CHAPTER 3

MATERIALS AND METHODS

3.1 *Chlorella vulgaris* UMACC 001

Species of *Chlorella* are widespread in freshwater and in the sea, air and soil. Phang and Chu (2004) reported a total of 150 microalgal strains are deposited in the University of Malaya Algae Culture Collection (UMACC) and *Chlorella vulgaris* UMACC 001 is one of the most studied species. Strain *C. vulgaris* UMACC 001 (Beijerink strain), from the UMACC, was isolated from ponds containing digested palm oil mill effluent. This microalgal strain has the ability to grow under autotrophic, mixotrophic and heterotrophic conditions (Phang and Chu, 2004). Phang and Chu (2004) reported that *C. vulgaris* UMACC 001 is also tolerant to high concentrations of heavy metals like manganese, chromium, zinc and cadmium; therefore it has potential applications in wastewater treatment and removal of metals. Besides that, *C. vulgaris* UMACC 001 was reported to be tolerant to high levels of nitrogen (Phang,1990), which showed that this microalgal strain can be used in the treatment of effluents in rubber production. *C. vulgaris* UMACC 001 is the strain used in this research.

3.2 Growth Characterization of *C. vulgaris*

Chlorella vulgaris was cultured and maintained in the Algae Research Laboratory, Institute of Postgraduate Studies, University of Malaya. The microalgal culture was cultured in Bold's Basal Medium (BBM) (Nichols and Bold, 1965) (Appendix 1) at 25°C and 40 µmol $m^{-2}s^{-1}$. The microalgal growth was studied by monitoring the cell density as OD_{620} using a spectrophotometer (Shimadzu Corporation, Japan), and performing cell count using a brightfield microscope (Leica Microsystem Ac, Germany). Based on the OD_{620} and cell count data of the cell biomass, a semi logarithmic growth curve was plotted. The specific growth rate of this strain was calculated by determining the exponential phase from the plotted growth curve. Two points of the each end of the exponential phase were identified and substituted into the equation below.

$$\text{Specific Growth Rate, } \mu = \frac{\ln X_1 - \ln X_2}{t_1 - t_2}$$

Whereby X_1 and X_2 is the cell biomass (OD_{620} or cell count) at time 1 (t_1) and time 2 (t_2) respectively.

The generation time was also calculated once the specific growth rate was known by using the equation below.

$$\text{Generation Time} = \frac{\text{Ln } 2}{\mu}$$

3.3 Chloroamphenicol Inhibition Test of *C. vulgaris*

Chlorella vulgaris at the exponential phase was exposed to chloroamphenicol (Sigma Aldrich, USA), at various concentrations ranging from 50 µg mL^{-1} to 400 µg mL^{-1} in liquid and agar BBM medium to determine the optimum antibiotic concentration for selection after transformation. The cells on agar plates and in liquid medium were observed ten days. The chloroamphenicol inhibition test was performed in triplicates for each concentration and LC_{50} values were determined based in OD_{620} and cell count data. LC_{50} is the median concentration that is lethal to 50% of the test organism and the Probit Method (Ashton, 1972) was used to calculate this value which is a method based on regression of the probit of mortality on the log of concentration.

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

3.4.1 Sample Preparation

Prior to genetic transformation, *C. vulgaris* was cultured until it reached exponential phase (OD_{620} = 0.2). Then, 50 mL of this *C. vulgaris* culture was harvested onto a 0.2 µm nitrate cellulose membrane (Whatman, USA) using a millipore filter apparatus. The filtered samples were kept in moist condition by dripping a few drops of BBM medium before bombardment.

3.4.2 Plasmid Isolation

The pCAT-HBSAg plasmid was used to transform *Chlorella vulgaris* UMACC 001 to study the integration and expression of hepatitis B surface antigen gene in *C. vulgaris*. The fusion plasmid pCAT-HBSAg (6150 bp) was derived from the pCAT-control vector (Promega, USA), with an insertion of a 1.38 Kb sized hepatitis B surface antigen (HBSAg) gene located at the down stream of the chloroamphenicol acetyl transferase (CAT) gene, which was driven by the SV40 promoter (Figure 3.1).

