CHAPTER III

RESULTS

3.1 Isolation of *A. halophytica* chromosomal DNA

The chromosomal DNA of *A. halophytica* was prepared by the protocol described in section 2.7. The DNA was shown as a high molecular weight band of greater than 23.1 kb when analyzed with agarose gel electrophoresis as shown in Fig. 3.1. The A_{260}/A_{280} ratio was higher than 1.8 indicating high purity of DNA.

3.2 Construction of the *A. halophytica* chromosomal DNA library

In order to construct a chromosomal DNA library, the *A. halophytica* chromosomal DNA was subjected to *Sau*3AI partial digestion. One half microgram of *A. halophytica* chromosomal DNA was partially digested with 1 unit of *Sau*3AI at 37°C for 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes. Agarose gel electrophoresis analysis of the digests as shown in Fig. 3.2 indicated that the 10 minute digestion in lane 3 gave smeary pattern with more or less equal intensity within the range of 3-6 kb. The 10 minutes digestion was chosen for the scale-up digestion. The smeary DNA bands between 3-6 kb were eluted from the agarose gel by electroelution. The yield of recovered DNA was estimated to be 100 ng/μl and used for DNA library construction. *A. halophytica* chromosomal DNA library was constructed by ligation of the 3-6 kb of *A. halophytica* DNA fragment into *Bam*HI site of pUC18 vector. The recombinant plasmids were transformed into *E. coli* JM109 by electroporation. The ampicillin resistance transformants were tested for the presence of *bet* genes as described in the following section.

Figure 3. 1 Chromosomal DNA preparation of *A. halophytica*

Lane 1 λ/*Hin*dIII

Lane 2 *A. halophytica* chromosomal DNA

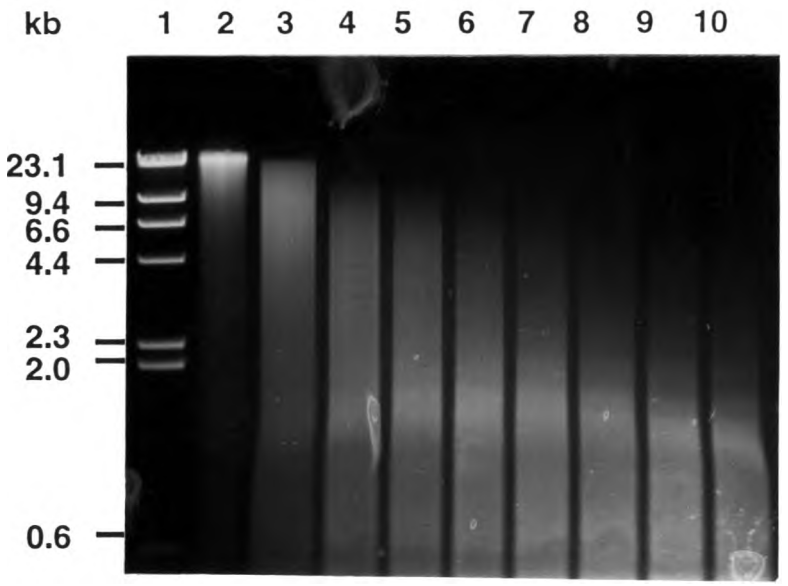

Figure 3.2 Analysis of partially digested of *A. halophytica* chromosomal DNA with *Sau*3AI on 0.7% agarose gel electrophoresis.

Lane 1 λ/*Hin*dIII

Lane 2-10 Chromosomal DNA partially digested with *Sau3AI* for 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes, respectively.

