# CHAPTER IV
# RESULTS

## 4.1 Production of *D. salina* using simple media for the substitution of original J/1 medium

Efficiency of protein production through the processes of gene expression in green algae required the system of both algae cell proliferation and gene expression

In the first part of the study, the media for *D. salina* production was investigated. The improve media for production at low cost for the substitution of original expensive media using an affordable formular based on chemical fertilizer was carried out.

JR media containing equivalent nutrients calibrated based on major chemical components to that of original J/1 using fertilizer was created and prepared. With an aim to determine its proper concentration for use in *D. salina* production, appropiate level of concentration of media was determined.

Among 5 conditions tested (concentration of media at 0.25, 0.5, 1, 2.5 and 5X), it was shown in figure 4.1 that at the 0.25X concentration, cells of *D. salina* were rapidly multiplied and could be detected at first 10 days. This concentration provided highest cell accumulation among the concentration tested. The increasing number of cells of *D. salina* was also observed even further to 30 days. The rest of fomulars including 0.5X 1X and 2.5X provided results of slower growth with delay rate approximately 5 days than the initials. No results of growth could be observed when the concentation of media was increased to 5X.

The 0.25X formular promoted fastest growth among treatments focused significantly. The 5x formular showed an obvious decrease in cell proliteration resulting in cell death. This suggested that 0.25X JR was more effective for rapid growth induction with higher in yield in term of cells numbers.

