CHAPTER 4

RESULTS

4.1 Growth Characterization of *C. vulgaris*

4.1.1 Optical Density

Growth study of *Chlorella vulgaris* based on optical density at 620 nm (OD_{620}) showed that all three replicates had similar growth trends (Figure 4.1) ($p<0.05$; $p=0.0000$) (Appendix 2a, c and e). Figure 4.1 shows that *C. vulgaris* was in a lag phase for two days after the first subculture, then it entered into an exponential phase on the second day and remained in that phase until the tenth day. After the tenth day, the growth reached stationary phase. The specific growth rate (μ) of this microalga was 0.2041 ± 0.0439 ($p<0.05$; $p=0.0151$) (Appendix 3a and b(i)). Therefore, the generation time for one cell divide to become two cells calculated from the OD_{620} data obtained was three days.

4.1.2 Cell Count

Growth study of *Chlorella vulgaris* based on cell count data also showed that all three replicates had similar growth trends (Figure 4.2) ($p<0.05$; $p=0.0000$) (Appendix 2b, d and f). Based on Figure 4.2, *C. vulgaris* was in a lag phase for two days after the first subculture. The microalga cells entered into an exponential phase on the second day remained in that phase until the tenth day. After the tenth day, the cells growth became stationary. The specific growth rate (μ) of this alga was 0.2867 ± 0.0279 ($p<0.05$; $p=0.0031$) (Appendix 3a and b

(ii)). Therefore, the generation time for one cell divide to become two cells calculated from the cell count data obtained was two days. This generation time (two days) was used to determine the generation time of *Chlorella vulgaris* cultures for further the subsequent analyses ; DNA, RNA and protein analyses.

4.1.3 Correlation between OD_{620} and Cell Count

According to Figure 4.3, there was a strong correlation of r = 0.9873 between the growth study data of *Chlorella vulgaris* based on OD_{620} and the cell count (Appendix 2g).

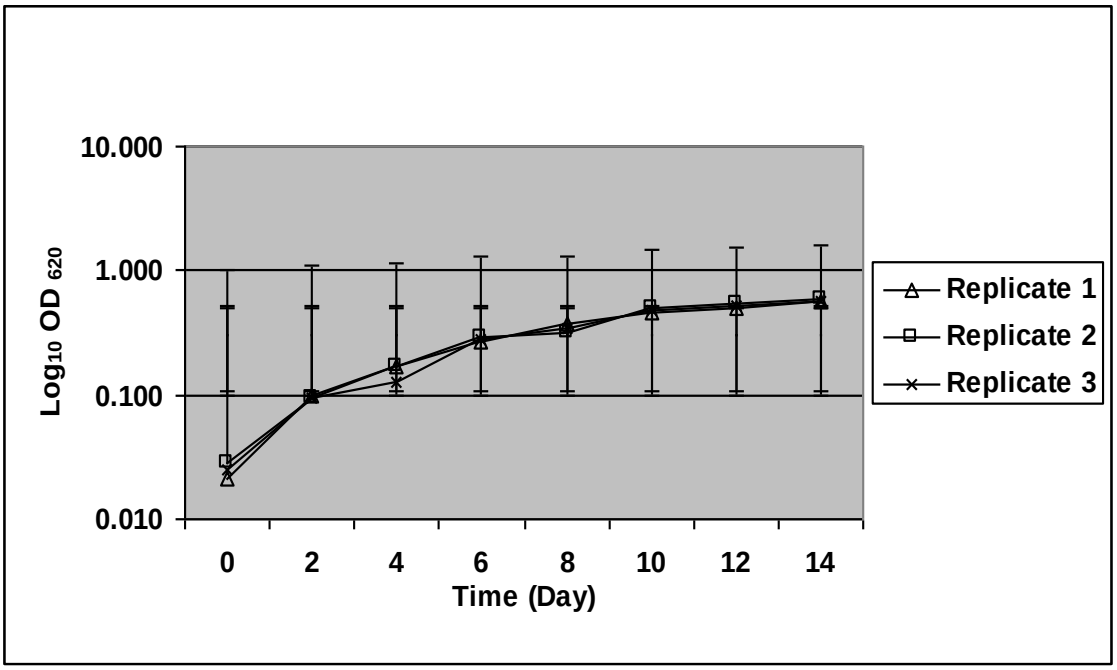


Figure 4.1 Semi-logarithmic growth curves of *Chlorella vulgaris* based on OD_{620}. *C. vulgaris* was in a lag phase for two days before entering into the exponential phase. The microalga remained in the exponential phase for eight days (second day until the tenth day) and finally entered into the stationary phase from the tenth day onwards.

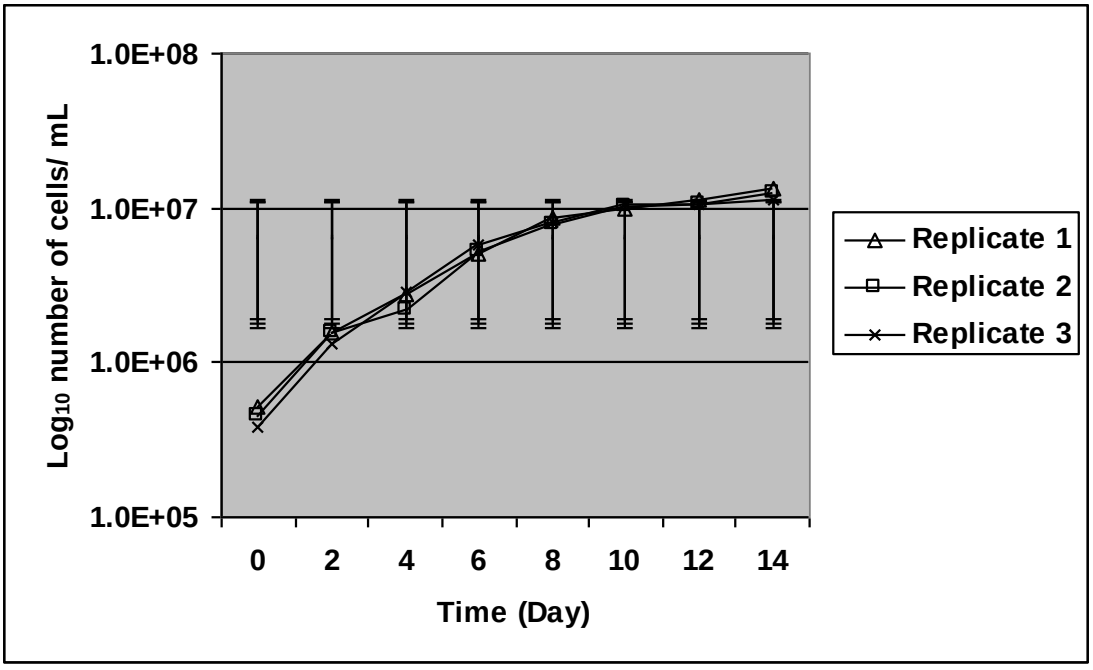


Figure 4.2 Semi-logarithmic growth curves of *Chlorella vulgaris* based on cell count. *C. vulgaris* was in a lag phase for two days before entering into the exponential phase. The microalga remained in the exponential phase for eight days (second day until the tenth day) and finally entered into the stationary phase from the tenth day onwards.

