CHAPTER 5

DISCUSSION

5.1 Growth Characterization of *C. vulgaris*

Chlorella vulgaris was in an exponential growth phase after a lag of two days (Figures 4.1 and 4.2). Walker and Rapley (2000) defined the lag phase as a period of adaptation of a particular microorganism, to the new culture conditions. During the exponential phase, the *C. vulgaris* growth rate gradually increased and the cells grew at a constant and maximum rate (Walker and Rapley, 2000). This phase was identified as the best period for particle bombardment, when nucleic acids and proteins were abundant in the cells which were most actively dividing.

The graphs that were plotted based on the natural logarithm of optical density (OD_{620}) and cell count against time (day) in Figures 4.1 and 4.2, gave a straight line at the exponential phase. The slope at this phase was calculated as the maximum specific growth rate of *Chlorella vulgaris*. The specific growth rates (μ) of *C. vulgaris* ranged between 0.2041 $\pm$ 0.0439 ($p<0.05$; $p=0.0151$) to 0.2867 $\pm$ 0.0279 ($p<0.05$; $p=0.0031$). This suggested that *C. vulgaris* took almost two to three days to reach one cell generation or in other words, it took two to three days for one cell to divide into two cells. Phang and Chu (2004) also reported that *C. vulgaris* has a generation time of two days. According to Chow and Tung (1999), *Chlorella* has a faster growth rate

compared to *Chlamydomonas* which has always been a common interest of researchers.

According to Calvin (1999), correlation is defined as a method of measuring the association between two variables. Figure 4.3 shows that there was a strong correlation of r=0.9873 between OD_{620} and cell count. This indicated that there was a strong direction of linear relationship between these two random variables.

5.2 Determination of Chloroamphenicol as a Selectable Marker

One of the common drawbacks of *Chlorella* transformation is the natural resistance towards antibiotics (Hawkins and Nakamura 1999). In this research, when non-transformed *Chlorella vulgaris* was exposed to chloroamphenicol at various concentrations, 200 µg mL^{-1} of chloroamphenicol was found to be sufficient enough to completely inhibit the cell's growth. Chloroamphenicol inhibits translation by blocking peptidyl tranferase on the 50s ribosomal subunit and if used at higher concentrations, it could inhibit eukaryotic DNA synthesis (Sambrook and Russel, 2001). Figures 4.4 and 4.5 show that there was a slight decrease in the growth when *C. vulgaris* was exposed to 100 µg ml^{-1} but the growth was totally inhibited at 200 µg mL^{-1} or higher concentration of chloroamphenicol. Besides that, Figure 4.6 and Figure 4.7 show drastic negative response in the specific growth rates when *C. vulgaris* cultures were exposed to 200 µg mL^{-1} and 400 µg mL^{-1} of chloroamphenicol, respectively. The main reason why 200 µg mL^{-1} was chosen over 400 µg mL^{-1} of chloroamphenicol as a concentration for selection because it was sufficient to completely inhibit the cell's growth and it was much cheaper to use a lower

concentration of antibiotics for selection. In addition, a slowing killing was better than the immediate kill as it allowed transgenic cells to undergo multiplication (Gan, 2005). Moreover, the LC_{50} analysis showed that 102.62 µg mL^{-1} of chloroamphenicol could cause 50% cell mortality. In order to ensure that all the colonies that grew on the selection plates were transformants, 200 µg mL^{-1} was chosen over 100 µg mL^{-1} .

Hawkins and Nakamura (1999) reported that there was still some growth of *Chlorella* when chloroamphenicol at the concentration of 2000 µg mL^{-1} was used for selection. However, the present study showed that chloroamphenicol at 200 µg mL^{-1} was sufficient for selection as the plates studied showed no growth even after one month. This also indicated that the *Chlorella vulgaris* UMACC 001 strain was susceptible to lower concentration of chloroamphenicol compared to the *C. vulgaris* C-27 strain that was used in the research of Hawkins and Nakamura (1999). Strain selection was important as it played a crucial role in the cost and time of the research. For example, a strain which has faster specific growth rate and susceptible to lower concentration of antibiotics could reduce the cost and time of research by many folds. Hence, the present study suggested that chloroamphenicol could be one of the better choice of selectable marker that could be used in the transformation of *C. vulgaris*. Chloroamphenicol has also been successfully used as an antibiotic for selection in several transformation of seaweeds such as *Laminaria japonica* and *Undaria pinnatifida* as these seaweeds are only sensitive to very few antibiotics (Qin *et al*., 1999 and 2004).