The pCAT-HBSAg plasmid was isolated using chloroform-phenol. *Escherichia coli* strain DH5-α that carried the pCAT-HBSAg plasmid was cultured in LB medium containing (50 mg mL^{-1}) kanamycin and incubated at 37°C, for 16 hours under constant agitation. A total of 10 mL of bacterial culture was harvested by centrifugation at 13,000 rpm for 5 min at 4ºC and the supernatant was decanted. The pellet was then completely resuspended in 150 µL of Solution I before being lysed in 250 µL Solution II. Then, a volume of 250 µL Solution III was added and immediately mixed by inverting the tube four times. Finally, the bacterial lysate was centrifuged at 13,000 rpm for 10 min at room temperature.

The supernatant was transferred to a new 1.5 mL centrifuge tube containing 700 µL isopropanol, mixed and centrifuged at 13,000 rpm for 5 min at room temperature. Then, supernatant was discarded and the pellet was washed with 500 µL absolute ethanol and centrifuged at 13,000 rpm for 1 min. After decanting the supernatant, the pellet was left to dry in a vacuum desiccator.

Then, the pellet was resuspended in 80 µL of TE (pH 8.0) and 10 µL of RNase A (10mg/mL) was added to the suspension and incubated at 70°C for 10 min followed by another 30 min incubation at room temperature. Then, 10 µL 3N sodium acetate, 50 µL buffer saturated phenol and 50 µL chloroform isoamly alcohol were added to the mixture and vortexed for 20 sec before submitting to centrifugation at 13,000 rpm for 5 min. The aqueous solution was transferred to a new 1.5 mL centrifuge tube containing 100 µL of 70% Ethanol and centrifuged again at 13,000 rpm for 5 min. The supernatant was decanted and the plasmid was left to dry in a vacuum desiccator. Finally, the plasmid was eluted in 100 µL TE (pH 8.0) and was stored at 20ºC until used. The quantity and purity of the plasmid were determined by a biophotometer (Eppendorf, Germany) at OD_{260} and OD_{280}.

3.4.3 Particle Bombardment

Prior to particle bombardment, gold particle (Bio-Rad Laboratories, USA), which has a diameter size of 1.0 µm, was coated with the pCAT-HBSAg comprising the hepatitis B surface antigen gene. Fifty microliters of gold particle solution (60 mg mL^{-1}) was mixed with 2 µL of a plasmid DNA solution (1 µL $µg^{-1}$), 50 µL of 2.5 M $CaCl_2$, and 20 µL of 0.1M spermidine. The mixture was vortexed, resuspended in 250 µL 100% Ethanol and centrifuged briefly for 10 s. Finally, 10 µL of gold-DNA particle was layered on a macrocarrier for bombardment. *C. vulgaris* cultures that were at a the exponential phase were bombarded in duplicates using Bio-Rad PDS-1000/He Biolistic Particle Delivery System (Bio-Rad

Laboratories, USA) at rupture disc pressure of 900 psi and 1100 psi at distances of 6 cm and 9 cm.

3.4.4 Selection of Putative Clones using Chloroamphenicol

The bombarded and non-bombarded (control) *Chlorella vulgaris* were inoculated into BBM liquid medium devoid of antibiotics for 16 hours at 25°C in the dark. Then, the cells were concentrated by medium centrifugation at 1000 rpm for 10 min at room temperature, before spreading the cells onto BBM agar plates containing 200 µg mL^{-1} of chloroamphenicol. Both of these cultures (bombarded and non-bombarded *C. vulgaris*) were also streaked on BBM agar medium supplemented with 0.5% glucose to test for bacterial growth.

The selection plates were left to grow at 25°C for two and half weeks. The algae growth area which is defined as the percentage of coverage of the BBM with chloroamphenicol agar surface was calculated to determine the transformation efficiency for each bombarded parameter. Single colonies that appeared on the plates were picked and inoculated into BBM liquid medium devoid of antibiotics for genetic and expression analyses. The cultures were maintained for further stability and expression analyses.