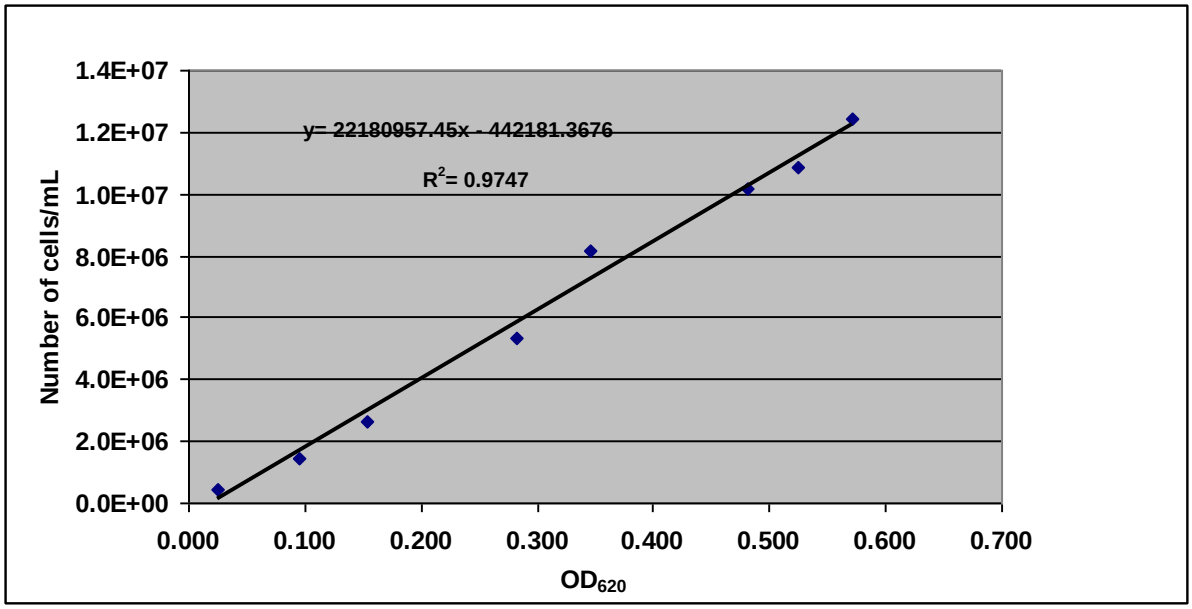


Figure 4.3 Correlation between OD_{620} and cell count (r=0.9873). There was a strong correlation (r=0.9873) between the both variables, OD_{620} and cell count.

4.2 Chloroamphenicol Inhibition Test

Chlorella vulgaris was exposed to 25 ug mL^{-1}, 50 ug mL^{-1}, 100 ug mL^{-1}, 200 ug mL^{-1} and 400 ug mL^{-1} of chloroamphenicol so that the exact concentration for selection after transformation could be determined. Table 4.1 shows that, the negative control (0 ug mL^{-1}) BBM plates had positive growth of *C. vulgaris* after the fourth day. This similar positive growth was also observed in BBM plates containing 25 ug mL^{-1}, 50 ug mL^{-1} and 100 ug mL^{-1} of chloroamphenicol. However, when *C. vulgaris* was exposed to BBM plates containg 200 ug mL^{-1} and 400 ug mL^{-1} of chloroamphenicol, there was completely no growth of *C. vulgaris* from the first day until the tenth day. These plates were left to grow for one month with no growth of *C. vulgaris* observed on the plates containing 200 ug $mL^{-1}$and 400 ug mL^{-1} of chloroamphenicol. Therefore, the inhibition results suggested that *C. vulgaris* was completely inhibited when exposed to chloroamphenicol concentration higher than 200 ug mL^{-1} inhibition when *C. vulgaris* was exposed to 200 ug mL^{-1} and 400 ug mL^{-1} of chloroamphenicol even after one month of observation.

Table 4.1 Growth observation of *Chlorella vulgaris* in triplicates during chloroamphenicol inhibition test.

Concentration of chloroamphenicol	**Day**					
	0	**2**	**4**	**6**	**8**	**10**
Negative control (0 ug mL^{-1})	---	+-+	+++	+++	+++	+++
25 ug mL^{-1}	---	+-+	+++	+++	+++	+++
50 ug mL^{-1}	---	++-	+++	+++	+++	+++
100 ug mL^{-1}	---	---	+++	+++	+++	+++
200 ug mL^{-1}	---	---	---	---	---	---
400 ug mL^{-1}	---	---	---	---	---	---

***** **+**: growth comparable to that of negative control grown in the absence of antibiotic;

-: no growth

The chloroamphenicol inhibition test performed in liquid BBM medium also showed similar results to the test performed using solid BBM medium. Figures 4.4 and 4.5 show the growth study of *Chlorella vulgaris* exposed to chloroamphenicol based on OD_{620} ($p<0.05$; $p=0.0213$) (Appendix 4a, c and e) and cell count ($p<0.05$; $p=0.0005$) (Appendix 4b, d and f). Both of these figures showed similar growth trend for all the different concentrations of chloroamphenicol tested. The results obtained from the chloroamphenicol inhibition test performed using BBM plates were merely based on observation and not quantitative. Nevertheless, the OD_{620} and cell count data obtained from the chloroamphenicol inhibition test performed using liquid BBM medium showed the gradual decrease in *C. vulgaris* growth as the concentration of chloroamphenicol increased (Figures 4.4and 4.5). When 25 ug mL^{-1} and 50 ug mL^{-1} were used, the growth of *C. vulgaris* was almost similar to the negative control (0 ug mL^{-1}). The growth of *C. vulgaris* decreased slightly at 100 ug mL^{-1} of chloroamphenicol and; at 200 ug mL^{-1} and 400 ug mL^{-1}, there was no growth of *C. vulgaris* detected. Figures 4.6 and 4.7 show that the specific growth rate of the cells decreased as the concentration of chloroamphenicol increased, based on data obtained from OD_{620} ($p<0.05$; $p=0.0151$) and the number of the cells ($p<0.05$; $p=0.0031$)(Appendix 5).

Based on the growth trends (Figures 4.4 and 4.5) and observations on chloroamphenicol-BBM agar plates (Table 4.1), the growth of *C. vulgaris* was inhibited before it reached its second cell generation (day 4) when it was exposed to 200 ug mL^{-1} and 400 ug mL^{-1} of chloroamphenicol. On the other hand, the LC_{50} analysis (Figure 4.8) suggested that 102.62 ug mL^{-1} of chloroamphenicol was the exact concentration that could lead to 50% of cell mortality (Appendix 4g).

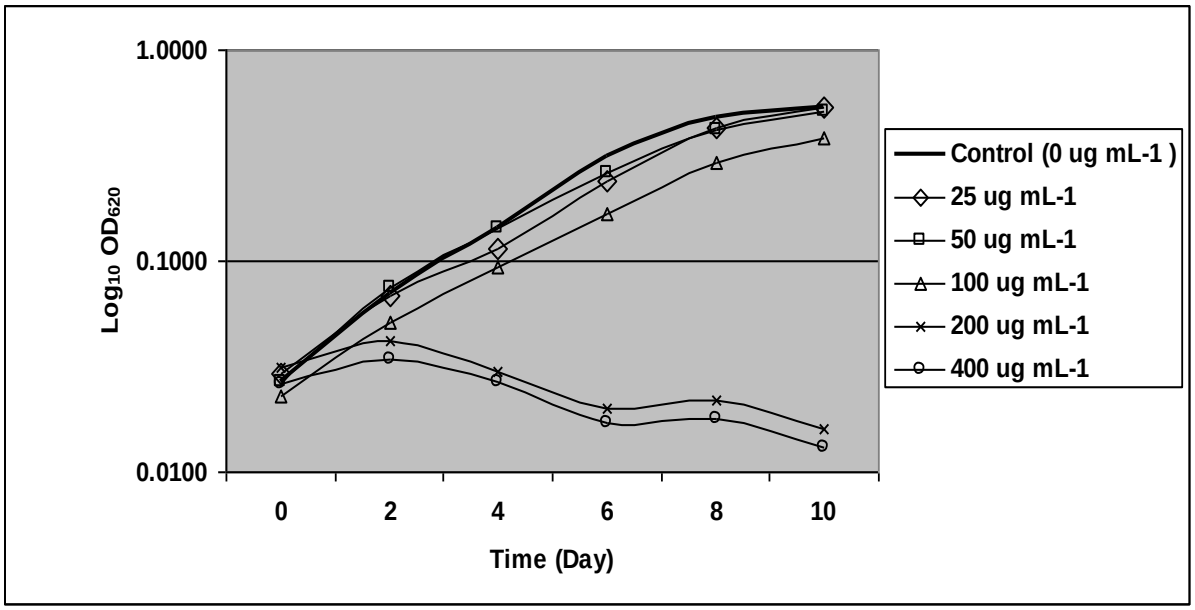


Figure 4.4 Semi-logarithmic growth curves of *Chlorella vulgaris* exposed to chloroamphenicol based on OD_{620}. The growth of *C. vulgaris* exposed 25 ug mL^{-1}, 50 ug mL^{-1} and 100 ug $mL^{-1}$of chloroamphenicol were almost similar to the negative control (0 ug mL^{-1}). There was a drastic decrease in the growth of *C. vulgaris* when it was exposed to 200 ug mL^{1} and 400 ug mL^{-1} of chloroamphenicol.