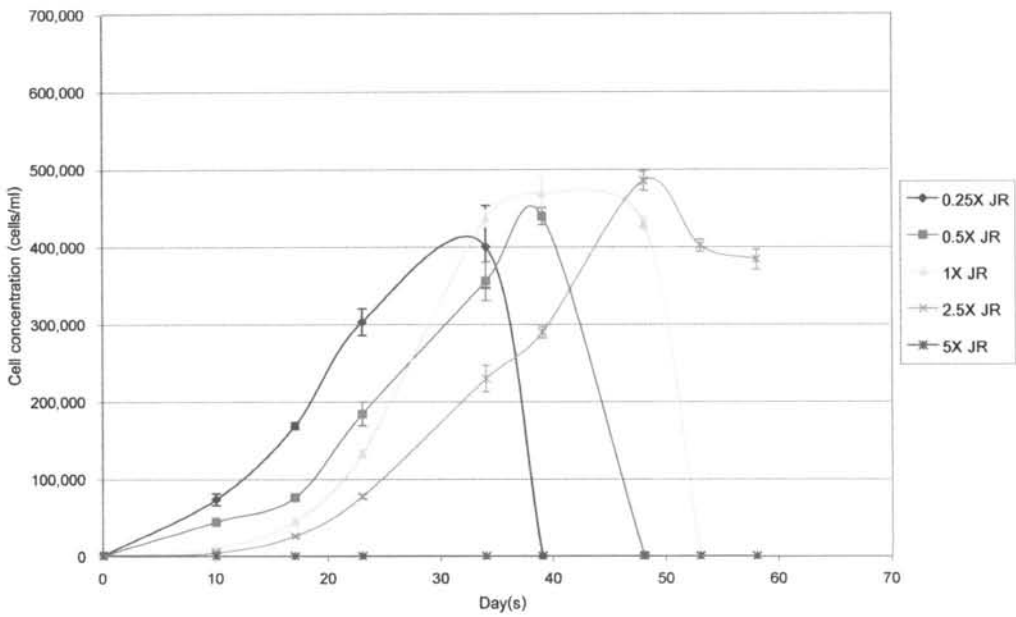



**Figure 4.1** Growth rate of *D. salina* in various concentrations of JR media

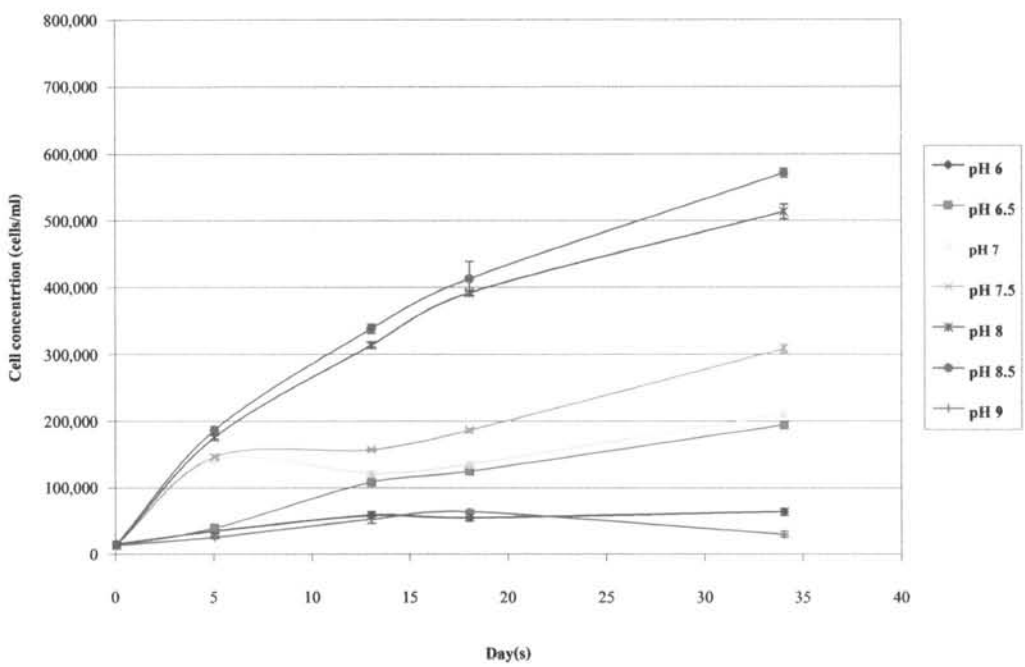



**Figure 4.2** Growth rate of *D. salina* in various pH

When cells was cultured at the 0.25X concentration, of media formulars having variation in pH conditions ranging from pH 6, 6.5, 7, 7.5 to 8.0 respectively were reviewed for

their effect on growth of *D. salina*. Results revealed that at pH 8.5, JR media supported growth at higher rate than those the rest. JR medium also provide better cell proliferation at pH 8.5 , however when the pH was kept increasing at pH 9.0 the lowest cell proliferation would be reached.

These results indicated that suitable pH for appropriate growth of *D. salina* was at 8.5. This pH will be used for pH arrangement throughout the studies. With these described conditions *D. salina* population would be increase to 45 times within 30 days (figure 4.2).

The changing of media J/1 to 0.25X JR aimed to reduce the chemical cost since major components of the media is based on the substitution of the chemicals with fertilizer used in agriculture applications which was cheaper in their costs and easier to access, whereas minor components are substituted with Unilate (table 4.1).

**Table 4.1** Comparison of costs between J/1 and JR media (personal data)

| **J/1 medium** (Borowitzka, 1988) | | | **JR medium** | | |
|---|---|---|---|---|---|
| Chemicals | Concentration (g/L) | Cost (Baht) | Chemicals | Concentration (g/L) | Cost (Baht) |
| NaCl | 30 | 6 | NaCl | 30 | 0.6 |
| KCl | 0.2 | 0.092 | 16-16-16 | 2.19 | 0.0438 |
| $KNO_3$ | 1 | 0.765 | Fertilizer | | |
| $K_2HPO_4$ | 0.035 | 0.0245 | | | |
| $MgSO_4.7H_2O$ | 0.5 | 0.65 | $MgSO_4.7H_2O$ | 0.5 | 0.65 |
| $CaCl_2.2H_2O$ | 0.2 | 0.127 | $CaCl_2.2H_2O$ | 0.2 | 0.127 |
| $NaHCO_3$ | 0.043 | 0.01376 | Unilate | 0.86 | 0.387 |
| $H_3BO_3$ | 0.0061 | 0.03355 | | | |
| $NH_{4(6)}Mo_7O_{27}.4$ | 0.0038 | 0.0912 | | | |
| $H_2O$ | 0.00244 | 0.366 | | | |
| $FeCl_3.6\ H_2O$ | 0.00006 | 0.0045 | | | |
| $CuSO_4.5\ H_2O$ | 0.000051 | 0.02856 | | | |
| $CoCl_2.6\ H_2O$ | 0.000041 | 0.008528 | | | |
| $ZnCl_2$ | 0.000041 | 0.006396 | | | |
| $MnCl_2.4\ H_2O$ | | | | | |

Estimation of cost was investigated. Changing of media from J/1 to 0.25X JR reduced the chemical cost from 8.21฿/L to 0.45฿/ L , that is, 18 times saved.