Although the ampicilin resistance gene was present in the pCAT-HBSAg plasmid, but it could not be used in this research as the EC50 value (concentration that

was effective to kill 50% of organism) of ampicilin on *Chlorella vulgaris* was above 1000 mg mL^{-1} (Eguchi *et al*., 2004). The susceptibility of an organism towards an antibiotic plays an important role in determining the choice of the selectable marker for selection of transformants (Sambrook and Russel, 2001). An antibiotic sensitivity test of *Chlorella* performed by Hawkins and Nakamura (1999) also suggested that *Chlorella* showed minimal effect towards ampicilin and kanamycin but was partially susceptible towards spectinomycin or streptomycin at high concentration (10 000 µg mL^{-1}).

Besides chloroamphenicol, there were other antibiotics that could be used in selecting transformants. The *Sh ble* gene could also be used as a selectable marker instead of the CAT gene. The *Sh ble* gene was originally cloned from *Streptoalloteichus hindustamus* and encodes a small protein that confers resistance to tallysomycin, bleomycin, phleomycin, and zeomycin by binding to the antibiotics and inhibiting their DNA cleaving activities (Gatignol *et al*., 1988). Kim *et al.* (2002) conducted a test on phleomycin, and detected complete growth inhibition at the concentration of 1 µg mL^{-1}. Therefore, by introducing the *Sh ble* gene under the control of the *Chlamydomonas* RBCS2 gene promoter into the transformation vector, transformants could confer resistance to phleomycin. The *Sh ble* gene has been used as selectable marker in various eukaryotes, including, fungi, algae, plants, and animal (Kim *et al*., 2002). However, unlike chloroamphenicol which was a more stable antibiotic, phleomycin was reported to break down due to light and temperature which then enabled escapes to grow among the transformants (Walker *et al*., 2005). Other antibiotics used for the selection of transformed *Chlorella* included Geneticin (G418)

at a final concentration of 1 mg mL^{-1} (Hawkins and Nakamura, 1999) and Hygromycin B at a concentration of 50 µg mL^{1} (Chow and Tung, 1999).

5.3 Genetic Transformation of *C. vulgaris* with the pCAT-HBSAg Vector Containing the Hepatitis B Surface Antigen Gene

The average number of clones that gave positive PCR results was 16 clones out of 20 selected transformed clones. This shows that chloroamphenicol is a good selectable marker as the number of positive PCR clones was quite consistent. The two way ANOVA test that was performed revealed that there were no differences between all four parameters. This suggested that rupture disc pressure of 900 psi and 1100 psi at the distance of 6 cm and 9 cm were good parameters to use when transferring the pCAT-HBSAg plasmid into *Chlorella vulgaris*. Dawson *et al.* (1997) also used rupture disc pressure of 1100 psi but at a longer distance of 15 cm to stably transform *Chlorella sorokiniana* cells with 0.4 micron sized tungsten bead coated with the nitrate reductase gene. Since the bombardment of the cell wall deficient organism, *Chlamydomonas* was conducted at rupture disc pressure of 1100 psi, therefore we justified that *C. vulgaris* could also withstand this pressure of 1100 psi due to its thick cell wall (Bateman and Purton, 2000; Ishikura *et al.*, 1999).

According to Teng *et al.* (2002), when *Haematococcus pluvialis* was bombarded with the *lacZ* gene, there was no detection of the *lacZ* gene when the rupture disc pressure was increased above 900 psi due to its thin cell wall. However, El- Sheekh (1999) used only helium pressure 130 psi to transform *Chlorella kessleri*. In our research, we did not reduce the rupture disc pressure to below 900 psi, fearing that the pressure might not be able to deliver the pCAT-HBSAg plasmid into *C.*

vulgaris. At shorter distance like 6cm, the rupture disc pressure could be reduced to obtain an impact sufficient to deliver the pCAT-HBSAg plasmid into the microalga cell. Steinbrenner and Sandmann (2006) reported that higher pressure was vital in order to obtain stable nuclear transformation of *Haematococcus pluvialis*. They bombarded the *H. pluvialis* cells with rupture disc pressure as high as 1350 psi and distance of 7.5 cm with the phytoene desaturase gene (Steinbrenner and Sandmann, 2006). Since *C. vulgaris* has a more rigid cell wall than *H. pluvialis*, therefore rupture disc pressure of 1100 psi at both distances 6 cm and 9 cm in addition to 900 psi were used to transform *C. vulgaris*. As a result, the two way ANOVA tests revealed that all the four parameters could be used to genetically transform the green microalga, *C. vulgaris* as it showed no difference in the transformation efficiency results.