3.3 Effect of NaCl concentrations on the growth of *E. coli* JM109

The *bet* deletion mutant, *E. coli* JM109, was used as the host for transformation and bet phenotypic selection. To determine the NaCl concentration suitable for the phenotypic test, M63 medium containing 22 mM glucose was used as the minimal growth medium. *E. coli* JM109 was grown in M63 medium containing 0, 0.4, 0.6, 0.8, 1.0, and 1.2 M NaCl. Growth was monitored by measuring the optical density at 420 nm. The increase in the NaCl concentration of the medium resulted in the decrease in the growth rate of the cells. The concentrations of salt at 1.0 and 1.2 M NaCl, were shown to strongly inhibit growth. Lower NaCl concentrations showed some inhibitory effect on the growth rate (Fig. 3.3). To test whether the high salt concentrations have the same effect on the growth of *E. coli* JM109 cells on M63 agar plates, *E. coli* JM109 were spread on M63 agar containing 0.7, 0.75 and 0.8 M NaCl and incubated at 37°C. The *E. coli* JM109 colonies of 1 mm in diameter could be seen on day 3, 4 and 5 in the M63 agar plates containing 0.7, 0.75 and 0.8 M NaCl, respectively (data not shown). Since the salt concentration at 0.7 M NaCl did not dramatically suppress the growth rate of the cells, it was chosen for further study.

3.4 Effect of exogenous choline, betaine aldehyde and glycine betaine on the growth of *E. coli* JM109

It had been shown in many reports that choline, betaine aldehyde and glycine betaine were able to protect the suppressive effect of salt on growth rate of the cells (Styrvold et al., 1986; Andresen et al., 1988; Boch et al., 1996). To analyze the osmoprotective effect of exogenous choline, betaine aldehyde and glycine betaine on the growth of *E. coli* JM109, 1 mM each of these osmoprotectants was added to the M63 medium containing 0.7 M NaCl. It was found that the presence of glycine betaine strongly relieved the growth from the inhibitory effect of high salt

concentration on growth (Fig. 3.4). Choline and betaine aldehyde had no osmoprotective effect when compared with the control. Choline and betaine aldehyde function as the precursors of glycine betaine.

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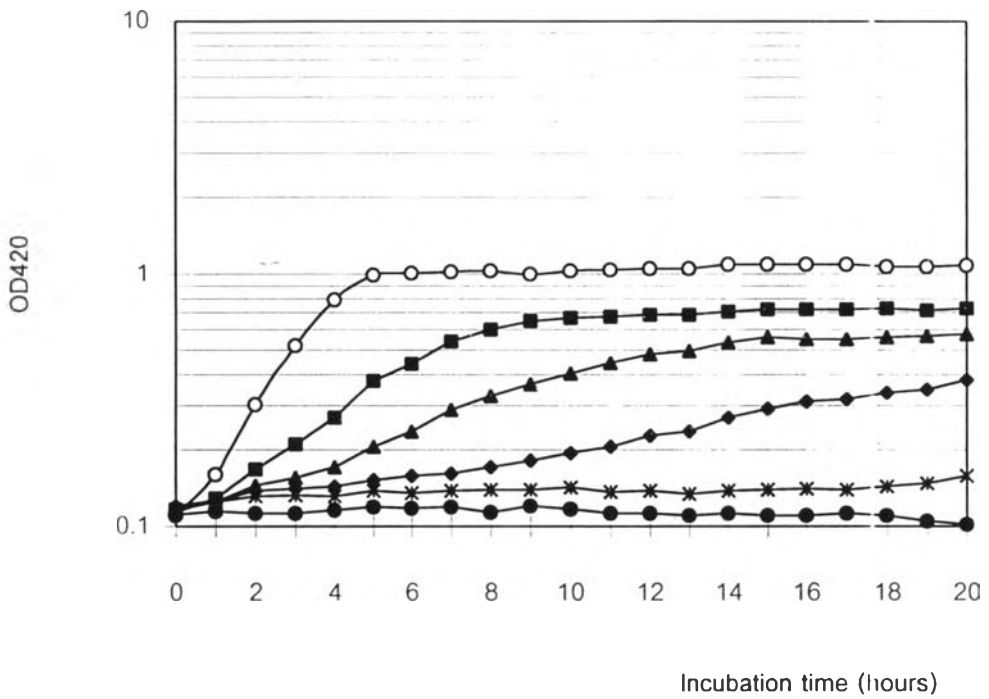

Figure 3.3 Effect of various NaCl concentration on the growth of *E. coli* JM109. Cells were grown at 37°C in the M63 medium (—⊖—) and M63 medium containing the NaCl concentration at 0.4 M (—■—), 0.6 M (—▲—), 0.8 M (—◆—), 1.0 M (—✱—) and 1.2 M (—●—).

