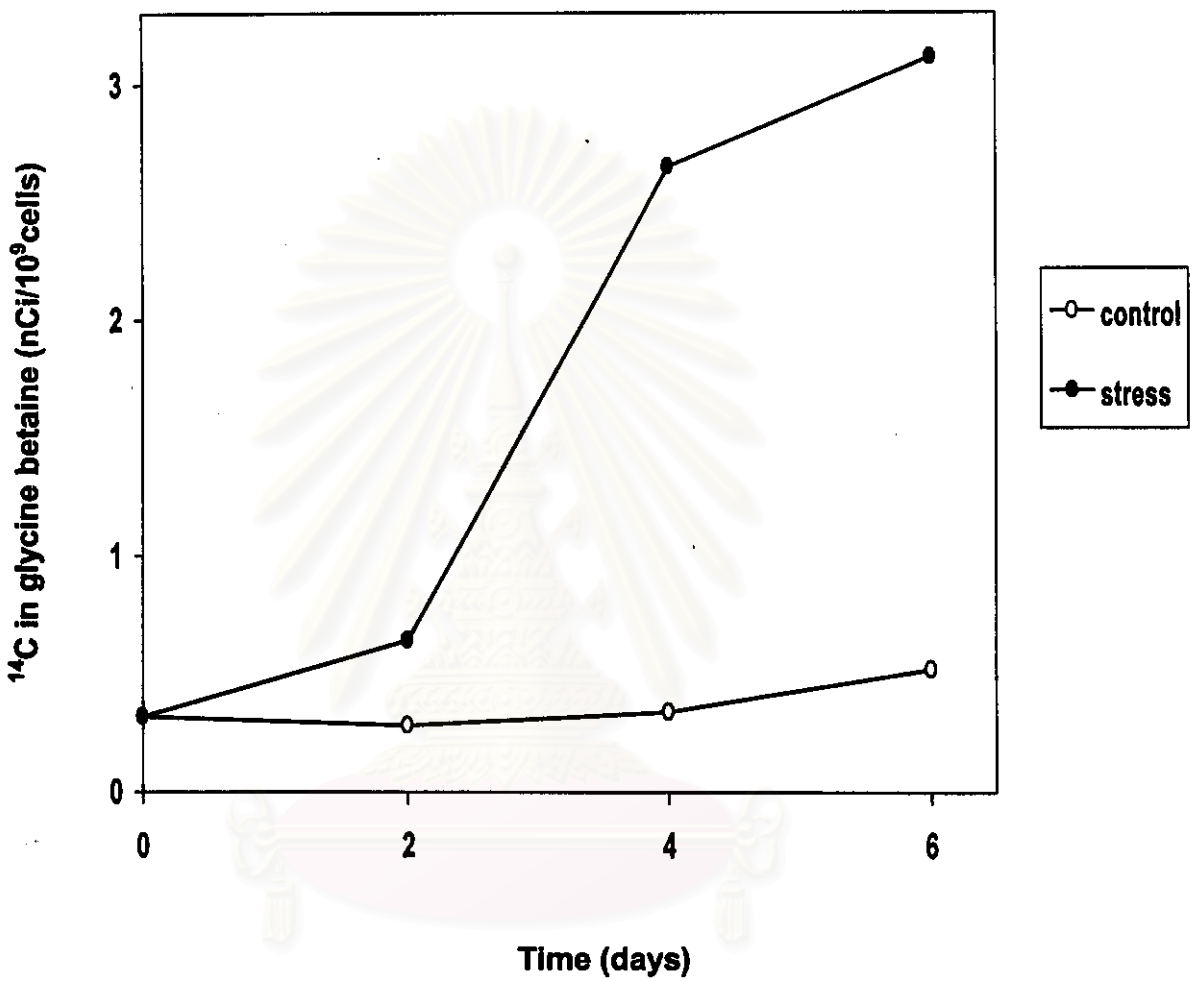

Figure 16 Effect of duration of salt stress on [^{14}C] glycine betaine biosynthesis. The reaction time for the synthesis was 3 h.