Since *Chlorella vulgaris* does not have the ability to move on its own, therefore it was a good candidate for transformation as transfection was made easy when the cells were not moving (Coll, 2006). According to Coll (2006), high mobility shown by *Chlamydomonas*, *Volvox*, *Euglena* and *Dunaliela* made transfection more difficult because of their movement. Furthermore, the Bio-Rad PDS-1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, USA) method was easy as there was no need to remove the cell wall and it was also proven to be successful in introducing the nitrate reductase and β-glucuronidase gene into *Chlorella* sp. (Dawson *et al.*, 1997; El-Sheekh, 2000).

The pCAT-HBSAg was encoated with 1.0 µm sized gold particle as mentioned in section 3.4.3. Gold particle was chosen over tungsten particles because of its uniform size and shape as well as the biological inertness. Although Dawson *et*

al. (1997), transformed *Chlorella* sp. using tungsten, but in the present study gold particles were used because tungsten particles were irregular in shape and heterogeneous in size, and tungsten particles could be toxic to cells. Moreover, these particles were subjected to surface oxidation that could alter DNA binding and overtime could catalytically degrade DNA bound to them (Sanford *et al*., 1993; Dawson *et al*., 1997).

According to Randolf-Anderson *et al*. (1997), small gold particles are less damaging to the cells and this was probably the main explanation for high efficiencies. Vasil (1999) also reported that, besides metal toxicity, the size of the microprojectile particle was a critical variable. Particle size interacts with other variables such as the DNA carrying capacity of the particle, the number of particles that impact the sample and the momentum of the particle (Vasil, 1999).

5.4 DNA Analysis of *C. vulgaris* Clones Transformed with the pCAT-HBSAg Vector

Hind III that cuts the pCAT-HBSAg plasmid at two sites (605 nt and 3893 nt) was employed to fragmentize the genomic DNA samples of *C. vulgaris*. The Hind III digestion of the pCAT-HBSAg plamid, produced two bands sized 3288bp (containing the HBSAg and CAT genes) and 2862 bp (containing the pCAT-HBSAg without the HBSAg and CAT genes). Figure 4.13 showed that genomic band was detected by the biotin labelled HBSAg probe from the Hind III digested genomic DNA. This was probably due to the partial digestion of Hind III which caused some remaining genomic band to be detected by the biotin labelled HBSAg probe due to the shorter incubation period (three days). According to Kim *et al*. (2002), a larger DNA

band was detected from the non digested samples, when *Chlorella ellipsoidea* was transformed with the flounder growth hormone.

It was very difficult to detect the integration of the HBSAg gene in *Chlorella vulgaris* using non-radioactive approach like North2south biotin chemiluminescent kit because the colorimetric assay was not sensitive enough like the ^{32}P radioactive (Hawkins and Nakamura, 1999). Even Kim *et al*. (2002) mentioned that the detection of foreign gene in transformed *Chlorella* was problematic because of the low copy number. According to Chow and Tung (1999), one of the reasons that makes *Chlorella* less desirable for genetic transformation, was that it was not easy to detect the gene of interests derived from the host genome.

5.5 Expression Analysis of *C. vulgaris* Clones Transformed with the pCAT-HBSAg Vector

The RT-PCR results in the Figure 4.15 showed that the expected 271 bp of the HBSAg gene was obtained from transformed *Chlorella vulgaris* cDNA at the 10^{th} cell generations. The PCR products were further analyzed via southern blot using the biotin labelled HBSAg probe. Figure 4.16 shows that the expected bands were obtained in the X-omat blue film after southern blotting. This proved that the HBSAg gene was successfully translated into mRNA in the host genome.

A 47 kDa was detected in all the transformed *Chlorella vulgaris* protein samples using the western blot probe which was the Mouse Anti-pre S2 (Hepatitis B Virus) Monoclonal Antibody (Figure 4.18). However, the expected band should be 24kDa as observed by Geng *et al.* (2003) from the crude protein samples isolated

from the *Dunaliella salina* transformed with HBSAg gene. According to Mason *et al.* (1992), the infectious HBSAg viral particle (Dane particle) is a 43-nm double-shelled sphere that consists of a core containing the 3.2 Kb DNA genome bound to a core protein, surrounded by the viral envelope containing phospholipids and the major surface antigen (HBSAg). In addition to Dane particles, the serum of infected individuals also contains 22nm subviral particles in great excess over virions. These non-infectious particles contain the elements of the viral envelope, including the major 24kDa peptide that occurs in glycosylated and unglycosylated forms (Mason *et al*., 1992).

