CHAPTER III

EXPERIMENTS

The determination of optimal conditions in this work was performed in the 1.5L cone bottom bubble column photobioreactor. This design helps prevent cell precipitation which is likely to occur for *Haematococcus pluvialis* at encystment stage where cells lose flagella and do not have the ability to move by themselves. Two types of illumination were examined, i.e. ambient or outdoor condition and control or indoor condition (Figures 3.1 and 3.2) respectively. The induction of astaxanthin in the 2.7L airlift photobioreactor (Figure 3.3) was performed and compared to the performance of the bubble column. The extraction of astaxanthin from *H. pluvialis* was done by tissue grinder and the amount of astaxanthin was analyzed by spectrometer. Figure 3.4 shows the photos of the mature cyst and the filtration of such cells.

3.1 Experimental set up

3.1.1 Set up of the photobioreactor for astaxanthin production

The cone bottom bubble columns were made of polyethylene terephthalate bottle 1.5 L (diameter 8 cm, height 32 cm) and were sterilized by ozone for 30 min. The liquid culture in the bubble column was agitated by rising air bubble into the base of the bubble column through the sparger. Ambient air from an aquarium pump was metered through a flow meter, sterilized with a polyethylene filter and 0.45 μm Gelman autoclavable filter, the ambient air was passed into the culture at the base of the bioreactor. For the indoor experiments, the light source used to supply the required intensity was the 18 and 36 W fluorescent lamps. The intensity was measured with a digital LX-5 Lux (see below for more detail).

For the experiment with airlift photobioreactor, the 2.7L airlift photobioreactor was illuminated with the vertical 18W fluorescent lamps. The illumination intensity incident to the airlift photobioreactor outer surface was measured with a digital LX-5 Lux meter, where photon flux density and irradiance could be readily interconverted (Thimijan et al., 1982) as:

$$1\ \mu mol\ (m^{-2}s^{-1}) = 74\ Lux \qquad (3.1)$$

3.1.2 Preparation of the bioreactor

1. set up the bioreactor as described in Section 3.2.1
2. fill 2.7 l of tap water into the bioreactor
3. sparge 200 $\times 10^3$ ml/min of ozone through the 0.45µm Gelman autoclave filter and a flow meter into the water at the base of reactor for 1h in order to clean the whole system
4. sparge the air through the 0.45µm Gelman autoclave filter and a flow meter into the water at the base of reactor for 3-4 h to remove residual ozone in the water

3.2 Experimental procedure

3.2.1 Outdoor experiments

3.2.1.1 Effect of nutrient concentration

1. filter the cells by Nylon filter (diameter of 10 µm), when the algal cells reached the stationary phase
2. dilute the filtrate by 5, 10 and 15 times with distilled water. The water is sterilized by autoclave at 121°C for 20 min
3. transfer the cells (1×10^5 cells/ml) and the culture in Step 2 to the bioreactor in Section 3.1.1 (the initial cells in each bioreactor is equal)
4. place the bioreactors in the middle of the sunlight (outdoor)
5. measure the cells (motile cells, non-motile cells, immature cyst, mature cyst) as described in Section 3.3.1
6. take 5 ml of sample for the determination of astaxanthin content
7. take sample and measure the cells until all cells turn red

3.2.1.2 Effect of cell concentration

1. repeat Step 1 in Section 3.2.1.1
2. prepare the cell solution with the dilution of 10X, 20X and 25X by adding distilled water into the stock cell solution at 1×10^5 cells/ml. The distilled water is sterilized by autoclave at 121°C for 20 min

3. transfer the cells in Step 2 and the culture to the bioreactor in Section 3.1.1 (the medium concentration in each bioreactor is equaled)
4. repeat Steps 4-7 in Section 3.2.1.1

3.2.2 Indoor experiments

3.2.2.1 Effect of light intensity with initial cell concentration

1. repeat Step 1 in Section 3.2.1.1 with optimal condition obtained from Sections 3.2.1.1 (optimal diluted medium concentration)
2. prepare the cell solution with the dilution of 10X, 20X and 25X by adding distilled water into the stock cell solution $1x10^5$ cells/ml. The distilled water is sterilized by autoclave at 121°C for 20 min.
3. transfer the cells in Step 2 and the optimal diluted medium concentration to the bioreactor in Section 3.1.1 (the initial cells in each bioreactor is equal)
4. provide light to the bioreactors using fluorescent lamps at predefined intensities. The light intensity is altered by controlling the number of fluorescent lamps (0, 2, 4 and 6 lamps).
5. repeat Steps 5-7 in Section 3.2.1.1

Note: The light intensity was measured with a digital LX-5 Lux meter.