**Table 4.2** Comparison on costs between J/1 and JR media calculated based on standard prices.

| J/1 medium (1X) | JR medium (1X) | JR medium (0.25x) |
|---|---|---|
| 8.21Baht/L | 1.8 Baht/L | 0.45 Bath/L |

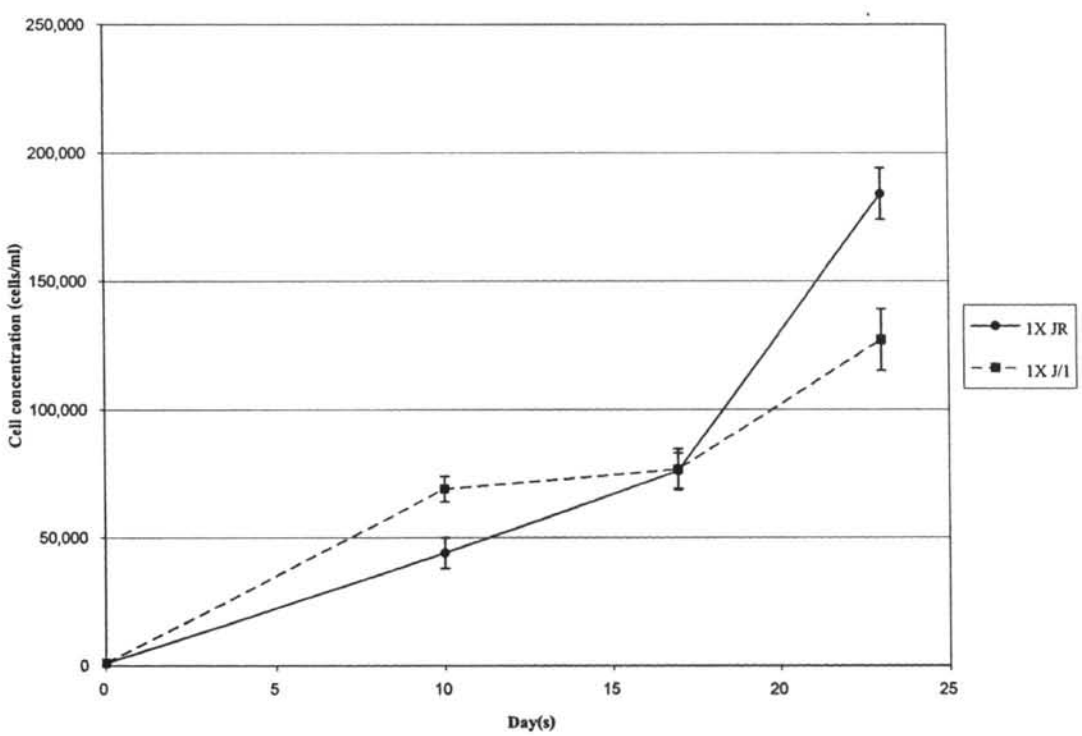



**Figure 4.3** Growth rate of *D. salina* in 1X JR and 1X J/1 media

The 1X JR and 1X J/1 was compared in term of the efficiency of growth promotion with J/1 medium corresponded for higher number of cells than JR in the first 17 days. However, after 17 days, cell number of JR increased over J/1 and reached 1.82 X $10^4$ cells/ml on day 23, whereas in J/1 cell number reached at 1.25X$10^3$ cells/ml (figure 4.3). The

result revealed that JR medium when used at equal concentration was more efficient than J/1 in term of cell proliferation. This supported the use of JR as a substitution for J/1 medium.

### 4.2 Selection condition for herbicide resistance

In order to prepare the system for gene transformation in *D. salina*, three main issues, the screening system, the transformation system and the evaluation system for the target gene expression, had been investigated. For the screening approach, the bar gene system which conferred resistance to herbicide bialaphos was used as resistant marker. First, to determined if *D. salina* could be survived in media supplemented with variation of doses of bialaphose. In detail, these concentrations of herbicide bialaphos ranging from 5 6 7 8 9 10 11 and 12 ppm were investigated using 1 mL of JR media condition mixed with herbicide and *D. salina* cell of $1.5X10^5$ cells. A day after treatment, rapid decrease in *D. salina*'s population as shown in Figure 4.4 was observed. Most of the cells were disrupted or bursted due to the stress of bialaphos (figure 4.5). While control experiment supplemented with no Bialaphos, normal cell proliferation was detected.

