MATERIALS & METHODS

3.0 Material and Methods

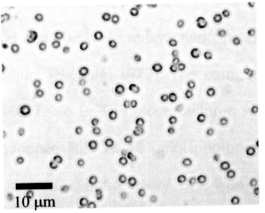
3.1 Preparation of glassware

All the glassware used in this study was soaked 15 minutes in tap water, washed with detergent, rinsed in tap water and followed by three rinses in glass-distilled water. The glassware was then dried and kept for use. The culture medium and glassware were sterilised by autoclaving (120 p.s.i, 15 minutes) before use. All solutions were made with distilled deionised water which was free of nitrate, ammonium or phosphate.

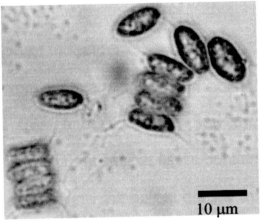
3.2 Microalgal cultures

3.2.1 Test organisms

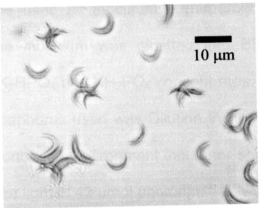
All microalgae were obtained from the University of Malaya Algal Culture Collection (UMACC) at the Institute of Postgraduate Studies, University of Malaya. Three axenic freshwater microalgae species were used in this study. The three species tested were *Chlorella vulgaris* UMACC 001, *Scenedesmus quadricauda* UMACC 041 and *Ankistrodesmus convolutus* UMACC 101 (Figure 2). *Chlorella vulgaris* UMACC 001 and *Scenedesmus quadricauda* UMACC 041 were isolated from a fish tank at the University of Malaya farm, while *Ankistrodesmus convolutus* UMACC 101 was isolated from Tasik Aman, a freshwater lake (Phang and Chu, 1999).



a) *Chlorella vulgaris*-UMACC 001



b) *Scenedesmus quadricauda* UMACC 041



c) *Ankistrodesmus convolutus* UMACC 101

Figure 2. Microalgae used in the present study.

3.2.2 Maintenance of stock cultures

Stock algal cultures were maintained on agar slants (2% w/v) containing Bold's Basal Medium (Nichols, 1973). The composition of Bold's Basal Medium (BBM) is shown in Table 9. The stock cultures were grown at 25°± 1°C under cool-white fluorescence light at 42 µmol photon m^{-2} s^{-1} with 12: 12 light: dark cycles. Subculturing was done every two weeks to maintain the viability of cultures.

3.2.3 Preparation of inoculum

The cells from the agar slant were inoculated aseptically into the test tube containing liquid BBM and allowed to grow for three days then inoculated aseptically again into 250 ml conical flasks containing 100 ml BBM and allowed to grow for five days, or until the optical density at 620 nm (OD_{620}) reached 0.2, as measured with a spectrophotometer Shimadzu UV-160. The cultures were then ready to be used as inoculum. Ten percent inoculum was used in all experiments. The inoculum was pipetted into BBM spiked with a series of $NaNO_3$, NH_4Cl, K_2HPO_4 and KH_2PO_4 concentrates. The minimal medium without nitrogen and phosphorus used was Dilution Water (Table 10). All cultures were incubated in a controlled-environment incubator shaker at 25°± 1°C under cool-white fluorescence light at 42 µmol photon m^{-2} s^{-1} with 12:12 light: dark cycles.

Table 9: Composition of Bold Basal's Medium (BBM) (Nichols, 1973).

Stock Solutions		
1	$NaNo_3$	10.0 g per 400ml
2	$MgSO_4.7H_2O$	3.0 g per 400ml
3	NaCl	1.0 g per 400ml
4	K_2HPO_4	3.0 g per 400ml
5	KH_2PO_4	7.0 g per 400ml
6	$CaCl_2.2H_2O$	1.0 g per 400ml
7	Trace Element Solution	
	- $ZnSO_4.7H_2O$	8.82 g per litre
	- $MnCl_2.4H_2O$	1.44 g per litre
	- MoO_3	0.71 g per litre
	- $CuSO_4.5H_2O$	1.57 g per litre
	- $Co(NO_3)_2.6H_2O$	0.49 g per litre
8	H_3BO_3	11.42 g per litre
9	EDTA-KOH Solution	
	- EDTA	50.0 g per litre
	- KOH	31.0 g per litre
10	$FeSO_4.7H_2O$	4.98 g per litre
	H_2SO_4 (conc)	1.0 ml
Adjust pH to 7 with 3N HCl or 1M KOH		
Final solutions:		
Stock solution 1-6	10 ml each	
Stock solution 7-10	1 ml each	
Make up to 1 litre with glass distilled and deionised water		

Table 10: Composition of Dilution Water (ASTM, 1993).