3.2.2.2 Effect of light intensity with diluted medium concentration

1. repeat Step 1 in Section 3.2.1.1 with optimal condition obtained from Sections 3.2.2.1 (optimal diluted initial cell concentration)
2. dilute the filtrate by 5, 10 and 15 times with distilled water. The distilled water is sterilized by autoclave at 121°C for 20 min
3. transfer the cells (optimal diluted initial cell concentration) and the culture in Step 2 to the bioreactor in Section 3.1.1 (the initial cells in each bioreactor is equal)
3. repeat Steps 3-4 in Section 3.2.2.1

3.3 Determination of growth

Algal cell growth was determined by monitoring changes in cell density with time. The productivity of astaxanthin in each reactor condition was also investigated.

3.3.1 Determination of cell density

Cell density was measured by microscope and the counting of cells was performed using an improved Neubauer haemacytometer (Figures 3.5 and 3.6).

1. take two 25 µl drops of culture and place them on a clean haemacytometer
2. place clean cover slip on the drop so that the drop is evenly dispersed under the cover slip
3. count cell under a microscope (objective 100x)
4. calculate the number of cells as follows:

$$N = \left[\frac{n_1 + n_2}{18}\right] x10^4 \tag{3.2}$$

where N = cell number (cell mL^{-1})

n_1 and n_2 = number of cells count in upper and lower grid (cells)

3.3.2 Determination of astaxanthin productivity

The astaxanthin productivity was calculated by the following equation:

$$\text{Astaxanthin productivity} = \frac{C_2 - C_1}{t_2 - t_1} \tag{3.3}$$

where C_1 and C_2 = astaxanthin at t_1 and t_2 (g)

t = time (day)

3.3.3 Determination of cell dry weight

Cell dry weight was done when the cell reached maturation stage where most cells accumulate astaxanthin.

1. count the cells by haemacytometer by fix volume is 200 ml
2. filter the cells by membrane filter (diameter of 10 µm), which is dried by oven 24 h
3. dry the membrane filter and cells in oven for 24 h
4. bring the dried membrane filter and cells it into the dessiccator until it cools down

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5. weigh the sample by four position digital balance
6. calculate %dry weight by the following equation:

$$\%\text{Dry weight} = \frac{C_1 x V_1}{G} x100 \tag{3.4}$$

where

C_1	=	number of cells (cells/ml)
V_1	=	volume (ml)
G	=	mass of membrane filter and cells – mass of membrane filter

3.4 Analytical measurement

3.4.1 Determination of astaxanthin

1. centrifuge 3-5 ml of culture sample at 2,500 rpm for 20 min
2. add 2-3 ml of MeOH (methanol) into the remaining the cell pellet
3. break cells by manual homogenizer in order to extract the total pigment from the cells
4. repeat the extraction procedure until the cells pellet becomes white in color
5. separate the mixture by centrifugation at 2,500 rpm for 15 min
6. take 10 ml of the supernatant by MeOH (methanol)
7. collect the supernatant containing pigment

Note that Steps 1-7 must be conducted in dark condition

8. fill both spectrometer cell with MeOH and found cell-to-cell blank
9. fill both spectrometer cell with the supernatant containing pigment determined astaxanthin content at 665 nm, 645 nm and 473 nm respectively
10. collect the measured absorbance by calculate the concentration of pigment from Strickland and Person (1997) equation
11. calculate the concentration of pigment

Calculate the concentration of astaxanthin according to:

$$\text{g pigment} = \frac{AV}{E_{1cm}^{1\%} x100} \quad (3.5)$$

where A = light absorbance at wavelength of maximum absorption (Table 3.1)