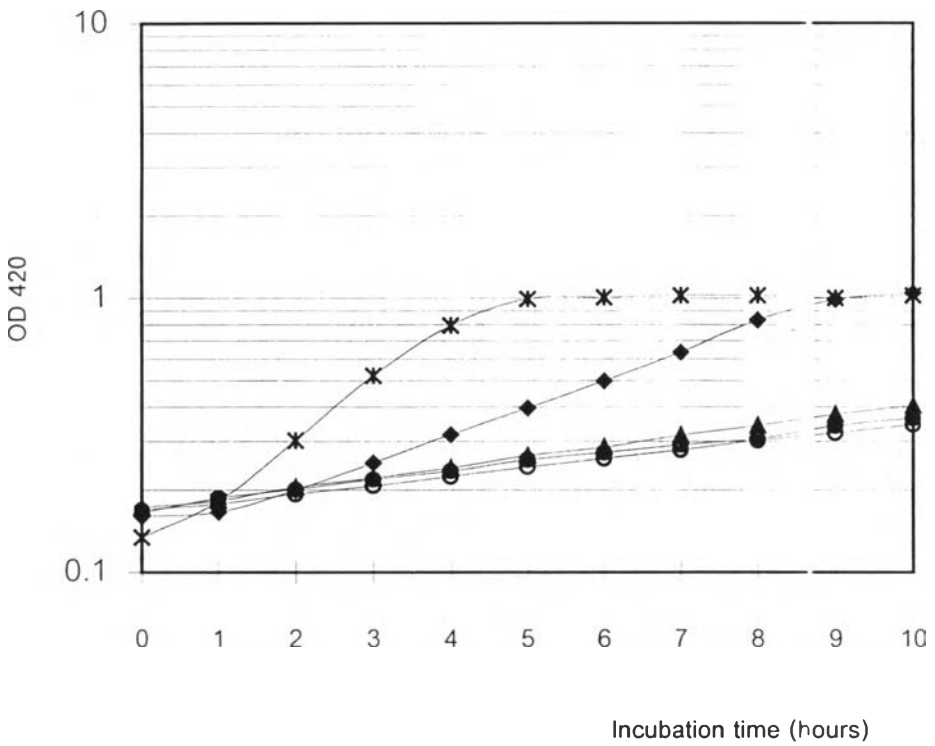

Figure 3.4 Effect of exogenous choline, betaine aldehyde and glycine betaine on the growth of *E. coli* JM109. Cells were grown at 37°C in the M63 medium (✱) and M63 medium containing 0.7 M NaCl in the absence of osmoprotectant (○), in the presence of 1 mM choline (●), betaine aldehyde (▲) or glycine betaine (◆).

3.5 Bet phenotypic selection

In *E. coli* wild type strain, choline and betaine aldehyde were oxidized to glycine betaine which enabled the cells to become salt tolerant. If plasmid with *bet* gene was provided, the cells might be able to overcome the suppressive effect of salt. *E. coli* JM109 cells were used as host in the construction of the *A. halophytica* chromosomal DNA library. The deletion of the *bet* genes from *E. coli* JM109 chromosome completely prevented the oxidation of choline and betaine aldehyde to glycine betaine. The transformants were selected for the bet phenotype on the M63 agar containing 0.7 M NaCl. 1 mM choline or betaine aldehyde was added to the salted agar as the precursor of glycine betaine. The transformants that harbored the *bet* genes should be able to convert choline or betaine aldehyde to glycine betaine and should be able to grow on this medium. Unfortunately, none of the *bet* transformant could be found.

3.6 Colony hybridization

The failure to recover the *A. halophytica* gene encoding the glycine betaine synthesis enzyme by phenotypic selection led to the search for an alternative method. Instead of selecting for *bet* containing transformant that was able to convert choline or betaine aldehyde to the osmoprotectant glycine betaine, the colony hybridization technique was attempted.