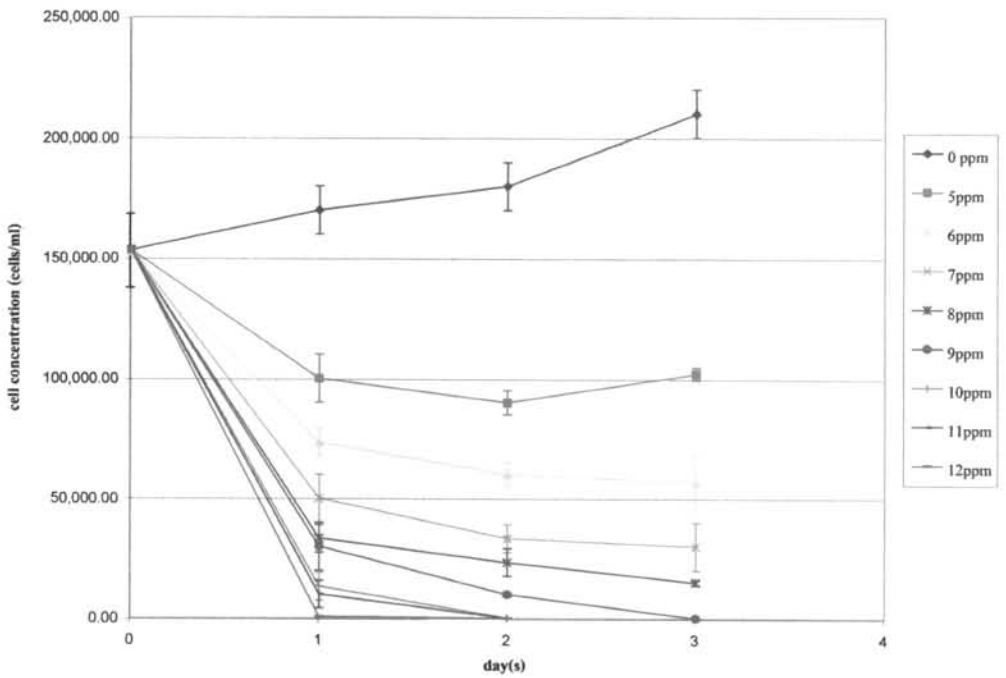
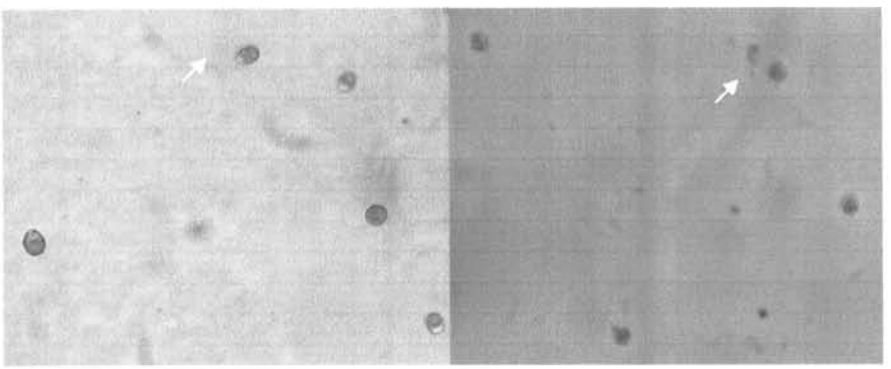



**Figure 4.4** Bialaphos screening of *D. salina* ranging from 5 – 12 ppm



(a) (b)

**Figure 4.5** Effect of the treatment of *D. salina* with bialaphos with (a) control normal cell without bialaphos and (b) disrupted cell after bialaphos treatment (arrowed)