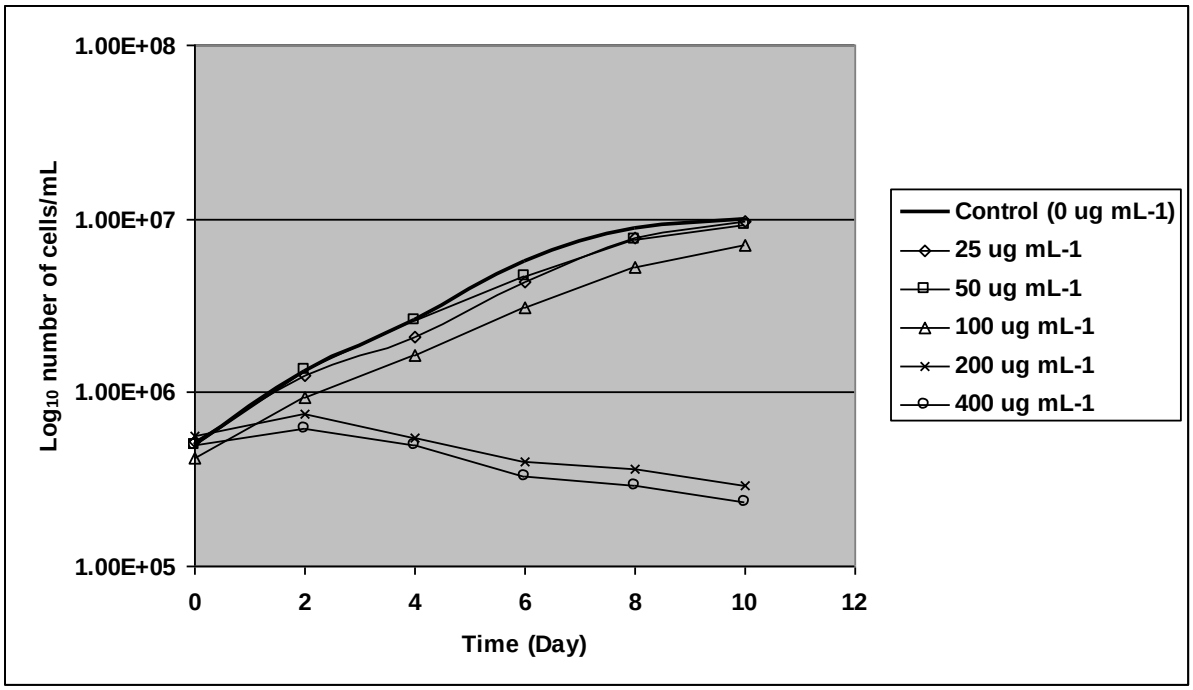


Figure 4.5 Semi-logarithmic growth curves of *Chlorella vulgaris* exposed to chloroamphenicol based on cell count. The growth of *C. vulgaris* exposed 25 ug mL^{-1}, 50 ug mL^{-1} and 100 ug mL^{-1} of chloroamphenicol were almost similar to the negative control (0 ug mL^{-1}). There was a drastic decrease in the growth of *C. vulgaris* when it was exposed to 200 ug $mL^{-1}$and 400 ug $mL^{-1}$of chloroamphenicol.

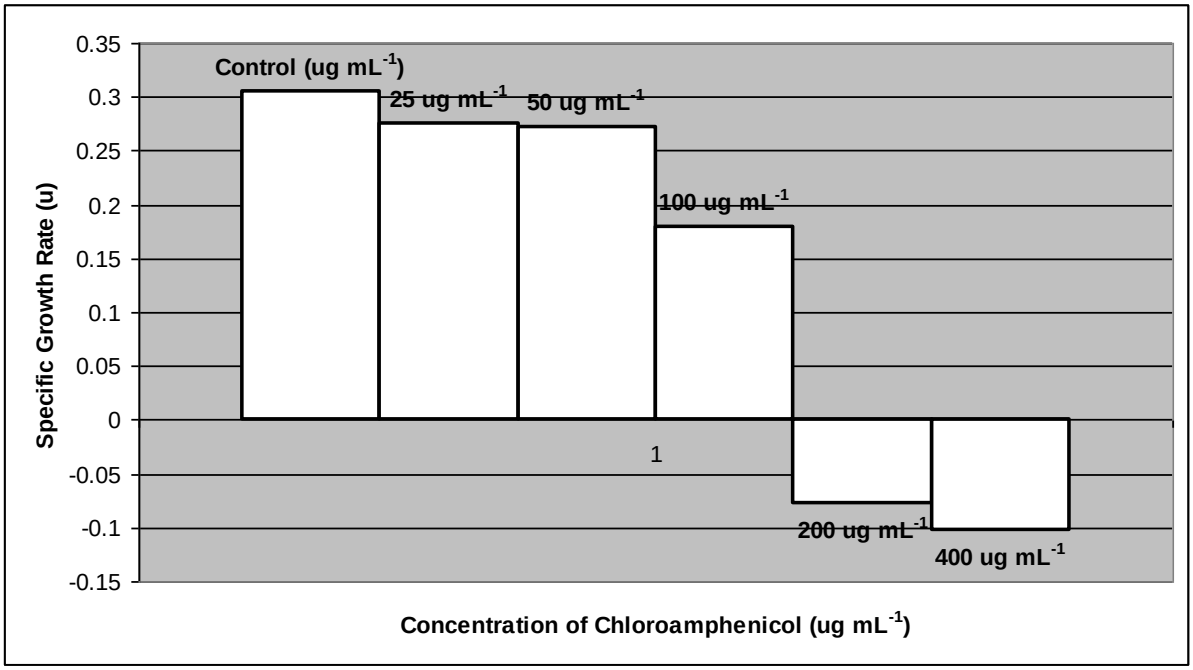


Figure 4.6 Specific growth rates of *Chlorella vulgaris* exposed to chloroamphenicol based on OD_{620}. Positive specific growth rates that was lower than the negative control (0 ug mL^{-1}) was obtained when *C. vulgaris* was exposed to 25 ug mL^{-1}, 50 ug $mL^{-1}$and 100 ug mL^{-1} of chloroamphenicol. There was no growth detected when *C. vulgaris* was exposed to 200 ug mL^{-1} and 400 ug mL^{-1}.