Stock solution		
1	$NaHCO_3$	0.096 g
2	$CaSO_4.2H_2O$	0.06 g
3	$MgSO_4.7H_2O$	0.06 g
4	KCl	0.004 g
5	4-(2-Hydroxyethyl)-Piperazine-1-Ethane-Sulfonic Acid (HEPES)	2.38 g
Dissolve all the stock solutions into 1 litre of distilled water		
Adjust pH to 7.0		

3.3. Preparation of stock solutions

The $NaNO_3$, NH_4Cl, K_2HPO_4 and KH_2PO_4 stock solutions were prepared 10 times more concentrated than the final concentrations used. The stock solutions were added aseptically into BBM without the respective nitrogen or phosphorus source. All medium contained 10 mM 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid (HEPES) as a buffer agent.

For the nitrogen experiment, a concentrated stock solution of 187.5 mM $NaNO_3$ or NH_4Cl was prepared. This stock was serially diluted to give $NaNO_3$ or NH_4Cl solutions of 0.3 mM, 1.5 mM, 7.5 mM and 37.5 mM.

From this series of stock solutions, 10 ml was pipetted from each concentration to give the final concentrations of 0.03, 0.15, 0.75, 3.75 and 18.75 mM of $NaNO_3$ or NH_4Cl.

For the phosphate experiment, a concentrated stock solution of phosphate containing 375 mM K_2HPO_4 and 875 mM KH_2PO_4 was prepared. This stock was serially diluted to give a series of phosphate solutions containing 0.6 mM K_2HPO_4 and 1.4 mM KH_2PO_4, 7 mM K_2HPO_4 and 3 mM KH_2PO_4, 35 mM K_2HPO_4 and 15 mM KH_2PO_4, 75 mM K_2HPO_4 and 175 mM KH_2PO_4. From this series of stock solutions, 10 ml was pipetted from each concentration to give the final concentrations of 0.02, 0.1, 0.5, 2.5 and 12.5 mM of total phosphate.

3.4. Test procedures

The three chlorophytes were used for all the experiments, except the one on selection of culture medium, where only *Chlorella vulgaris* UMACC 001 was used. The inoculum used (10%) (for all the experiments) was from exponential phase cultures was used. The inoculum was centrifuged (3000 rpm for 10 minutes at 4°C), washed and resuspended in Dilution Water and OD_{620} was adjusted to 0.2. The inoculum was pipetted into all BBM experimental growth medium (80 ml) spiked with a series of NO_3^- , NH_4^+, or PO_4^{3-} (10 ml) concentrates. Triplicate cultures were used for each treatment. Growth of the cultures was monitored by cell count and OD_{620}.

3.4.1. Experiment 1: Selection of minimal culture medium

This test was conducted to determine the range of nutrient concentrations to be used in the subsequent experiments. Four different media were used in this preliminary test: Dilution Water (Table 8), BBM 100 %, BBM 10 % and BBM 1 %. The chlorophyte *Chlorella vulgaris* UMACC 001 was used as the test organism. 10 ml inoculum was inoculated into conical flasks (250 ml) containing 90 ml test medium. Triplicate cultures were used for each treatment. The cultures were grown in a controlled-environment shaker (150 rpm) set at 25°C, 42 µmol $m^{-2}s^{-1}$ with 12:12 h light-dark cycles for ten days. Every two days (0, 2, 4, 6, 8, and 10), 2 ml samples were withdrawn for OD_{620} measurement.

3.4.2. Experiments 2 & 3: Effects of $NaN0_3$ and NH_4Cl levels on short term growth (96 h) of three chlorophytes

In this test, 10 ml cultures were inoculated into conical flasks (250 ml) containing 80 ml of culture medium (BBM without $NaNO_3$), buffered with 10 mM HEPES and 10 ml of $NaNO_3$ or NH_4Cl of different concentrations The cells were washed with nitrogen-free medium before inoculation, which was done by centrifuging (3000 rpm, 10 minutes) the inoculum to spin down the cells with the supernatant removed and replaced with nitrogen-free medium.

For each concentration, triplicate flask cultures were used. The range of $NaNO_3$ or NH_4Cl concentrations used was 0 (Dilution Water), 0.03, 0.15, 0.75, 3.75 to 18.75 mM.

