CHAPTER V
CONCLUSIONS AND DISCUSSIONS

Medium for culturing microalgae *D. salina* had been developed with the aim of lowering cost but its efficiency in *D. salina* proliferation. JR medium was modified comparing with widely-used J/1 medium (Borowitzka, 1988). While J/1 contained expensive chemicals and minerals, JR composed of 16-16-16 fertilizer, Unilate and simple salt. Since those fertilizers were widely accessible at local, this mede total production of *D. salina* cheaper in their cost and easier to apply. In addition, culturing of *D. salina* in JR media provided difference in total cell numbers than that of a J/1. Cost estimation from the experiment showed, JR media use about 10 times cheaper when determine at same concentration (1X) and at 40 times cheaper when determine at 0.25X JR. This encouraged JR medium application a substitution for J/1 medium in culturing this microalgae.

A suitable pH for JR medium was also investigated. In 0.25X JR medium, the most effective pH were 8 - 8.5 which *D. salina* could increase to 45 times within 30 days. Combining of JR concentration and pH would promote the higher growth rate of *D. salina* not only in large-scale culture but also in small-scale production. The condition could also be used as buffer in PEG-mediated transformation which will be discussed further.

Several selective reagents were used in algal transformations. Hawkins and Nakamura (1999) reported several uses of antibiotics in *Chlorella* sp. High concentrations (10 mg/L) of ampicillin or kanamycin had minimal effect while spectinomycin, streptomycin and chloramphenicol was more inhibitory at 2 mg/ml. Geneticin was found to be completely inhibitory at 1 mg/L.

However in *D. salina*, De-Gui *et al.* (2004) reported that streptomycin, kanamycin, hygromycin and geneticin did not inhibit the growth while chloramphenicol, incontrast, completely inhibited *D. salina* at 60 µg/ml but it was expensive to purchase.

Bialaphos had become a selective reagent recently (Altenburger *et al.*, 2006) especially in this experiment since it was cheap and had rapid effect to microalgae. It involved in inhibition of glutamine synthase enzyme. The immediate effect of inhibiting glutamine synthase is the accumulation of ammonia to toxic levels, which rapidly damage the

algae cell (Slater *et al.*, 2003). Bialaphos was employed and set at 8 concentration, 5 -12 ppm. When treated with bialaphos, cell concentration of *D. salina* decreased sharply in various level after 1 day. The concentration at 6 ppm was estimated as an LD 50; however, the actual concentration recommended for stringent condition was set as 8 ppm to meet more efficiency in screening of transformants.

Several researches are conducted on method inducing transgenic microalgae. Most of them focused on particle bombardment (Apt, 1996, Ishikura *et al.*, 1999; Poulsen and Kroger, 2005 and Boynton, 1998), electroporation (Shimogawara *et al.*, 1998; Chen *et al.*, 2001 and De-Gui *et al.*, 2004) and silicon-carbide whisker (Nagatani *et al.*, 1997; Dunahay, 1993 and Lohuis and Miller, 1998). In 1999, Hawkins and Nakamura reported the expression of human growth hormone in *Chlorella* sp. using sea sand mediated PEG, they found a transient expression of this hormone in transformant.

In this experiment PEG-mediated transformation was performed in order to introduce pBicBar into *D. salina* cells. The concentration of PEG affecting transformation efficiency was determined and concentration of 0.08% PEG was the most effective concentration select based on the increasing number of cells in selection condition of 8 ppm bialaphos and presence of *bar* gene via PCR and RT-PCR analysis. Our genetic ransformation of *D. salina* had been perform via electroporation; however, the experiment have revealed our success on using only PEG with simple solution to mediated gene transform in *D. salina.* Transformation system in *D. salina* were to develop a transformation system for human glucocerebrosidase gene expression in *D. salina* the conduction of DNA materials were performed.

According to our experiment, DNA construct having a connection between 35S promoter GBA were employed. The whole portion was ligated to pBicBar containing an already existed 35S terminator at *Eco*RI site. Chen *et al.* (2001) reported the use of CaMV 35S promoter to express rabbit neutrophil peptide-1 gene in *Chlorella* sp. as evidence by RNA expression and translation. This again confirmed the possible use of 35S promoter for green microalgae *D. salina.*

The vector containing GBA gene was transformed using PEG mediated into *D. salina* culture in JR medium for 1 month. The transformants were obtained in high amount at 5×10^4

cells/ml. PCR analysis of DNA from transformants via special 35S forward primer and 5' GBA portion's reverse primer, results revealed 800 nt DNA bands corresponding to the expected gene indicating the integration of 35S/GBA fragment into *D. salina* genome.

Expression of GBA gene in term of RNA synthesis was observed via RT-PCR. Results confirmed the GBA gene expression as evidence by fragment of 570 nt RT-PCR products detection. However, the experiment did not investigate the expression of GBA protein producing due to the unaccessible of an antiGBA antiserum.

Glucocerebrosidase is an enzyme used in Gaucher disease treatment. At present, the widely use of glucocerebrosidase in enzyme replacement therapy is Cerezyme® (Futerman, 2004). Cerezyme®, a recombinant protein is produced in mammalian cell, making it expensive in fermentation, low yields, high in operating costs, difficult to scale up to large volume and contaminated by viruses and prions (Gomord and Faye, 2004).

In contrast, *D. salina* can grow in cheap medium with rapid proliferation equal to the widely used one, operate in low cost, able to produce in large-scale and easy to purify for protein. These advantages encourage *D. salina* an alternative protein production system among other choice of organisms.

Recommendations

This experiment might show a new trend for producing recombinant protein; however, this experiment didn't include the protein-detection part due to glucocerebrosidase unaccessible in time. Thus, detection for glucocerebrosidase protein should be carried out further to confirm its translation. The study of molecular structure and enzyme activity is also necessary, comparing with natural and other recombinant glucocerebrosidase. In addition, sea-water transgenic *D. salina* should be an in-land culture with well-sealed container to provide safety and avoid GMO's contamination.