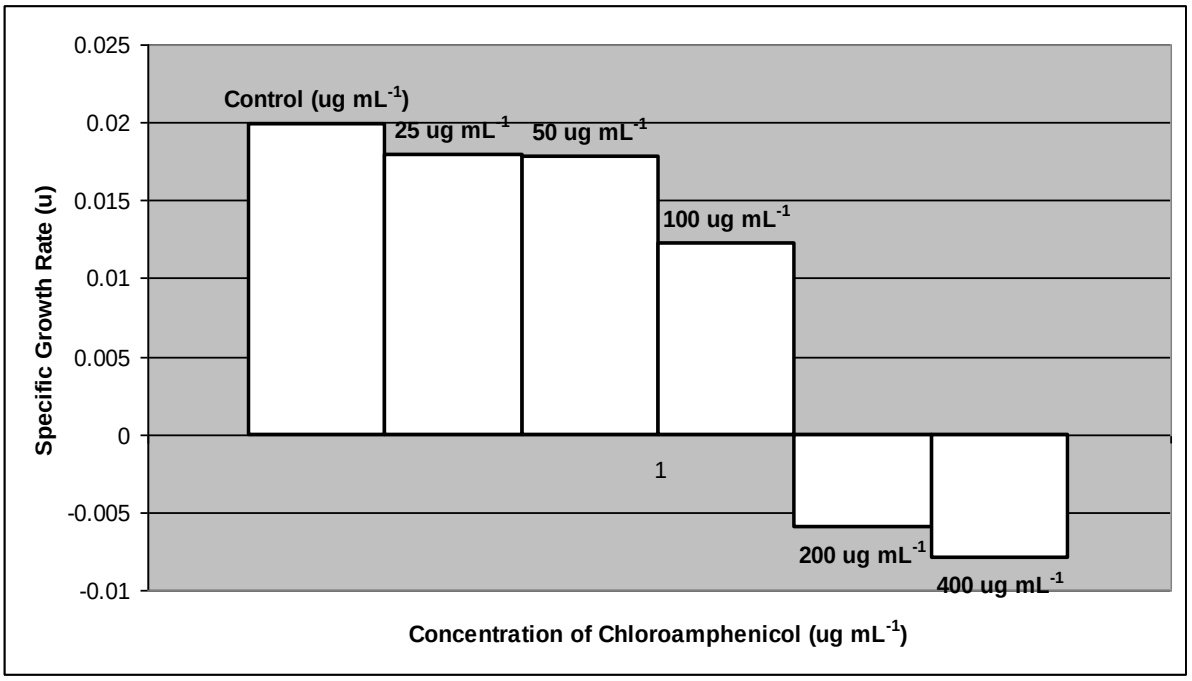


Figure 4.7 Specific growth rates of *Chlorella vulgaris* exposed to chloroamphenicol based on cell count. Positive specific growth rates that was lower than the negative control (0 ug/mL) was obtained when *C. vulgaris* was exposed to 25 ug mL^{-1}, 50 ug mL^{-1} and 100 ug mL^{-1} of chloroamphenicol. There was no growth detected when *C. vulgaris* was exposed to 200 ug mL^{-1} and 400 ug mL^{-1}.

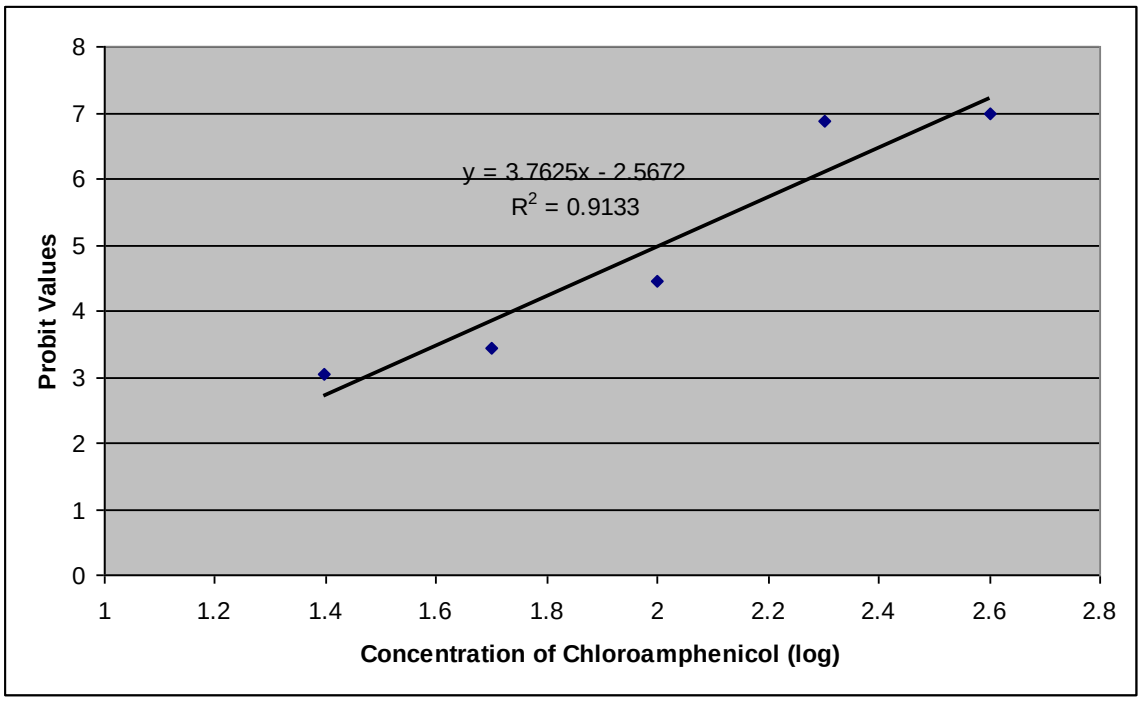


Figure 4.8 LC_{50} test of *Chlorella vulgaris* exposed to 25 ug mL^{-1}, 50 ug mL^{-1}, 100 ug mL^{1}, 200 ug $mL^{-1}$and 400 ug mL^{-1} of chloroamphenicol. Chloroamphenicol at the concentration of 102.62 ug mL^{-1}could cause 50% of cell mortality.

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

4.3.1 pCAT-HBSAg Vector

The fusion plasmid pCAT-HBSAg (6150 bp) with an insertion of a 1.38 Kb sized hepatitis B surface antigen (HBSAg) was successfully isolated using chloroform-phenol extraction method. The purity of the isolated plasmid using this method was between the ranges of 1.80 to 2.00. Figure 4.9 show the quality of the isolated pCAT-HBSAg plasmid sized of 6150 bp analyzed on 1.0 % (w/v) agarose gel electrophoresis in 1× TAE at 90 V for 45 min, respectively.

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

The bombarded and non-bombarded *Chlorella vulgaris* culture was bacterial-free because it showed no bacterial presence in the glucose test (section 3.4.4). Genomic DNA of the transformed *C. vulgaris* was successfully isolated using the chloroform-phenol extraction method. The purity of all the sample was between the range of 1.63 to 2.13 ±0.1662 ($p<0.05$; $p=0.0000$) (Appendix 6a); while the total yield of the genomic DNA isolated from 100 mg of sample ranged between 6.1577 µg to 184.5600 µg ±47.5430 ($p<0.05$; $p=0.0000$) (Appendix 6b). The average purity and total DNA yield of the samples were 2.01 ±0.1662 ($p<0.05$; $p=0.0000$) (Appendix 6a) and 54.3523 µg/100mg ±47.5430 ($p<0.05$; $p=0.0000$) (Appendix 6b), respectively. The quality and integrity of DNA samples were verified with 1.0% (w/v)

agarose gel electrophoresis in 1× TAE at 90 V for 45 min. A distinct genomic DNA band with no smearing was successfully obtained (Figure 4.10).