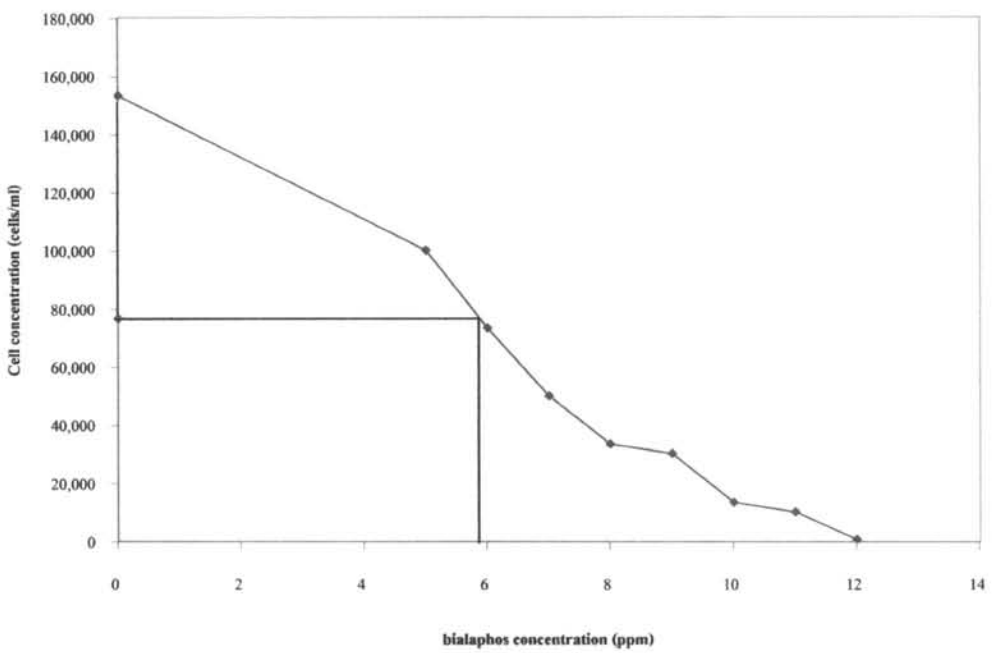



**Figure 4.6** LD 50 of *D.salina* a day after treating with bialaphos

Calibration of the number of disrupted cells in Bialaphos treatments during the course of observation rendered a relationship shown in figure 4.6. At 5 ppm concentration, rapid disruption of cells took place with ratio 65%. The more the concentration of bialaphose were added, the more situation of cells disruption were detected. When the bialaphos concentration was determined based on the of 50% reduction of cells servival, an LD 50

could be estimated (figure 4.6). Here the calculation base on the LD50 reiationship indicated that at the concentration of 6 ppm, the survival cells would render 7.3 x $10^4$ cells, which was equally to 50%. However, since our purpose was focused on selection at stringent condition, a concentration of bialaphose used was set at higher dose than that of estimated LD 50. In this case value at 8 ppm of bialaphase was employed to make sure of providing much efficiency for the herbicide screening.

**4.3 Method for gene transformation in *D. salina***

Next step in preparing the whole system for gene transformation in *D. salina* is the method for DNA transformation. To develop this basic system, a complete construction of pBICBar, containing 35S promoter with Bialaphos herbicide resistant gene was used as target DNA for the study of conditions for DNA transformation as well as for screening for transformant selection. When plasmid DNA was transferred to *D. salina* cells, only transformants having introduced gene in stable form, could survive bialaphose herbicide pressure at long period of time. In the experiment, pBICBar was transformed into *E. coli* DH5α strain and the overnight was collected and used for plasmid DNA isolation. During manipulation, plasmid DNA was checked for both presentation of marker gene bar, 35S promoter and, terminator via PCR for their gene status confirmation (data not shown).

Transformation was based on PEG-mediated gene transformation principle. For the optimization of system via PEG-mediation, 7 level of concentrations of PEG ranging from 0% to 0.1% w/v that had effective on tissue transfomation were comparatively examined. The ability of PEG to induce DNA transformation into *D. salina* was revealed (figure 4.7). In the experiment the fixed amount of DNA at 30 µg was mixed with 5 x $10^4$cells/mL of *D. salina* in 1 mL buffer and incubated for 1 hour. After transformation, buffer for transformation was all replaced after centrifugation with fresh JR medium containing 8 ppm of bialaphos. At 1 week after transformation, cells in control treatment transformed with 0.1% PEG without treating with DNA and bialaphos were still provided a normal growth with 1.9 x $10^5$ cells/mL were expected. The treatment with 0% PEG and bialaphos screening inhibited cell growth. When both PEG and DNA were added, results revealed that lower of PEG (0.02% and 0.04%) did not induce cell transformation at better ratios than the higher concentration (0.06% and

0.10%). The maximum ratio of cell transformations was detected when cells were transformed with 30mg of DNA and 0.08% PEG. The condition obtained here would be used for GBA transformation in further study.