3.6.1 The design of oligonucleotide probes

The oligonucleotide probes were used for colony hybridization. The sequences of the oligonucleotide probes were designed from the published sequence of *betB* related gene in various bacteria, *E. coli*, *B. subtilis* and *R. meliloti*, and plants;

S. bicolor, *H. vulgare*, *A. hortensis*, *S. oleracea*, *B. vulgaris* and *A. hypochondriacus*. The sequences were aligned by using a ClustalX(1.64b) program. The sequence of the genes coded for betaine aldehyde dehydrogenase program as shown in Fig. 3.5. The highly homologous sequences were selected. The two oligonucleotides were designed and called no. 4402 and 4403, respectively. The sequences of probe no. 4402 and 4403 were 5′TGGAACTTGGCGGTAAAA 3′ and 5′ GC CCCTGCGCTGG CCGCTGG 3′, respectively. The oligonucleotide probes were chemically synthesized and end-labeled with $[\gamma\text{-}^{32}P]$ATP using T_4 polynucleotide kinase.

3.6.2 Screening of *betB* gene by colony hybridization

After ligation of the 3-6 kb DNA fragments into *Bam*HI site of pUC18, the recombinant plasmids were transformed into *E. coli* JM109 by electroporation. The transformants were selected on LB agar containing 100 µg/ml ampicillin. The colonies were transferred to new plates by using steriled toothpicks. The plates were incubated until the colonies were approximately 1 mm in diameter. The colonies were blotted onto the filter papers and hybridized with the radioactive labeled probe no. 4402 at 30°C overnight. The initial washing step was performed at 37°C. The stringency of hybridization was increased by raising the washing temperature until the radioactivity of the control colonies, *E. coli* harboring pUC18, were low (Fig. 3.6). The 53 colonies that gave hybridization signal were picked up from the master plates and grew in LB medium. The recombinant plasmids were extracted and digested with restriction enzymes, *Eco*RI and *Hin*dIII, to cleave the DNA insert. The digests were analyzed by agarose gel electrophoresis and transferred to nylon membranes by Southern blotting. The membranes were hybridized with the radiolabeled probe no. 4402 at 30°C overnight and washed at 37°C. Unfortunately, autoradiographs did not show any signals of hybridization (data not shown). To test the ability of

hybridization of the oligonucleotide probes, *A. halophytica* chromosomal DNA was run on 0.7% agarose gel and transferred to nylon membrane by Southern blotting and hybridized with radioactive labeled probe no. 4402 or 4403 at 30°C overnight and washed at 37°C. No hybridization signal was observed on the film. Therefore it was concluded that the oligonucleotide probes no. 4402 and 4403 could not be used to select the *betB* containing colony and the control.