Expression of an exogenous gene can be very low or non-existent, even though all the elements required for optimal transcription and translation like promoters, introns and other regulatory regions have been included in the chimeric gene construction (Leon-Banares *et al*., 2004). The SV 40 promoter that was used in the present study was able to drive the expression of the CAT and the HBSAg genes. A 47 kDa was detected in all the transformed *Chlorella vulgaris* protein samples using the western blot probe which was the Mouse Anti-pre S2 (Hepatitis B Virus) Monoclonal Antibody (Figure 4.18). However, the expected band should be 24kDa as observed by Geng *et al.* (2003) from the crude protein samples isolated from the *Dunaliella salina* transformed with HBSAg gene. According to Mason *et al.* (1992), the infectious HBSAg viral particle (Dane particle) is a 43-nm double-shelled sphere that consists of a core containing the 3.2 Kb DNA genome bound to a core protein, surrounded by the viral envelope containing phospholipids and the major surface antigen (HBSAg). In addition to Dane particles, the serum of infected individuals also contains 22nm subviral particles in great excess over virions. These non-infectious

particles contain the elements of the viral envelope, including the major 24kDa peptide that occurs in glycosylated and unglycosylated forms (Mason *et al*., 1992).

One possible probability that could have led to the observation of 47kDa instead of 24kDa (Figure 4.18) was that the HBSAg protein and the neighbouring CAT protein could also be expressing as a fused protein through a disulfide bond. The HBSAg gene was supposed to encode a 24 kDa protein and the CAT gene was supposed to encode another 24 kDa protein, which means the fusion protein could be 48 kDa in size (Mason *et al*., 1992; Basu *et al*., 2005). The lack of protein reducing agent like beta-mercaptoethanol (BME), dithiothreitol (DTT), glutathione, cysteine, Tris(2-carboxyethyl) and phosphine hydrochloride (TCEP.HCl) in extraction buffers could have caused the formation of fusion protein. A fusion protein is a product of joining genes or two portions or peptides together. This process could occur naturally or could be created artificially together. According to Noutoshi *et al.* (1997), *Chlorella vulgaris* has an average GC content of 52.7%. The GC percentage of the whole pCAT-HBSAg plasmid is 47.4%, which was almost half of the total nucleotide in the plasmid. *C. vulgaris* was reported to show codon biasness and the GC content of the pCAT-HBSAg plasmid was also high. Therefore, there was a high chance that the high GC content could have lead to high cysteine content in the crude protein extracts. Cysteine (UGU, UGC) is a naturally occurring sulfur containing amino acid that is found in most proteins. According to Mckee and Mckee (2003), the sulfhydryl group of cysteine is highly reactive. The most common reaction to this group is a reversible oxidation that forms a disulfide. Oxidation of two molecules of cysteine forms cysteine, a molecule that contains a disulfide bond. When two cysteine residues form such a bond, it is referred to as a disulfide bridge. This bond can occur in a

single chain to form a ring or between two separate chains to form an intermolecular bridge. These disulfide bridges are strong covalent bonds that help stabilize many polypeptides and proteins (Mckee and Mckee, 2003). Thus, the disulfide bond between the fusion protein (HBSAg protein and CAT protein) could have been difficult break by the reducing agent, beta-mercaptoethanol.

The 47 kDa protein was detected in the 42nd (day 84) and 50th (day 100) cell generations (Figure 4.18) but not after the 50th cell generation (day 100) (results not shown). Possible explanation for this failure include the silencing of introduced genes by methylation, inappropriate codon usage in the foreign gene compared to the highly selective codon usage seen in *Chlorella vulgaris* genes, ectopic effects and lack of introns or other coding elements (Stevens *et al*., 1996). According to Primrose *et al*. (2001), transgene silencing is a complex phenomenon, occurring in all eukaryotes, caused by the introduction of foreign nucleic acid into the cell. Typically, expression of the affected transgene is reduced or abolished, associated with increased methylation at the transgenic locus. An understanding of transgene silencing is important, first, because it is a serious impediment to the use of animal and plant systems for the expression of foreign genes and second, because if a transgene is homologous to an endogenous gene, the endogenous gene can also be silences by co suppression (Primrose *et al*., 2001).