$E_{1cm}^{1\%}$ = mass extinction coefficient at wavelength of maximum absorption (Table 3.1) (http://www.fda.gov/ohrms/dockets/dailys/.pdf)

Table 3.1 Extinction coefficient and the wavelength of maximum absorption

Solvent	Wavelength of maximum absorption (nm)	Mass extinction coefficient ($100mlg^{-1}cm^{-1}$)
DMSO	492	1980.7
Acetone	477	2177.4
MeOH	477	2306.6

Remark: It was assumed here that total carotenoids extracted from the cell consisted mostly of astaxanthin and light at 473 nm. Hence, the process originally designed for the measurement of total carotenoids could be applied accordingly.

Figure 3.1 Astaxanthin production in 1.5L bubble column photobioreactor (Outdoor experiments)

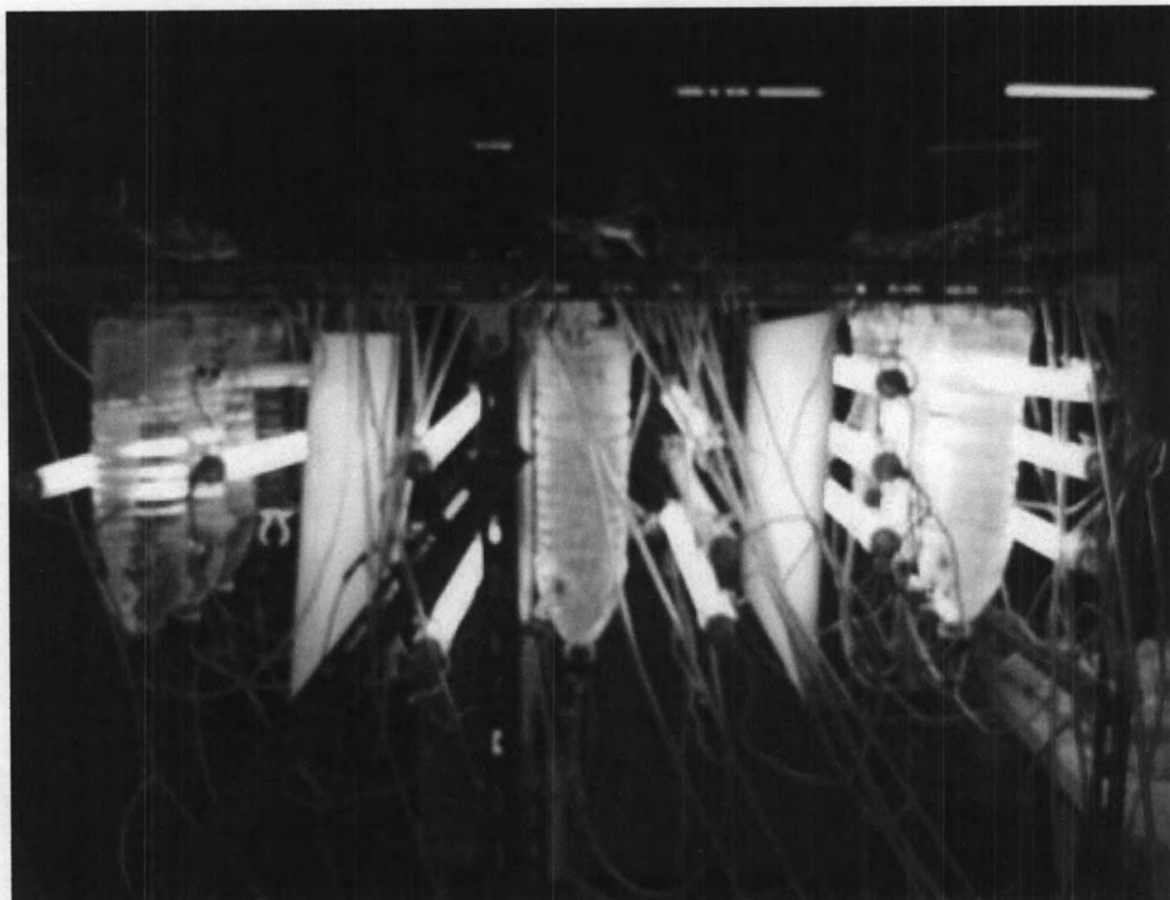

Figure 3.2 Astaxanthin production in 1.5L bubble column photobioreactor (Indoor experiments)