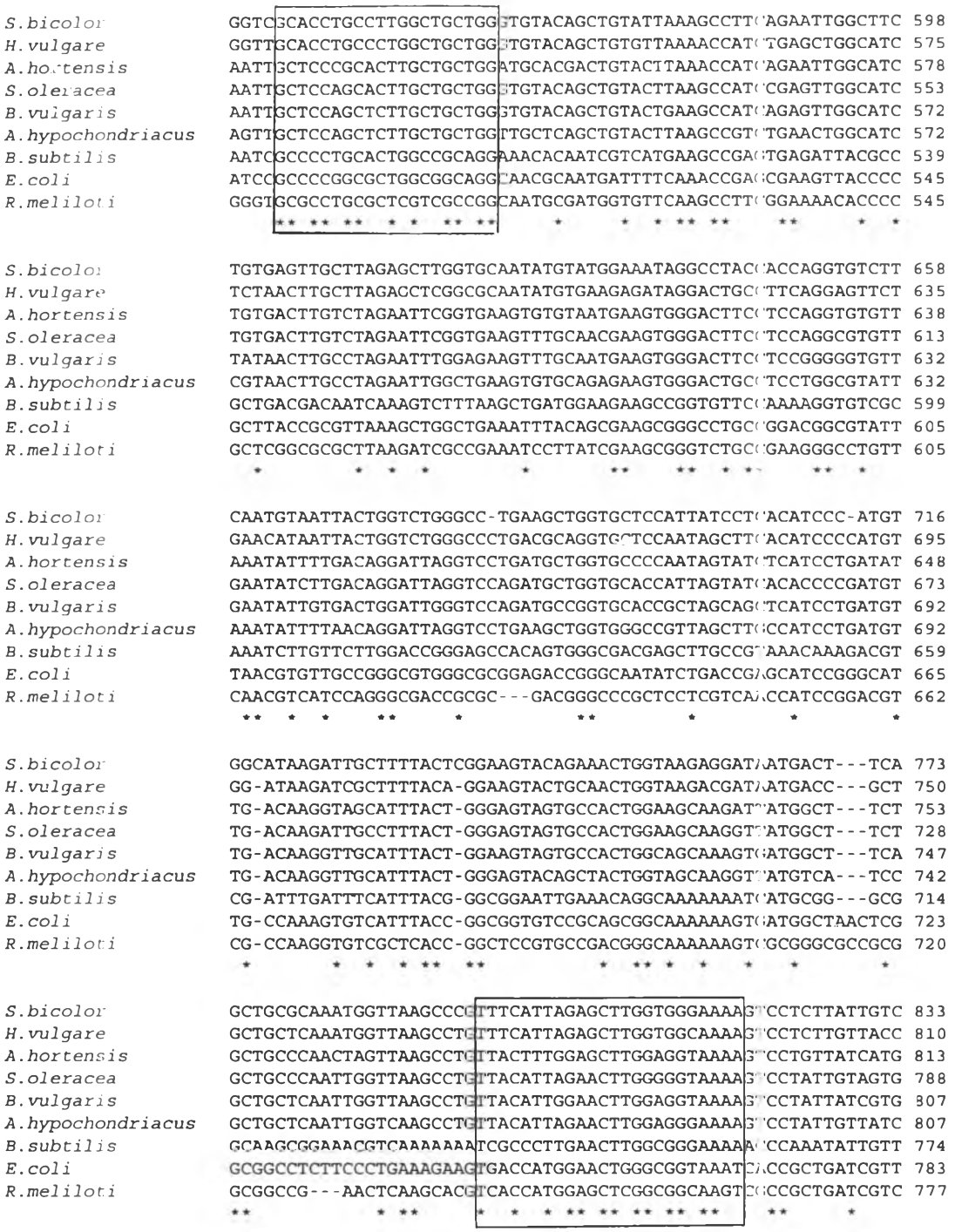

Figure 3.5 Sequence comparison of the genes coded for betaine aldehyde dehydrogenase from bacteria; *E. coli*, *B. subtilis and R. meliloti*, and plants ; *S. bicolor*, *H. vulgare*, *A. hortensis*, *S. oleracea*, *B. vulgaris* and *A. hypochondriacus*. Identical nucleotides in all organisms are marked by asterisks. Homologous sequences, used to design the synthetic oligonucleotides, were surrounded by rectangles. The numbers indicate positions in nucleotide sequences.