In the experiments bialaphos resistant transformants were confirmed after incubated for 1 month for their gene insertion stability in their genome via cell direct PCR. Results of PCR amplification using primers, specific to bar gene in figure 4.8 revealed the success in PCR amplification. Bands of 400 nucleotides of expected size were detected in all cell lines transformed with various concentration of PEG (0.02-0.1%).

Results of both bialaphos resistant and the DNA detection confirmed the accomplishment in DNA transformation in *D. salina* via PEG mediation.

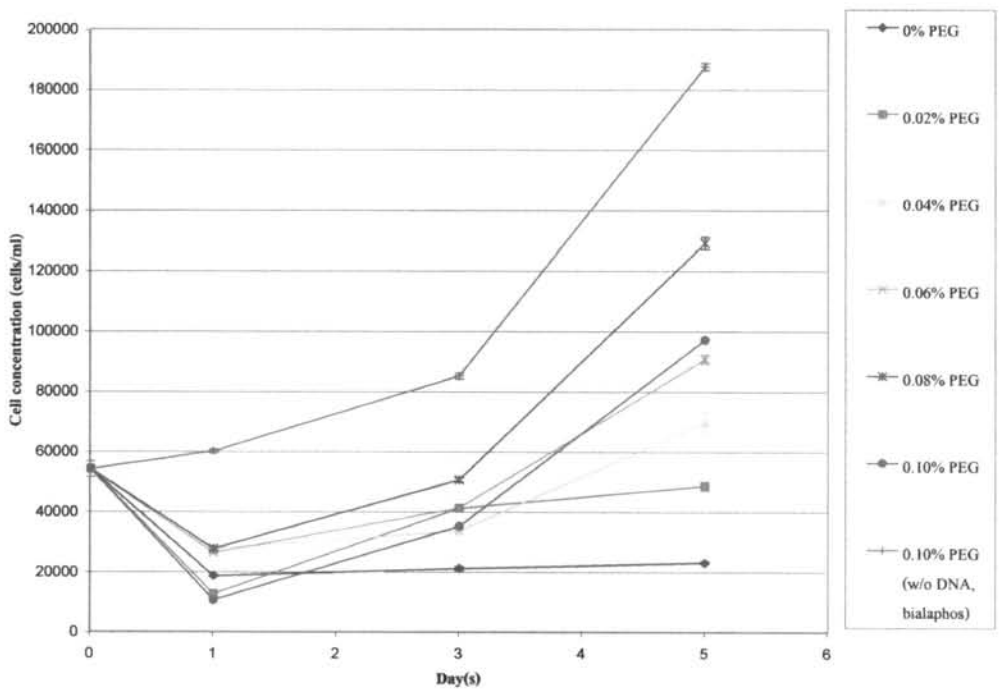



Figure 4.7 Efficiency of pBic Bar transformation in various PEG concentration

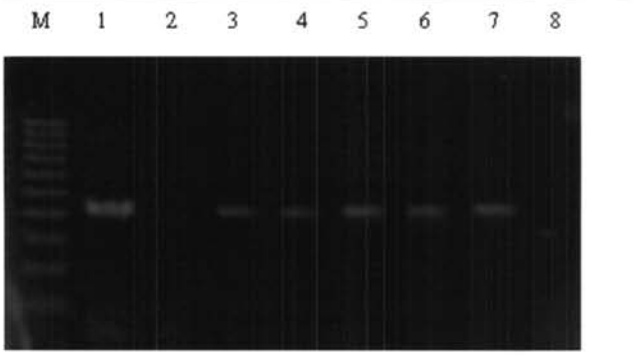



**Figure 4.8** PCR analysis of *bar* gene using genomic DNA obtaining from transformant using different concentration of PEG.