5.6 Stable Integration Analysis of the pCAT-HBSAg Vector in Transformed *C. vulgaris* Clones

The presence of the HBSAg gene was detected until the 92nd cell generation (day 184) via PCR. Southern blot analysis showed that the HBSAg gene was still

integrated in the *Chlorella vulgaris* genome even at 82nd cell generation (day 164). The successful employment of heterologous constructs in *C. vulgaris* bypasses the requirement for complementation mutants and/or characterisation of homologous promoters. According to Ladygin (2004), generally unstable trait manifestation is governed at any of three levels which are DNA, RNA and protein. The probable causes of instability of a foreign gene integrated sequence, factors affecting mRNA transcription and processing, and/or factors influencing the synthesis of a protein product of the gene in question and the further stability of this protein (Ladygin, 2004).

The present study has proven that the *Chlorella vulgaris* can be transformed via particle bombardment. The introduced HBSAg gene was successfully integrated, transcribed and translated in the host genome. This raised the possibility that *Chlorella* could be used as a host for the production of recombinant proteins. This was particularly appealing since this unicellular eukaryote is capable of photoautotrophic growth (or heterotrophic growth using cheap carbon source acetate) and could be easily cultured in large quantity.

5.7 Project Appraisal

The hepatitis B virus causes high mortalities increase globally. Currently, the method used to deliver the hepatitis B vaccine to a patient is expensive. Hence, there is an urgent need to produce and deliver these vaccines in a more affordable and painless manner, so that the number of deaths in poorer countries related to this infectious disease could be reduced. There have been much research aimed at

exploring a strategies of producing and delivering oral vaccines as constituents of transgenic, edible plants such as potatoes, tomatoes or bananas (Thanavala *et al*., 2005). There have even been attempts to transform the seaweed, *Laminaria japonica* and green microalga, *Chlamydomonas reindahardtii* with the hepatitis B surface antigen gene (Jiang *et al*., 2002; Geng *et al*., 2003) Nevertheless, it is very tedious and time consuming, to genetically transform higher plants and seaweeds compared to the unicellular green microalga, like the *Chlorella vulgaris*. The present study shows that this is the first transformation done on *C. vulgaris* using the hepatitis B surface antigen gene. The simple and fast growing *C. vulgaris* can be transformed with the hepatitis B surface antigen without undergoing tedious tissue culture process. It will only take less than a week to prepare the microalga culture and transform them. The transformed *C. vulgaris* can be easily mass cultured using bioreactors. The cost of maintaining transformed *C. vulgaris* can be cheap too. Most importantly, the mortality rate of microlaga transformants was much lower compared to higher plants and seaweeds. Hence, with further research on the expression level of the hepatitis B surface antigen, like the ELISA analysis and clinical trials, these edible *C. vulgaris* that are transformed with the hepatitis B surface antigen gene could be used as an edible vaccine at a cheaper cost. However, the cost of the Biolistics (Bio-Rad Laboratories, USA) which is used to transform the *Chlorella* cell efficiently is very expensive and not many laboratories could afford it. Besides that, during southern blot analysis, the detection of hepatitis B surface antigen gene was difficult because the present study used a non-radioactive approach. It is suggested by many research papers, that the detection of foreign gene integration could be more sensitive if the ^{32}P was used labelled the probe.

5.8 Future Research

Modern science and technology has developed many foreign DNA delivery methods, to enable researchers to transform many species with foreign genes using standard protocols. However, each genetic transformation protocol has to be optimized for each species. The particle bombardment parameters determined in this study could be used to deliver other foreign genes besides the hepatitis B surface antigen gene, into *Chlorella vulgaris.* However, it would be beneficial, to test various plasmid with different promoters which can be used to drive the expression of the hepatitis B surface antigen gene. Besides that, the intercellular stimulus that is needed to switch on the inserted gene that has integrated into the host genome has to be identified. This is crucial in order to understand how each foreign gene functions in different host.

After transformation, the next step is to mass produce the culture. An optimized batch-cultured system in a photo-bioreactor is crucial to facilitate better culture control and higher productivity. Nevertheless, there are controversy regarding genetically modified organism because of the potential long-term risks to human health and the environment caused by the release of genetically modified organisms into the environment. Many people claim that genetically modified organism for foods are beneficial because of their higher nutrient value and because of their capacity to substantially increase food production to feed the world's growing population. On the other hand, there are critics who argue that the potential risks cannot be dismissed. Therefore this has to be conducted with strict adherence to the

biosafety protocols to avoid any genetically modified organism escape into the environment.