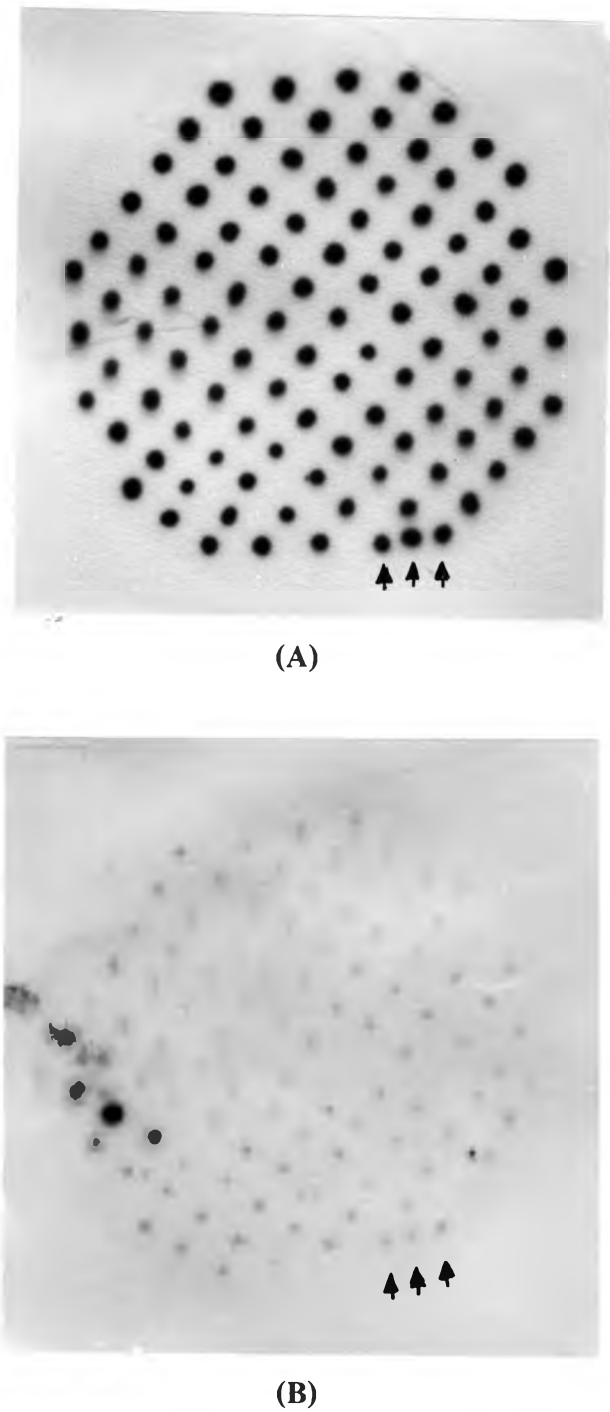

Figure 3.6 Examples of colony hybridization screening with the $[\gamma\text{-}^{32}P]$ATP labeled probe no. 4402 after washing at 45°C (A) and 52°C (B). Arrows indicated the control colonies, *E. coli* harboring pUC18.

3.7 Southern blot hybridization using *gbsA* probe

In an attempt to clone the *betB* gene from *A. halophytica*, the third alternative method, Southern blot hybridization, was used. The hybridization probe was derived from *gbsA* gene, a *betB* related gene from *B. subtilis* which was kindly provided by Dr. E. Bremer.

3.7.1 Preparation of *gbsA* probe

The recombinant plasmid, pJB007, harboring the *gbsAB* gene of *B. subtilis* was used. The *gbsA* codes for betaine aldehyde dehydrogenase and the *gbsB* codes for choline dehydrogenase. The pJB007 is a pSC101-based plasmid which is a low-copy number plasmid. The pJB007 was transformed into *E. coli* DH5α by electroporation. The plasmids were prepared by using an alkaline method. Digestion of pJB007 with 2 restriction enzymes, *Nde*I and *Pst*I gave 0.92 kb *gbsA* gene. The fragment was eluted from the agarose gel by using QUIquick gel extraction kit. The recovery was 80% as shown in Fig. 3.7. The 0.92 kb fragment was labeled with [α-^{32}P]dATP by nick translation.

3.7.2 Southern blot hybridization analysis

Four micrograms of *A. halophytica* chromosomal DNA was digested separately with 5 restriction endonucleases, *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Xba*I, and run on 0.7% agarose gel along with λ/*Hin*dIII marker (Fig 3.8). The DNA fragments were transferred from agarose gel onto nylon membrane. The *betB* gene in the chromosomal DNA of *A. halophytica* was traced by using *gbsA* probe. The results of hybridization showed that each of the *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I digested *A. halophytica* chromosomal DNA gave 1-2 hybridization bands (Fig. 3.9). The sizes of

the hybridization bands were approximately 9.4 kb and larger than 23.1 kb. These results implied that the *betB* gene existed in the *A. halophytica* chromosomal DNA.

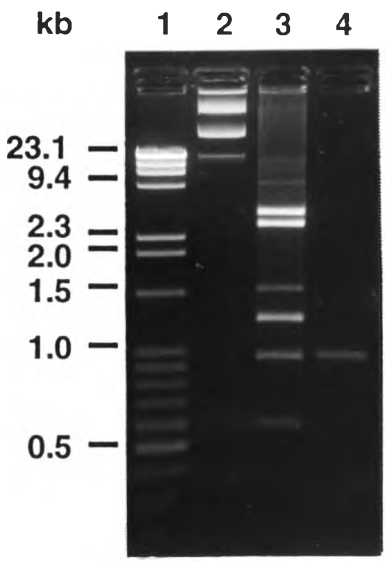

Figure 3. 7 Preparation of 0.92 kb of *gbsA* gene from the digestion of pJB007 with *Nde*I and *Pst*I