M: molecular marker

1: positive control (pBicBar)

2: 0% PEG

3: 0.02% PEG

4: 0.04% PEG

5: 0.06% PEG

6: 0.08% PEG

7: 0.1% PEG

8: 0.1% PEG (with out DNA and bialaphos)

### 4.4 Construction of transforming vector, containing GBA gene

In order to obtain a valid vector for *D. salina* transformation, The cDNA of GBA gene obtaining from blood specimen was employed. The cDNA corresponding with GBA gene was synthesis using primer specific to GBA 5' and 3' portion. Fullength connection of clone corresponding to GBA gene was based on ligation mediated DNA amplification. The RT-PCR products using two different primers results in DNA bands, 570 and 1338 nucleotide.

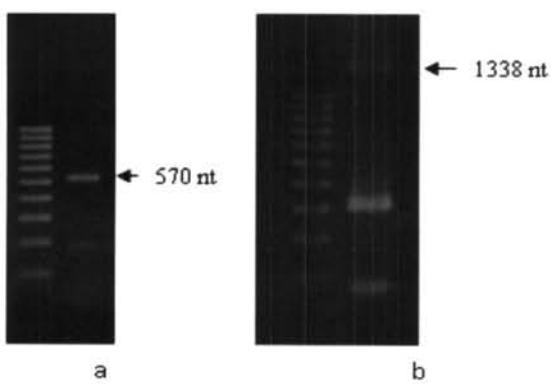



**Figure 4.9** Amplification of GBA gene with (a) 570 nt 5' portion of the gene and (b) 1338 nt 3' portion of the gene

The lower band of 570 nucleotide belonged to 5'portion of GBA and the other belonged to the 3'one (Figure 4.9 a). Both were overlapped between 423 nt to 643 nt and if there were theoretical combined, DNA product of 1610 nucleotide, a full length GBA would be rendered. After 5'portion was eluted out of agarose gel, connected with 35S promoter fragment, amplified from pBICBar using *Taq* DNA polymerase, via DNA ligation and re-amplification of the ligated products using *Taq* DNA polymerase with 5'primer of 35S and 3'primer of 5'portion of GBA, a target connected bands of about 800 nucleotide corresponding to 35S-GBA5'would result (Figure 4.10).

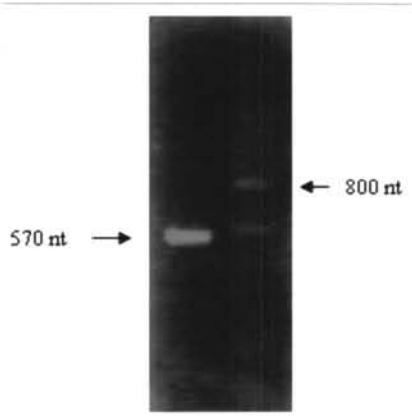



**Figure 4.10** Approximately 800 nt of 35S-GBA5' fusion obtaining after ligation-PCR amplification

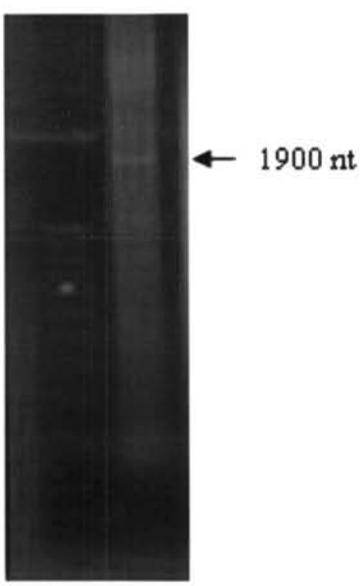



**Figure 4.11** Full-length GBA after 35S promoter connection

The obtaining DNA was then further connected with the 3'-1633 nucleotide-portion to generate a full length fragment containing, 35S promoter, GBA gene (Fig. 4.11) of 1900 nucleotides. The 35S was selected to regulate gene transcription in *D. salina* cells and the 3'primer specifically matched with 3'protion of GBA with *Eco*RI restriction recognition overhang was introduced to facilitate DNA ligation with pBICBar. Last step of construction

was concerning the connection of the resulting DNA from the previous step with pBICBar predigested with *Bam*HI / *Eco*RI using T4 DNA ligation and transform in to *E. coli* DH5α as a cloning system. Target plasmid, pBICBarGBA was screened base on correct DNA amplification of 35S–GBA genes using *Taq* DNA polymerase. Fig. 4.12 showed the results of screening selection in which the expected bands of 800 nucleotides were generated. The selected plasmid was harvested and purified for further use in GBA transformation.