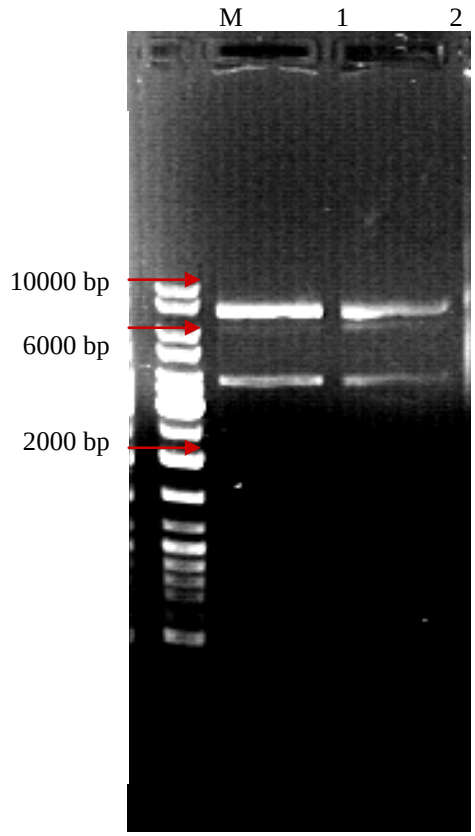


Figure 4.9 Agarose gel analysis of the isolated pCAT-HBSAg plasmid (6150 bp). Lanes 1 to 2 contained the isolated pCAT-HBSAg plasmid DNA; while M contained the 1Kb plus DNA marker (Crystalgen Incorporated, USA)

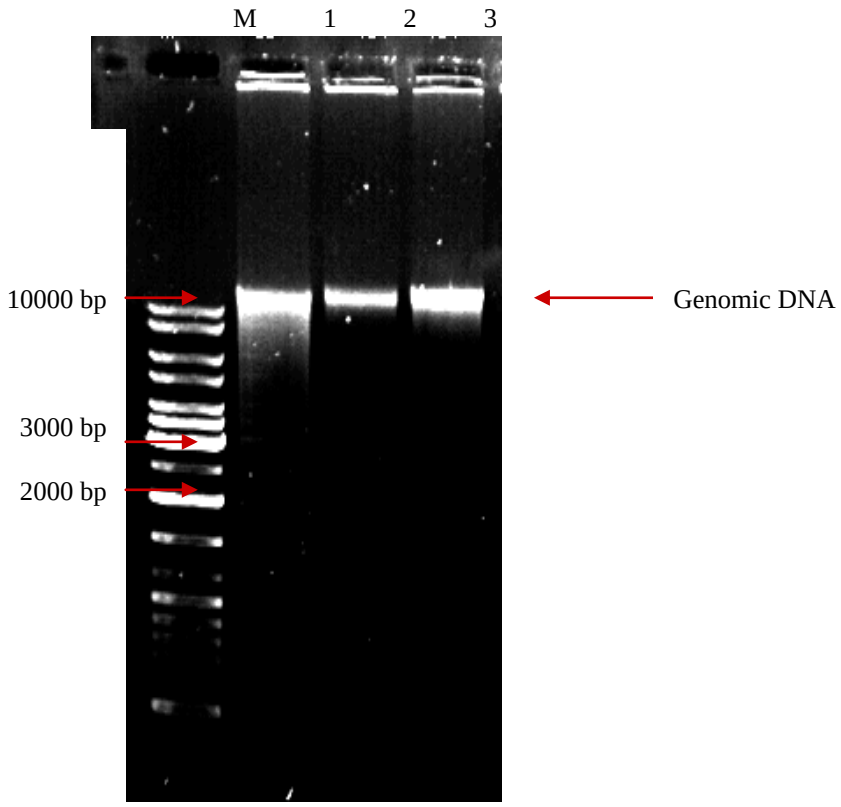


Figure 4.10 Agarose gel electrophoresis of the genomic DNA isolated from *Chlorella vulgaris.* A distinct genomic DNA band was successfully obtained using chloroform-phenol method in lanes 1 to 3; while M contained the 1 Kb plus DNA marker (Crystalgen Incorporated, USA).

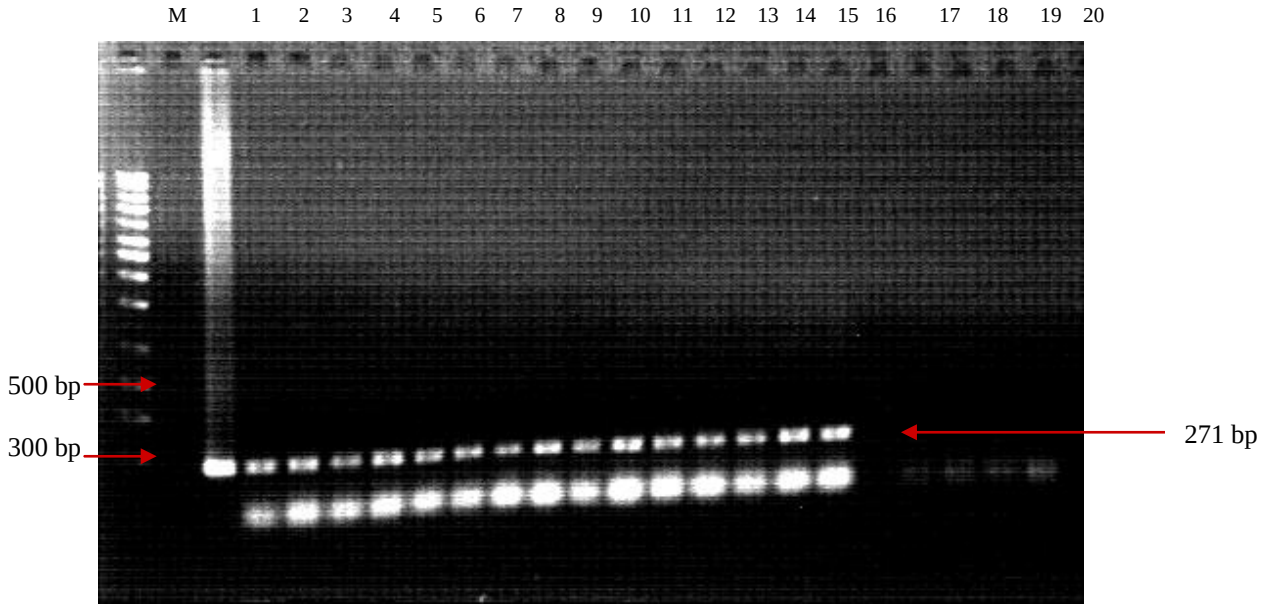
4.3.2.a Polymerase Chain Reaction (PCR) and Southern Blot Analysis

The presence of the gene of interest was verified through PCR using primers that amplified the partial sequence of hepatitis B surface antigen gene. Figure 4.11(a) shows the agarose gel electrophoresis verification of the putative PCR band sized 271 bp that was produced from the PCR amplification of primers F_{HBSAg} (5'-GTG GTG GAC TTC TCT CAA TTT TC-3') and R_{HBSAg} (5'-GCA GGT TTT GCA TGG TCC CGT AC-3'). Two negative controls were set up which were reaction mixtures of the non-transformed *C. vulgaris* DNA and reaction mixture without any DNA template; while the positive control was the HBSAg amplified product from the pCAT-HBSAg vector. The expected size PCR product of the HBSAg gene was obtained from only the genomic DNA of transformed *C. vulgaris*, but not from DNA of untransformed cells (Figure 4.11 (a)). These HBSAg PCR amplified bands were transferred to a nitrocellulose blot and detected via North2South Biotin Chemiluminescent Kit (Pierce Biotechnology, USA). The HBSAg PCR amplified product from the pCAT-HBSAg plasmid was labelled with biotin. The biotin labelled HBSAg probe was able to detect the HBSAg PCR product amplified from the transformed genomic DNA of *C. vulgaris* (Figure 4.11 (b)). There was no detection of the HBSAg gene in all the negative control lanes Figure 4.11 (b). Sequencing results further confirmed that HBSAg PCR products (271 bp) obtained were the HBSAg gene (Appendix 10a).

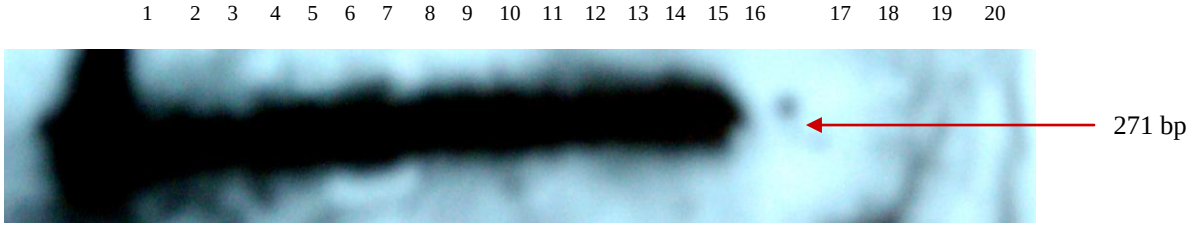
The transformation efficiency of each parameter used for bombardment was analysed by calculating the algae growth area which is the percentage of coverage of the BBM with chloroamphenicol agar surface (Appendix 7b). The two way ANOVA test suggested that there were no differences in the transformation efficiency when

different distances ($p<0.05$; $p=0.6523$) and rupture disk pressure ($p<0.05$; $p=0.8641$) or even the combination of both pressure and distance ($p<0.05$; $p=0.1056$) that were used during bombardment (Appendix 7c). Twenty transformed *Chlorella vulgaris* clones of each parameter were selected for PCR analysis. The average number of clones that gave positive PCR results was 16 clones. The presence of the HBSAG gene of these clones were detected in the 22nd (day 44), 32nd (day 64), 42nd (day 84), 53rd (day 106), 63rd (day 126), 69th (day 138), 72nd (day 144), 82nd (day 164) and until the 92nd (day 184) cell generation (Appendix 7a) via PCR analysis.