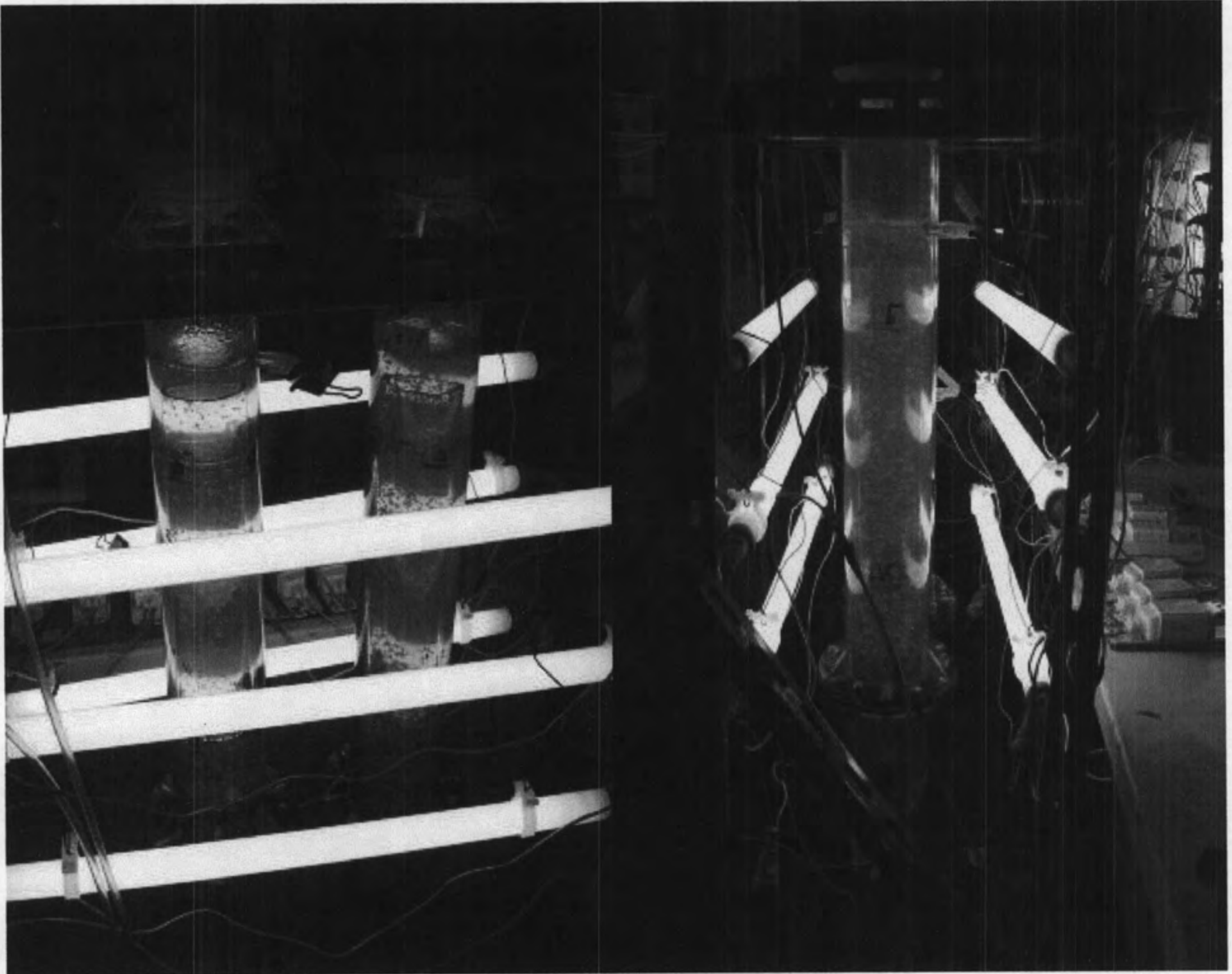

Figure 3.3 Astaxanthin production in 2.7L airlift photobioreactor (Indoor experiments)