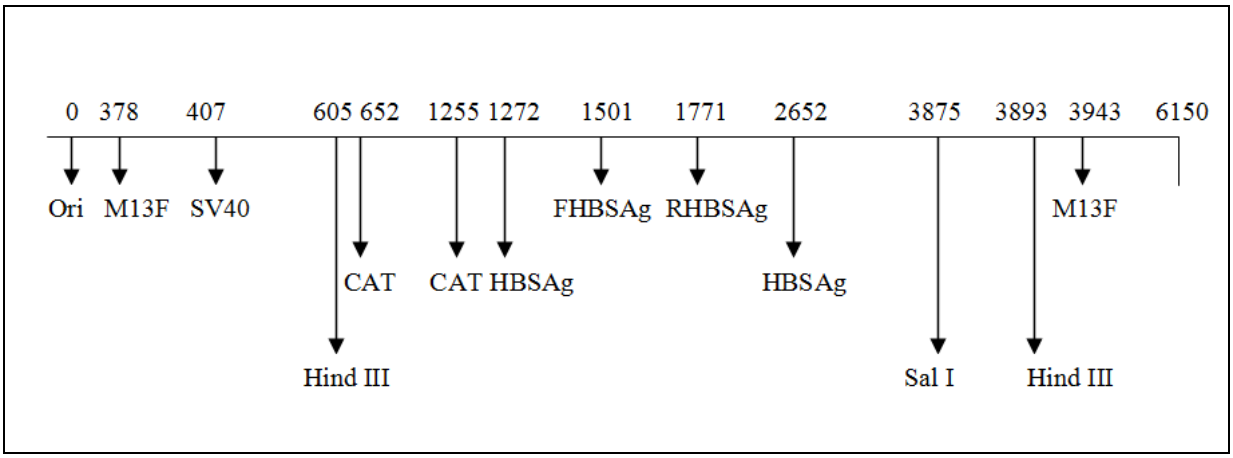

Figure 3.1 Schematic representation of the pCAT-HBSAg vector. The HBSAg gene and CAT gene in this vector was located at the downstream of the SV40 promoter (407 nt). The size of the HBSAg gene was 1.38 Kb (1272 nt – 2652 nt). The FHBSAg and RHBSAg (1501 nt – 1771 nt) were the forward and reverse primers that partially amplified the HBSAg gene.

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

3.5.1 Genomic DNA Extraction

Once the single colonies from section 3.4.4 were visible as green colored colonies, they were cultured in BBM medium until they reached exponential phase (OD_{620} = 0.2), which was normally on the fourth day. One hundred milligram of *Chlorella vulgaris* (OD_{620} = 0.2) was harvested by centrifugation at 10,000 rpm for 10 min at room temperature. The total DNA was lysed in 550 µL lysis buffer (0.1 M Tris-HCL, 0.05 M EDTA, 0.5 M NaCl and 1% BME) and homogenized by using a mortar and pestle for 3 min. Three microliter of RNase A (100 mg mL^{-1}) and 35 µL of 20% SDS were added to the lysate and the microcentrifuge tube was inverted for five times before incubating at 65ºC for 1 hr. The protein was precipitated with 170 µL 5 M KAc and the microcentrifuge tube was inverted slowly for five times before incubating again in ice for 20 min. Then, 600 µL of chloroform:isoamyl (24:1) was added to eliminate the protein and the microcentrifuge tube was inverted for five times until the contents were well mixed. The mixture was centrifuged at 10, 000 rpm at 4°C for 10 min. The supernatant that contained the DNA was transferred into a clean microcentrifuge tube containing 500 µL of chilled isopropanol. The solution was gently mixed by inversion until thread-like strands of DNA formed a visible mass followed by centrifugation at 10,000 rpm for 10 min at 4ºC. The supernatant was decanted and the pellet was washed with 500 µL of 70% ethanol at room temperature by gentle inversion. The DNA was recovered by centrifugation at 10,000 rpm for 3 min at 4ºC. The ethanol was carefully aspirated by using a micropipette before inverting the tube onto clean absorbent paper and air-drying the pellet for 30 min. Then, the DNA was dissolved in 50 µL TE (pH 8.0) at 65ºC. The DNA was stored at –20ºC until used.

The quantity and purity of the genomic DNA were determined by a biophotometer (Eppendorf, Germany) at OD_{260} and OD_{280}. The ratio between the absorbance values at 260 nm and 280 nm gave an estimate of the DNA purity. The quality and integrity of the DNA sample were also verified with 1.0 % (w/v) agarose gel electrophoresis in 1× TAE buffer at 90V for 45 min. The genomic bands were viewed and photographed using AlphaImager ™ 2200 (Alpha Innotech Corporation, USA).