Southern-blot analysis was conducted by digesting the genomic DNA of *C. vulgaris* with restriction enzyme, Hind III (Figures 4.12). When total DNA of the transformed *C. vulgaris* was digested with Hind III (Promega, USA) for three days, genomic DNA bands were detected from the digested transformed genomic samples, as a result of incomplete digestion (Figure 4.13). The probe that was used for this detection was the biotin labelled HBSAg gene. The transformed genomic DNA bands of bombarded *C. vulgaris* samples that were obtained in blots were in its 92nd cell generations (Figure 4.13).



(a)



(b)

Figure 4.11 PCR amplification and southern blot assay of the HBSAg gene amplified from the genomic DNA of *Chlorella vulgaris*. (a) Agarose gel electrophoresis verification of the HBSAg PCR products (271 bp). (b) Detection of the HBSAg gene using biotin labelled HBSAg gene as probes. Lane 1 was the positive control (pCAT-HBSAg), lanes 2 to 16 were the HBSAg gene detected from the transformed *C. vulgaris*, lanes 17 to 18 were the negative controls with untransformed DNA, lanes 19-20 were the negative controls without any DNA; while M represents the 1 Kb plus DNA marker (Crystalgen Incorporated, USA). The HBSAg PCR product (271 bp) was successfully amplified using the FHBSAg and RHBSAg primers, and detected using HBSAg biotin labelled probe.

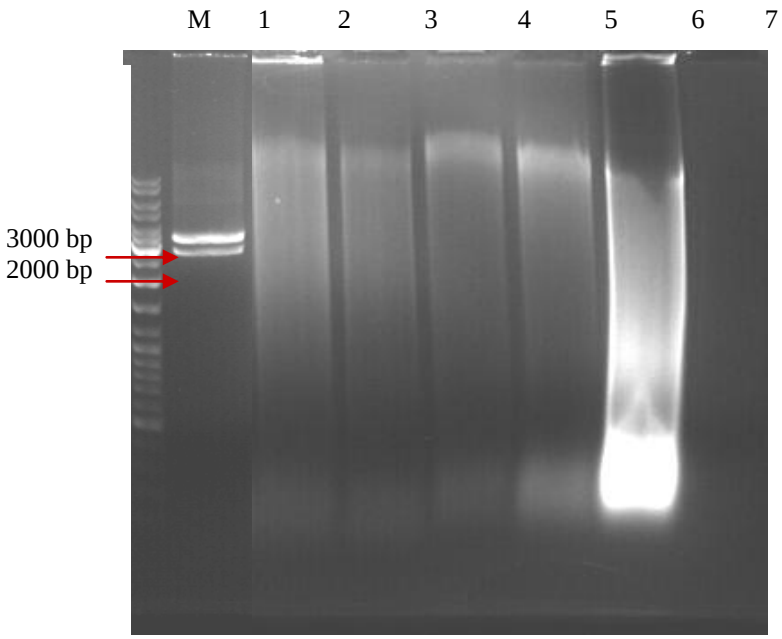


Figure 4.12 Hind III digestion of the genomic DNA of *Chlorella vulgaris* analyzed using agarose gel electrophoresis. Lane 1 was the undigested pCAT-HBSAg plasmid; lanes 2 to 6 were the Hind III digested transformed *C. vulgaris* samples; lane 7 was the Hind III digested untransformed *C. vulgaris* DNA; while M contained the 1 Kb plus DNA marker (Crystalgen Incorporation, USA).

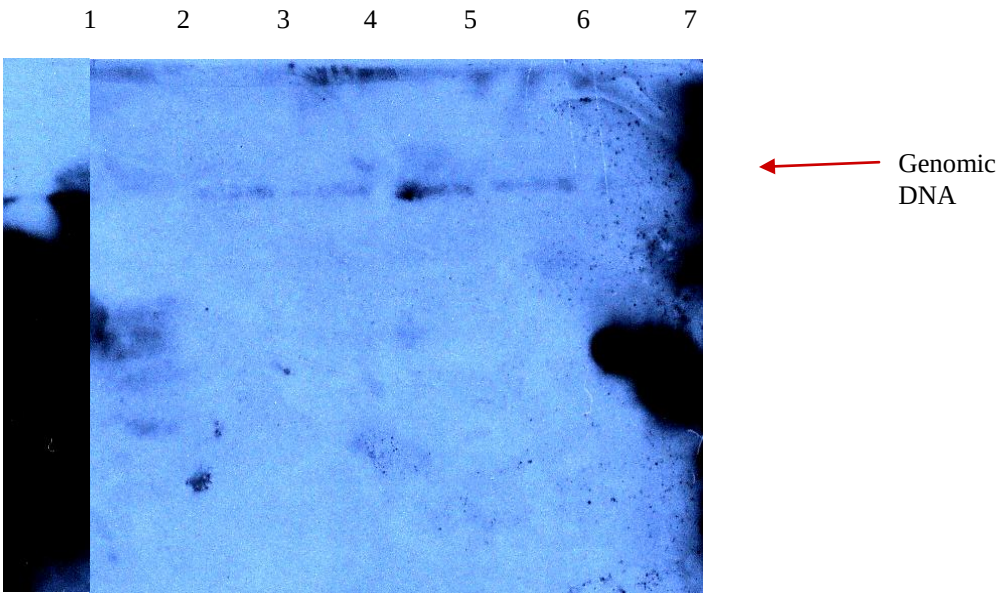


Figure 4.13 Southern blot analysis of the genomic DNA of *Chlorella vulgaris*. Lane 1 was the undigested pCAT-HBSAg plasmid; lanes 2 to 6 were the Hind III digested transformed *C. vulgaris* samples; and lane 7 was the Hind III digested untransformed *C. vulgaris* DNA. The genomic DNA band of four digested transformed samples (lanes 3 to10) were detected from the partially digested genomic DNA at the top of the membrane using biotin labelled HBSAg probe. The transformed and untransformed samples used for this blot were in the 92nd cell generations.

4.3.3 Total RNA Isolation and RT-PCR Analysis of *C. vulgaris* Clones Transformed with the pCAT-HBSAg Vector

Total RNA of transformed and untransformed *Chlorella vulgaris* were successfully isolated using the Trizol Reagent (Invitrogen Corporation, USA). The purity of the extracted samples were between the range of 1.51 to 1.96 ±0.1770 ($p<0.05$; $p=0.0000$) (Appendix 8a); while the total yield of the RNA ranged from 28.49 µg to 121.68 µg ($p<0.05$; $p=0.0010$) (Appendix 8b). The average purity and total RNA yield of *C. vulgaris* samples were 1.78±0.1770 ($p<0.05$; $p=0.0000$) and 64.4944 µg±33.2490 ($p<0.05$; $p=0.0010$), respectively (Appendix 8). The quality and integrity of the RNA samples were verified using 1.0 % agarose gel electrophoresis. Figure 4.14 shows that two distinct bands of 28S and 18S with no smearing were obtained. The total RNA isolated was subjected to a DNase treatment to eliminate DNA contamination prior to RT-PCR. RNA samples were transcribed to cDNA using Superscript III First Strand Synthesis System (Invitrogen Corporation, USA) and then a PCR was performed using the transcribed cDNA. A 271 bp PCR product of the HBSAg gene was obtained in all transformed samples (Figure 4.15). There was no amplification in the controls which consisted of DNase 1 treated total RNA of untransformed and transformed *C. vulgaris* samples as well as the untransformed cDNA samples. The HBSAg PCR product was also blotted onto a nitrocellulose membrane and detected using a biotin labelled HBSAg probe. Figure 4.16 shows that the 271 bp HBSAg gene was successfully detected using the biotin labelled HBSAg probe. Sequencing results of 271 bp HBSAg PCR band obtained from transformed cDNA samples further proved that it was the HBSAg gene (Appendix 10b). The RNA samples used in this research were extracted from *C. vulgaris* cultures that were at the 10^{th} cell generations (day 20).