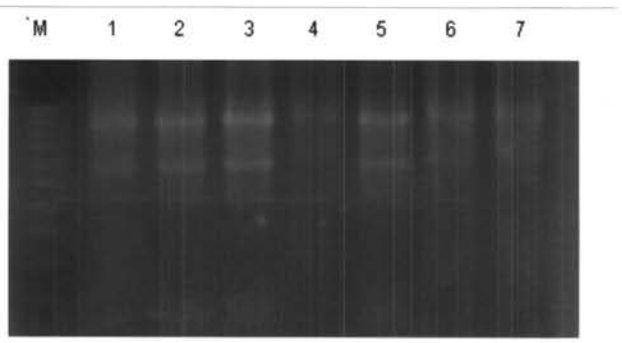



**Figure 4.12** Selection of pBicBarGBA

M: molecular marker

1-7: recombinant clone no. 1-7 respectively

**Transformation of GBA gene in *D. salina***

pBicBarGBA was transformed into *D. salina* with the condition discussed above. 0.08% PEG was performed along with 30 µg of plasmid DNA in 1 ml buffer. After transformation, transformants were incubated in JR medium containing 8 ppm bialaphos for 1 month and performed molecular analysis further. Number of transformants increased sharply to 3 x $10^5$ cells/ml and was extracted for genome using phenol extraction method (Sambrook *et al.*, 1989) and was analized by PCR using amplified primers of 5' portion GBA. The results revealed an expected 570 nt band of 5' portion GBA in transformants (figure 4.13) resulted in the success of transferring GBA to *D. salina*. The detection of RNA expression was further carried out using RT-PCR .

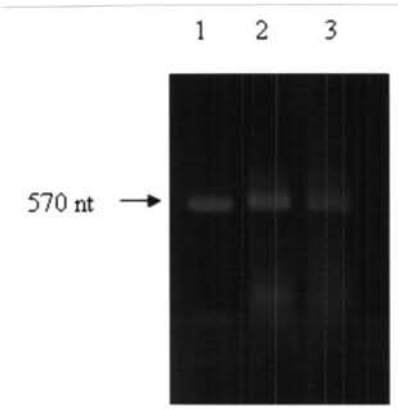



**Figure 4.13** PCR analysis of GBA5' portion in transformed *D. salina*

1: positive control (pBicBar GBA)

2: transformant 1

3: transformant 2

RT-PCR was performed to investigate the expression of RNA. Transformed *D. salina* was extracted for total RNA by standard phenol-extraction (Sambrook *et al.*, 1982) and was used as template to generate cDNA with Superscrip II (Invitrogen, USA). cDNA template was amplified using 5'GBA portion's to confirm the transcription (figure 4.14).

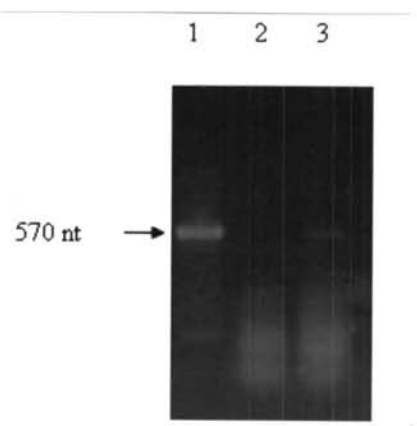



**Figure 4.14** RT-PCR analysis of GBA5' portion in transformed *D. salina*

1: positive control (pBicBarGBA)

2: transformant 1 3: transformant 2

RT-PCR analysis resulted a 570nt band in transformant 2 which is equal to the positive control (pBicBarGBA). According to previous result, transformant 1 was detected by PCR but not detected by RT-PCR. This supported that GBA had integrated to *D. salina* genome but no RNA expression were found while in transformant 2 the result encourage that GBA was integrated into *D. salina* genome and transcription had occurred revealed an RNA expression.