Lane 1	λ/*Hin*dIII and 100 bp marker
Lane 2	Uncut pJB007
Lane 3	pJB007 digested with *Nde*I and *Pst*I
Lane 4	Eluted 0.92 kb fragment containing *gbsA* gene

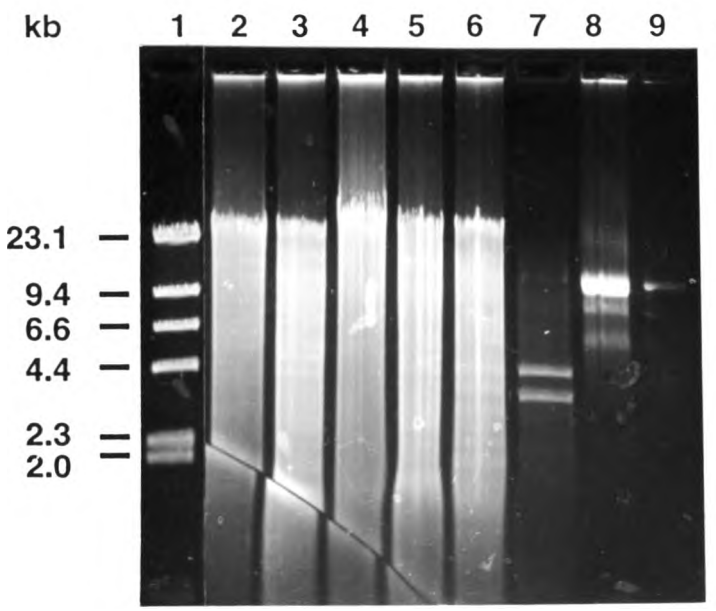

Figure 3.8 Digestion of *A. halophytica* chromosomal DNA with 5 restriction endonucleases

Lane 1	λ/*Hin*dIII
Lane 2	DNA digested with *Bam*HI
Lane 3	DNA digested with *Eco*RI
Lane 4	DNA digested with *Hin*dIII
Lane 5	DNA digested with *Pst*I
Lane 6	DNA digested with *Xba*I
Lane 7	pJB007 digested with *Hin*dIII
Lane 8	pJB007 partailly digested with *Nde*I
Lane 9	uncut pJB007

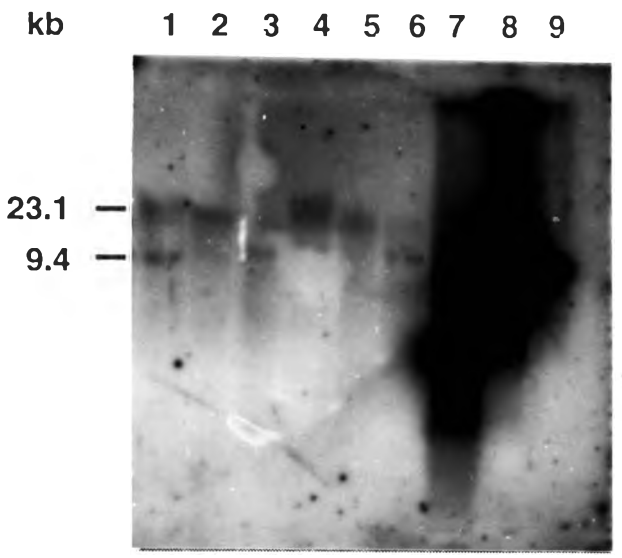

Figure 3.9 Southern-blot hybridization analysis of digested *A. halophytica* chromosomal DNA with *gbsA* probe

Lane 1	λ/*Hin*dIII
Lane 2	DNA digested with *Bam*HI
Lane 3	DNA digested with *Eco*RI
Lane 4	DNA digested with *Hin*dIII
Lane 5	DNA digested with *Pst*I
Lane 6	DNA digested with *Xba*I
Lane 7	pJB007 digested with *Hin*dIII
Lane 8	pJB007 partially digested with *Nde*I
Lane 9	uncut pJB007