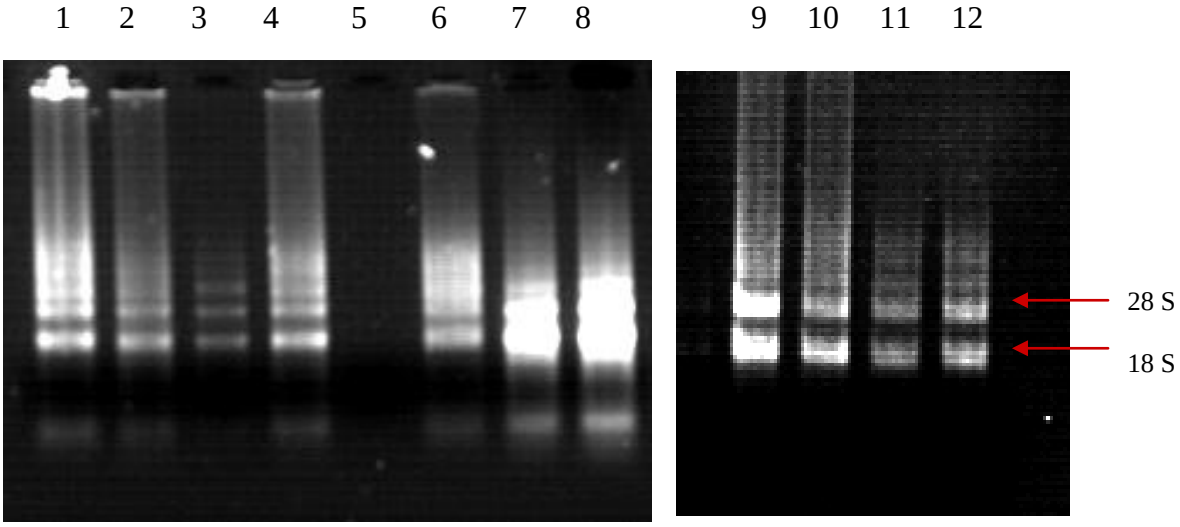


Figure 4.14 Agarose gel electrophoresis of the total RNA isolated from *Chlorella vulgaris.* Lanes 1 to 3 were the untransformed RNA samples; lanes 4 to 12 were the transformed RNA samples. Two distinct bands of the 28S and 18S were obtained using Trizol reagent (Invitrogen Corporation, USA).

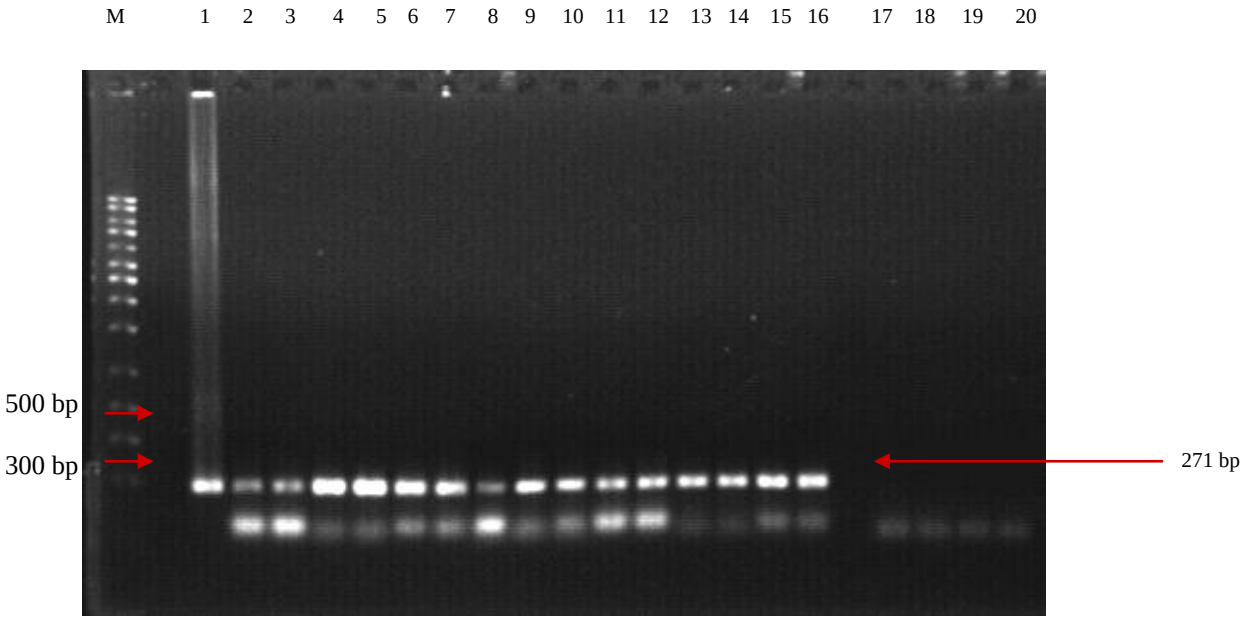


Figure 4.15 PCR amplification of the HBSAG gene (271bp) from cDNA of *Chlorella vulgaris*. Lane 1 was the positive control (pCAT-HBSAg), lanes 2 to 16 were the HBSAg gene detected from the transformed *C. vulgaris* samples, lane 17 to 18 were the negative control with untransformed DNA, lanes 19 to 20 was the negative control without any DNA; while M contained the 1 Kb plus DNA marker (Crystalgen Incorporated, USA). A 271 bp band of the HBSAg gene was successfully amplified from the transformed cDNA *C. vulgaris* samples (lanes 2 to 16). There was no HBSAg gene detected in any of the negative controls (lanes 17 to 20).

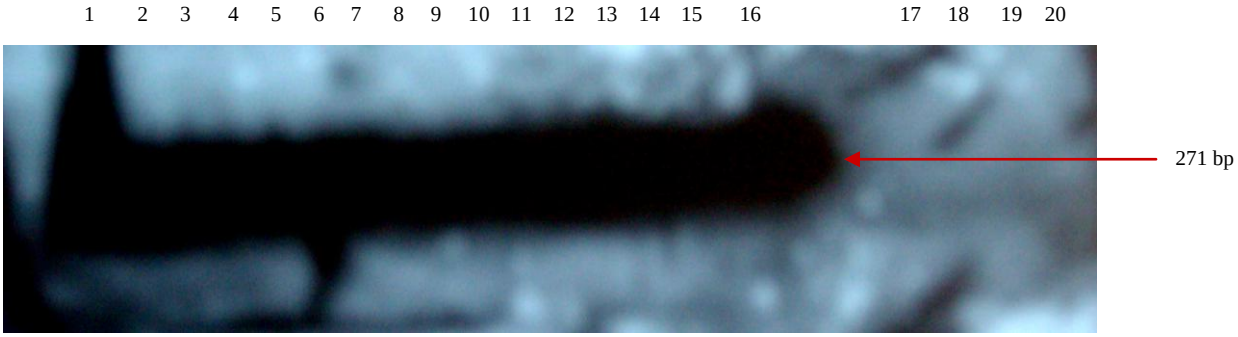


Figure 4.16 Southern blot assay of the HBSAg gene from cDNA of *Chlorella vulgaris*. Lane 1 was the positive control (pCAT-HBSAg), lanes 2 to 16 were the HBSAg gene detected from the transformed *C. vulgaris* samples, lane 17 to 18 were the negative control with untransformed DNA, lanes 19 to 20 was the negative control without any DNA. A 271 bp band of the HBSAg gene was successfully detected from the transformed cDNA *C. vulgaris* samples using a biotin labelled HBSAg probe (lanes 2 to 16). There was no HBSAg gene detected in any of the negative controls (lanes 17 to 20).