3.5.2 Polymerase Chain Reaction (PCR)

Two pairs of PCR primers were synthesized by Bio Basic Inc. (Malaysia). Partial HbsAg fragment (271 bp) was amplified by 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'). The 25 µL PCR reaction consisted of 2.5 µL 10× PCR buffer, 0.5 µL $MgCl_2$ (100 mM), 0.4 µL dNTP mix (10 mM) (Bioron, Germany), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2U *Taq* DNA Polymerase (Bioron, Germany), 1 µL genomic DNA (0.5 ng/µL) and 18.2 µL sterile deionized water. The PCR conditions were performed as follows: 5 min at 94ºC for pre-denaturation, 1 min at 94ºC to denature the double stranded DNA strand, 1 min at 55ºC to anneal the DNA and 2 min at 72ºC to extend the PCR amplified product. The denaturation, annealing and extension steps were repeated for 35 cycles. This was followed by a final extension at 72ºC for 10 min. The PCR products were analyzed with 1.2% (w/v) agarose gel electrophoresis in 1× TAE buffer at 90V for 45 min and viewed using AlphaImager ™ 2200 (Alpha Innotech Corporation, USA). Then, the putative PCR amplified bands were excised from the gel for further analysis.

The putative DNA fragments from PCR were purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to supplier's protocol. The excised gel slice was dissolved in Buffer QG at a ratio of 300 µL of solution per 100 mg of

agarose gel slice. The mixture was vortexed every few minutes during the 10 min incubation at 50ºC to increase the rate of agarose melting until the gel slice was completely dissolved.

Then, 1mL of isopropanol was added to the dissolved gel before transferring into the QIAquick spin column and centrifuged at 10,000 rpm for 1 min at room temperature. The flow through was discarded and the column was added with 500 µL Buffer QG again and centrifuged at 10,000 rpm for 1 min at room temperature. Then the column was washed with 750 µL of Buffer PE and followed by centrifugation at 10,000 rpm for 1 min at room temperature. Fifty microlitre of Buffer EB was applied to the column to elute the DNA and centrifuged at 10,000 rpm for 1 min at room temperature. Finally, the eluted DNA was sequenced (1st Base, Malaysia) using FHBSAg (5'-GTG GTG GAC TTC TCT CAA TTT TC-3') and RHBSAg (5'-GCA GGT TTT GCA TGG TCC CGT AC-3') primers to confirm that the amplified PCR fragment was the desired HBSAg gene.

The putative PCR products obtained were also blotted directly to a nitrocellulose positively charged membrane to perform PCR-Southern blot as described in the section 3.5.3. The probe that was used for detection was prepared from the PCR product of the pCAT-HBSAg vector using the FHBSAg (5'-GTG GTG GAC TTC TCT CAA TTT TC-3') and RHBSAg (5'-GCA GGT TTT GCA TGG TCC CGT AC-3') primers.

3.5.3 Southern Blot Analysis

Genomic DNA of the transformed positive clones and untransformed DNA were digested four days with restriction enzyme, Hind III (Promega, USA). Hind III cuts the pCAT-HBSAg vector at two sites at 605 nt and 3893 nt. Plasmid pCAT-HBSAg was also digested with Hind III (Promega, USA) to be used as a control. The digested products were separated

by electrophoresing on 0.8% (w/v) agarose gel in 1xTAE at 90V for 45 min and viewed using AlphaImager ^{TM} 2200 (Alpha Innotech Corporation, USA).

After DNA fractionation, the gel was trimmed to remove unused areas of the gel. A small triangular piece was cut off from the bottom left hand corner of the gel of the gel to simplify orientation during the succeeding operations. The DNA was depurinated in 0.2 M HCl for 10 min. Then, the DNA was denatured by soaking the gel twice in ten gel volumes of Denaturation Solution (1.5 M NaCl, 0.5 M NaOH) for 30 min with constant agitation. Then, the gel was briefly rinsed in deionized water, followed by soaking of the gel in Neutralization Buffer I (0.5 M Tris-Cl, 1 M NaCl, pH 7.4) for 30 min with constant agitation. Meanwhile, charged nylon membrane, Hybond N+ (Amersham, U.K) was soaked in deionized water for 30 sec prior to soaking it in Alkaline Transfer Buffer (0.4 M NaOH , 1 MNaCl) for 5 min.