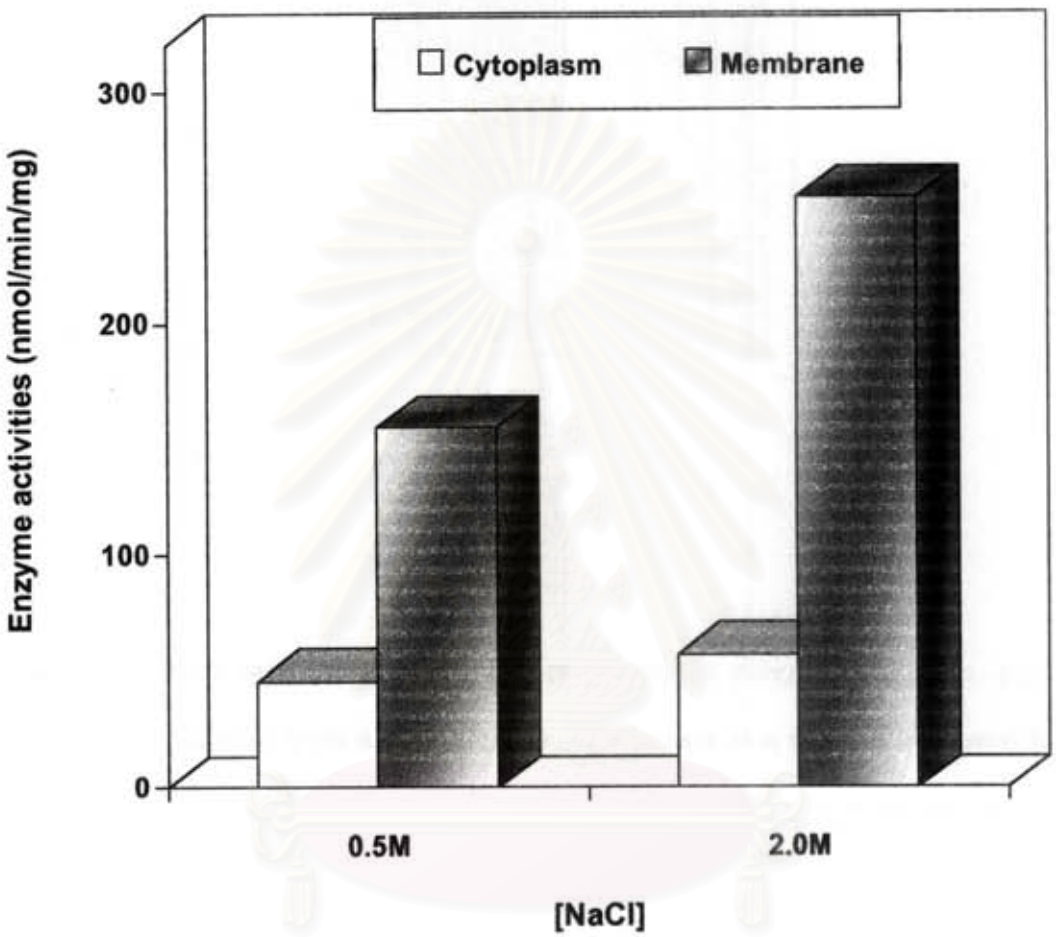

Figure 17 CDH activity of *A. halophytica* extracted from cytoplasm and membrane in low and high salinities

Figure 18 Non-denaturing PAGE pattern of choline dehydrogenase in membrane fraction from *A. halophytica*. Coomassie blue staining was used to detect protein (A). The native protein was stained for enzyme activity (B).

Lane 1: crude spinach leaves extract	30 μg
4: crude spinach leaves extract	100 μg
2,5: crude extract from membrane fraction in 0.5 M NaCl	30 μg
3,6: crude extract from membrane fraction in 2.0 M NaCl	30 μg

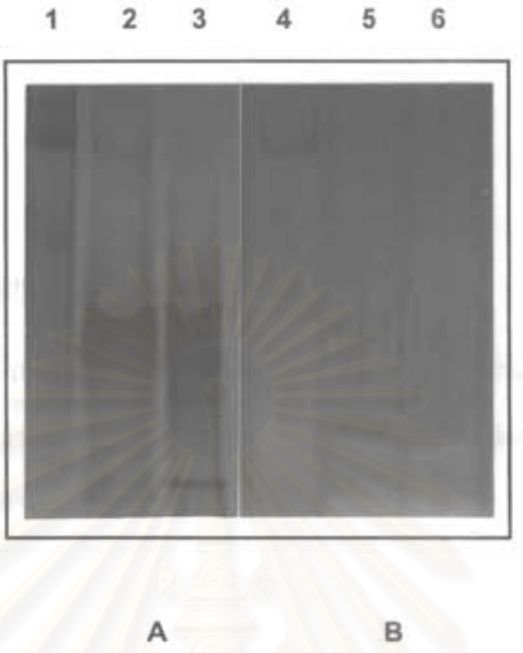

Figure 19 Non-denaturing PAGE pattern of choline dehydrogenase in cytoplasmic fraction from *A. halophytica.* Coomassie blue staining was used to detect protein (A). The native protein was stained for enzyme activity (B).

Lane 1:	crude spinach leaves extract	30 µg
4:	crude spinach leaves extract	100 µg
2:	crude extract from cytoplasmic fraction in 0.5 M NaCl	30 µg
5:	crude extract from cytoplasmic fraction in 0.5 M NaCl	100 µg
3:	crude extract from cytoplasmic fraction in 2.0 M NaCl	30 µg
6:	crude extract from cytoplasmic fraction in 2.0 M NaCl	100 µg