4.3.4 Protein Separation and Western Blot Analysis of *C. vulgaris* Clones Transformed with the pCAT-HBSAg Vector

The concentrations of crude proteins extracted from an average wet weight of 0.0927g of *Chlorella vulgaris* cultures (OD_{620} = 0.5) were between the range of 209.3750 ug to 310.9375 µg ±17.4226 ($p<0.05$; $p=0.0000$) (Appendix 9a). The average concentration of proteins from untransformed and transformed samples were 228.9065µg±16.5725 ($p<0.05$; $p=0.0326$) and 258.4375µg±36.5167, ($p<0.05$; $p=0.0000$) (Appendix 9b and c), respectively. Figure 4.17 shows the separated proteins of the transformed and untransformed *C. vulgaris* in a 12% SDS-PAGE gel. The HBSAg protein was observed by transferring the protein to a nitrocellulose membrane and detecting using a Mouse anti-pre S2 (Hepatitis B virus) monoclonal antibody (Chemicon International Incorporated, USA) which reacted with the large and middle HBSAg proteins that was amino acid positions 132-137, adw nomenclature. A single band sized 47 kDa was observed in the western blot analysis of protein isolated from transformed *C. vulgaris* at 42^{nd} (day 84) (Figure 4.18 (a)) and 50^{th} (day 100) (Figure 4.18 (b)) cell generations, but no bands were present in untransformed lanes.

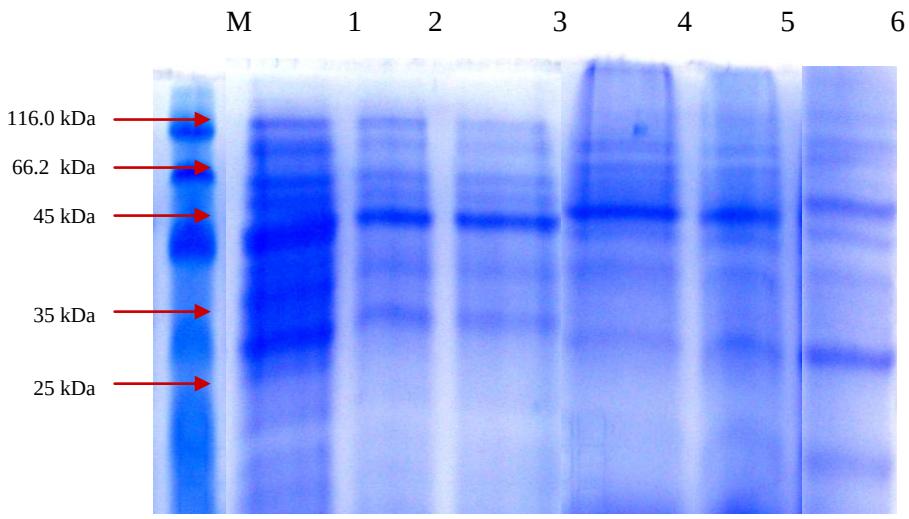
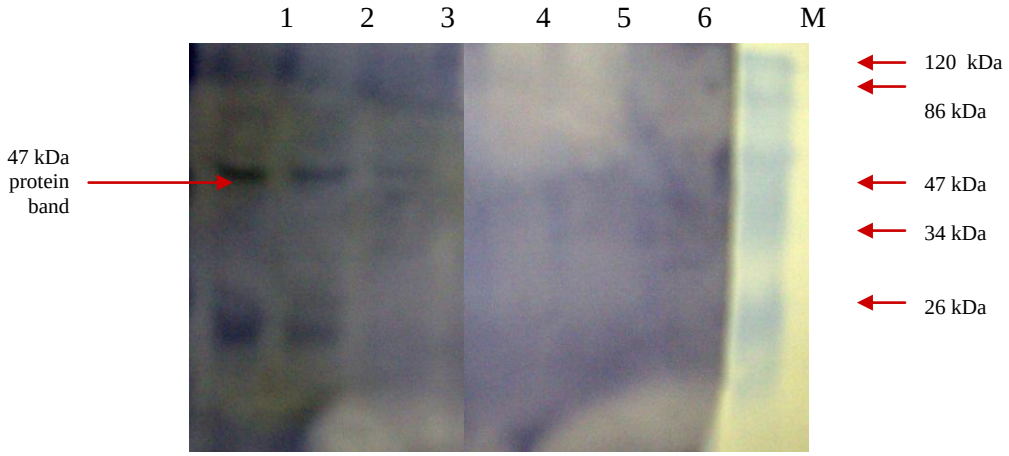
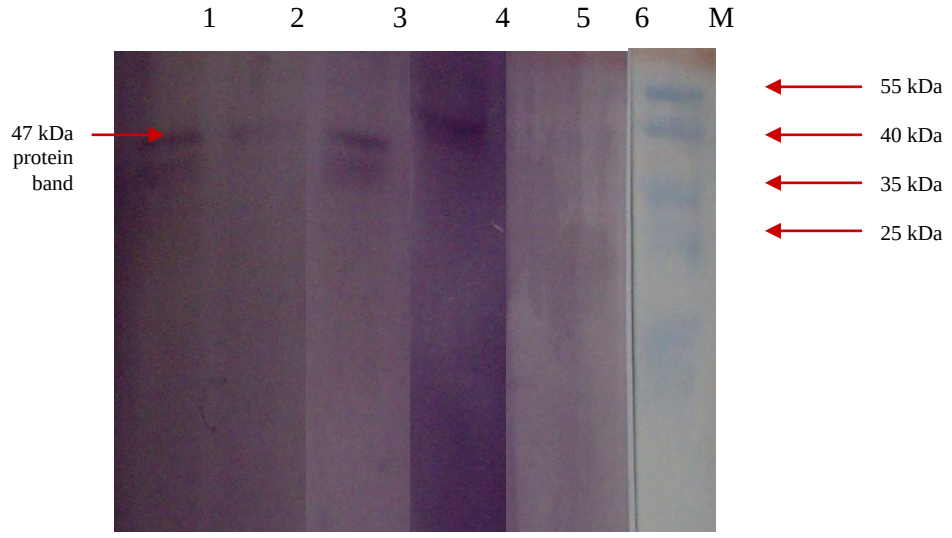


Figure 4.17 The separation of crude protein extract of untransformed and transformed *Chlorella vulgaris* using a 12% SDS-PAGE gel. (a) Lanes 1 to 3 were the untransformed *C. vulgaris* protein; lanes 4 to 6 were the transformed *C. vulgaris* protein; while M contained the unstained protein molecular weight marker (Fermentas International Incorporated, Canada). There was an equal accumulation of protein between the transformed and untransformed *C. vulgaris* protein sample.



(a)



(b)

Figure 4.18 Western Blot analysis of the protein transferred from SDS-PAGE gel to nitrocellulose membranes. (a) Lanes 1 to 3 were the transformed protein sample of *Chlorella vulgaris*; lanes 4 to 6 were the untransformed protein samples of *C. vulgaris*; while M contained the prestained protein molecular weight markers (Fermentas International Incorporated, Canada). (b) Lanes 1 to 4 were the transformed protein samples; lanes 5 to 6 were the untransformed protein samples; while M contained the Page Ruler Prestained Protein Ladder (Fermentas International Incorporated, Canada). A distinct 47kDa protein band was detected by the Mouse anti-pre S2 (Hepatitis B virus) monoclonal antibody (Chemicon International Incorporation, USA) in figure (a) lanes 1 to 3 and figure (b) lanes 1 to 4.