A 3mm filter paper (Whatman, USA) was placed on a plastic platform in a blotting reservoir that was wider and longer than the gel. The ends of the filter paper were left to drape over the edges of the platform. The reservoir was filled with Alkaline Transfer Buffer until the filter paper on the plastic platform became thoroughly wet, before smoothing out the air bubbles with a glass pipette. Then, the gel was removed from the solution and inverted so that its underside was at the uppermost and the gel was placed on the support. The top of the gel was moistened with Alkaline Transfer Buffer so that the moistened membrane could be placed on the gel. Two pieces of 3 mm filter paper (Whatman, USA) were wet in Alkaline Transfer Buffer and placed on the wet membrane avoiding any air bubble formation. Finally, paper towels (five to eight centimeters high) were cut and placed on the filter paper and the transfer of DNA was allowed for 16 to 24 hours.

Since, alkaline transfer results in covalent attachment of DNA to positively charged nylon membranes, there was no necessity to fix the DNA before hybridization. Therefore,

after the transfer, the membrane was soaked in Neutralization Buffer II for 20 min and hybridized at 55°C for 16 hours using the North2South Biotin Hybridization Buffer (Pierce Biotechnology, USA). The target DNA was detected using the North2South Biotin Detection Kit (Pierce Biotechnology, USA) according to manufacturers protocol. The probe that was used for detection was the pCAT-HBSAg plasmid digested with a single cutter, Sal I (Promega, USA) labeled using the North2South Biotin Labelling Kit (Pierce Biotechnology, USA) according to manufacturers protocol.

3.6 RNA Analysis of *C. vulgaris* Clones Transformed with the pCAT-HBSAg Vector

3.6.1 Total RNA Isolation

Total RNA was isolated from *Chlorella vulgaris* UMACC 001 using TRIzol® Reagent (Invitrogen Corporation, USA). A total of 100 mL *C. vulgaris* culture that was at an exponential phase (OD_{620} = 0.2) was pelleted by centrifugation at 4000 rpm, for 15 min at 4°C. The cells were homogenized using liquid nitrogen for 5 min. Then, 1ml of Trizol Reagent was added to the homogenized cells and incubated for 5 min at room temperature. A volume of 200 µL of chloroform was added to the homogenized samples and vortexed vigorously for 15 sec before incubating for 3 min at room temperature. The mixture was then centrifuged at 13,000 rpm for 15 min at 4°C before transferring the aqueous layer to a new tube that contained 250 µL of isopropanol and 250 µL of 0.8 N sodium citrate / 1.2 M NaCl. The mixture was incubated at room temperature for 10 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was aspirated and the RNA pellet was washed with 1 mL of 70% ethanol by centrifuging at 10,000 rpm for 5 min at 4°C. The pellet was left to dry for 10 min before incubating at 55°C for another 10 min by opening the centrifuge cap. The total RNA isolated was finally eluted with 20 µL of DEPC treated UHQ water (Eppendorf, Germany) and was stored in 80°C until used.

The quantity and purity of the RNA were determined by a biophotometer (Eppendorf, Germany) at OD_{260} and OD_{280}. The ratio between the absorbance values at 260 nm and 280 nm gave an estimate of the RNA purity. The quality and integrity of the RNA sample were also verified with 1.0 % (w/v) agarose gel electrophoresis in 1× TAE buffer at 80V for 30 min. All the extracted RNA samples were treated with DNAse I (New England Biolabs, U.K) to remove DNA contaminants before mRNA was isolated. PCR was performed directly using the DNAse 1 digested RNA samples, as described in section 3.6.2 without synthesizing the cDNA, to make sure that the digestion is complete.

3.6.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

A 10 µL of reaction volume which consisted of 1 µL of 50 µm oligo (dT_{20}), 8 µL of 1 pg to 5µg of mRNA, and 1 µL of 10mM dNTP mix was set up. The mixture was heated at 65ºC for 5 min and chilled quickly on ice. Then, 10µl of cDNA Synthesis Mix (2 µl RT buffer, 4 µl 25mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT and 1 µl SuperScript III RT) was added to each RNA/primer mixture and incubated for 50 min at 50 ºC. The reactions was terminated at 85 ºC for min and chilled on ice. The cDNA synthesis was stored in -20ºC prior to PCR.