The cultures were grown in a controlled-environment incubator shaker (150 rpm) set at 25°C, 42 µmol $m^{-2}s^{-1}$, with 12:12 h light-dark cycles. Every 12 hours (from 0 h until 96 h), 2 ml samples were withdrawn for OD_{620} measurement and cell count.

3.4.3. Experiments 4 & 5: Effects of phosphate (K_2HPO_4 and KH_2PO_4) levels on short term growth (96 h) of three chlorophytes in low- $NaNO_3$ and low- NH_4Cl medium

Two experiments were conducted whereby a series of phosphate levels was added to BBM containing low nitrogen level. In the first experiment, 0.03 mM $NaNO_3$ was used while in the second experiment, 0.03 mM NH_4Cl was used.

In this test, 10 ml exponential phase cultures were inoculated into conical flasks (250 ml) containing 80 ml of culture medium. The medium used was 1% BBM without K_2HPO_4 and KH_2PO_4, spiked with 10 ml of phosphate solutions (K_2HPO_4 and KH_2PO_4) of different concentrations The cells were washed with phosphate-free medium before inoculated, which was done by centrifuging (3000 rpm, 10 minutes) the inoculum to spin down the cells and the supernatant removed and replace with nitrogen free medium. For each concentration, triplicate flask cultures were used. The range of phosphate concentrations used was 0 (Dilution Water), 0.02, 0.1, 0.5, 2.5 to 12.5 mM. The cultures were grown in a controlled-environment incubator shaker (150 rpm) set at 25°C, 42 µmol $m^{-2}s^{-1}$, with 12:12 h light-dark cycles. Every 12 hours (from 0 h until 96 h), 2 ml samples were withdrawn for OD_{620} measurement and cell count.

3.4.4. Experiments 6 & 7 : Effects of phosphate (K_2HPO_4 and KH_2PO_4) levels on short term growth (96 h) of three chlorophytes in high-$NaNO_3$ and high- NH_4Cl medium

Two experiments were conducted whereby a series of phosphate levels was added to BBM containing high nitrogen level. In the first experiment, 18.75 mM $NaNO_3$ was used while in the second experiment, 18.75 mM NH_4Cl was used. In this test, 10 ml exponential phase cultures were inoculated into conical flasks (250 ml) containing 70 ml of culture medium. The medium used was 1% BBM without $NaNO_3$, K_2HPO_4 and KH_2PO_4, and spiked with 10 ml of phosphate solutions (K_2HPO_4 and KH_2PO_4) of different concentrations and 10 ml $NaNO_3$ or NH_4Cl solution (18.75 mM) (Before inoculation). The cells were washed with phosphate-free medium, which was done by centrifuging (3000 rpm, 10 minutes) the inoculum to spin down the cells, and the supernatant removed and replaced with nitrogen-free medium. For each concentration, triplicate flask cultures were used. The range of phosphate concentrations used was 0 (Dilution Water), 0.02, 0.1, 0.5, 2.5 to 12.5 mM. The cultures were grown in a controlled-environment incubator shaker (150 rpm) set at 25°C, 42 µmol $m^{-2}s^{-1}$, with 12:12 h light-dark cycles. Every 12 hours (from 0 h until 96 h), 2 ml samples were withdrawn for OD_{620} measurement and cell count.

3.5. Cell Count

Algal cell number was determined by counting the cells using a haemacytometer (Improved Double – Neubauer), and three counts were performed for each sample. For each count, cell numbers within the upper and lower counting chambers were obtained. The samples were diluted accordingly if the counts were more than 100 cells per counting chamber.

3.6. Specific growth rate

The specific growth rate (μ, day^{-1}) was determined using the following formula:

$$\mu = \frac{Ln\ N_1 - Ln\ N_2}{t_1 - t_2}$$

where N_1 and N_2 represent the cell number or OD_{620} at times t_1 and t_2 respectively, within the exponential phase.

3.7. Percentage Growth Enhancement

Growth obtained in Dilution Water was arbitrarily assigned as 100%. The Percentage Growth Enhancement (PGE) refers to percentage increase of growth in varying concentrations of nitrogen and phosphorus. Cell number and OD_{620} of the cultures were used for calculation of PGE, as shown below:

$$PGE = \left[\frac{\text{Cell no or } OD_{620} \text{ of Cultures grown in BBM containing N \& P} - \text{Cell no or } OD_{620} \text{ of cultures grown in Dilution Water}}{\text{Cell no or } OD_{620} \text{ of cultures grown in Dilution Water}} \right] \times 100\%$$

3.7. Linear regression analysis

Linear regression was used to test for correlation between the two growth parameters OD_{620} and cell number. The analysis was done using the MS Excel program.