A 25 µL of PCR reaction was set up comprising of 2.5 µL 10× PCR buffer, 0.5 µL $MgCl_2$ (100 mM), 0.4 µL dNTP mix (10 mM) (Bioron, Germany), 1 µL FHBSAg primer (5'-GTG GTG GAC TTC TCT CAA TTT TC-3') (10 µM), 1 µL RHBSAg primer (5'-GCA GGT TTT GCA TGG TCC CGT AC-3') (10 µM), 1U *Taq* DNA Polymerase, (Bioron, Germany), 1 µL cDNA from the first strand synthesis and 18.2 µL sterile deionized water. The PCR conditions were performed as follows: 2 min at 94ºC for pre-denaturation, 1 min at 94ºC to denature the double stranded DNA strand, 1 min at 57ºC to anneal the DNA and 2 min at 72ºC to extend the PCR amplified product. The denaturation, annealing and extension steps were

repeated for 35 cycles. This was followed by a final extension at 72ºC for 10 min. The PCR products were analyzed with 1.2% (w/v) agarose gel electrophoresis in 1× TAE buffer at 90V for 45 min. Finally, the putative PCR amplified bands were excised, purified and sequenced (1^{st} Base, Malaysia) using the FHBSAg (5'-GTG GTG GAC TTC TCT CAA TTT TC-3') and RHBSAg (5'-GCA GGT TTT GCA TGG TCC CGT AC-3') primers to confirm that the amplified PCR fragment was the desired HBSAg gene.

3.7 Protein Analysis of *C. vulgaris* Clones Transformed with the pCAT-HBSAg Vector

3.7.1 Preparation of Crude Protein Extracts

A volume 100 mL *Chlorella vulgaris* culture at the end of the exponential phase (OD_{620} = 0.5) was centrifuged and the pellet was resuspended in three volumes of PBS buffer (0.1370 M NaCl, 0.0027 M KCl, 0.0010 M Na_2HPO_4, 0.0018 M KH_2PO_4, 0.0020 M EDTA, pH 7.4). After washing, the lysis buffer (0.1370 M NaCl, 0.0027 M KCl, 0.0010 M Na_2HPO_4, 0.0018 M KH_2PO_4, 0.0020 M EDTA, 0.0020 M β-mercaptoethanol, pH 7.4) was added to the pellet. The mixture was sonicated for 30 sec at four intervals and incubated overnight at 4°C. The mixture was then centrifuged and the supernatant was separated using SDS-PAGE gel. The concentration of the total protein in the crude extract was estimated according to Bradford (1976).

3.7.2 SDS-PAGE Gel Electrophoresis

Proteins extracted from the transformed positive clones and untransformed *Chlorella vulgaris* were separated using 12% SDS-PAGE using the Mini Protein 3 (BioRad Laboratories, USA) at 120 V for two hours. Unstained and prestained protein molecular weight markers (Fermentas International Inc, Canada) were used to determine protein band

sizes. The gel was stained with Coomassie Brilliant Blue R-250 (Merk and Corporation, USA) and destained with methanol:acetic acid solution.

3.7.3 Western Blot Analysis

The separated proteins on polyacrylamide gel were immobilized onto PVDF (Amersham, UK) membrane by using Mini Trans Blot (BioRad Laboratories, USA) at 100 V for an hour. After the transfer, ProBlot AP System with Stabilized Substrate (Promega, USA) was used as an immunodetection system. Firstly, the membrane was blocked in TBST (Tris Buffer Saline with Tween-20) with 1% BSA for 16 hrs, 4°C to saturate non specific binding sites. Then, the blocking solution was replaced with TBST containing 1:2000 Mouse Anti-pre S2 (Hepatitis B Virus) Monoclonal Antibody (Chemicon International, USA) and incubated for 60 min at room temperature with gentle agitation. The membrane was then washed three times in TBST, 10 min for each wash to remove unbound antibody. After washing, the membrane was transferred to TBST solution containing 1:2500 anti-IgG AP conjugate and incubated for another 30 min with gentle agitation. The membrane was washed again three times in TBST, 10 min for each wash to remove unbound antibody. Then, it was washed with TBS solution to remove the Tween-20 from the membrane surface before incubating the membrane in Western Blue Stabilized Substrate until the bands of interest have reached the desired intensity. When the colour of the band had been developed to the desired intensity, the reaction was stopped by washing the membrane in deionized water for several minutes, by changing the water at least three times. Finally, the membrane was photographed while still moist by placing on top of a damp piece of filter paper.

3.8 Statistical Analysis

All the data obtained in this research which included optical density and cell count readings, DNA, RNA and protein quantification data and PCR results, were analyzed using statistical software, STATISTICA 7 (Statistica Software Incorporated, USA).