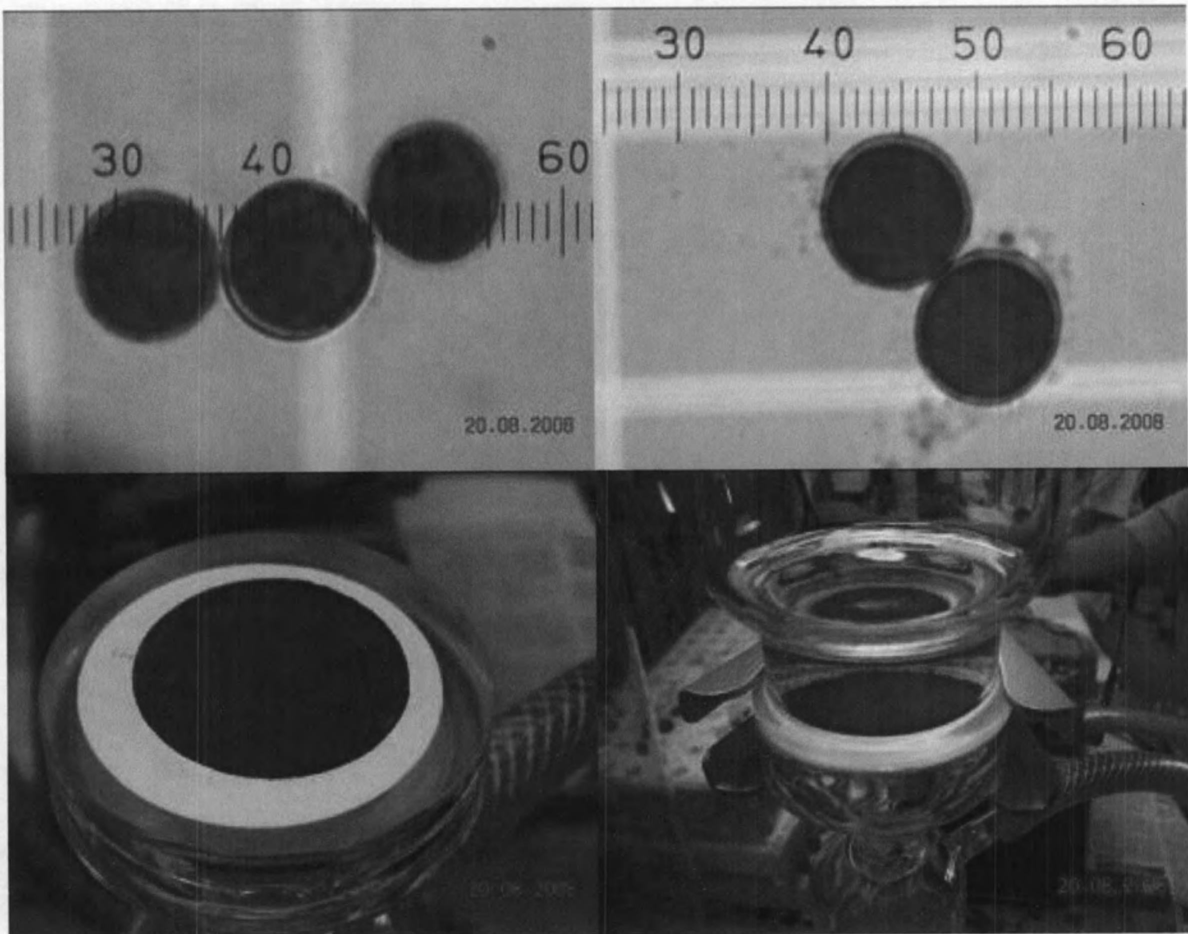

Figure 3.4 *Haematococcus pluvialis* and the filtration samples

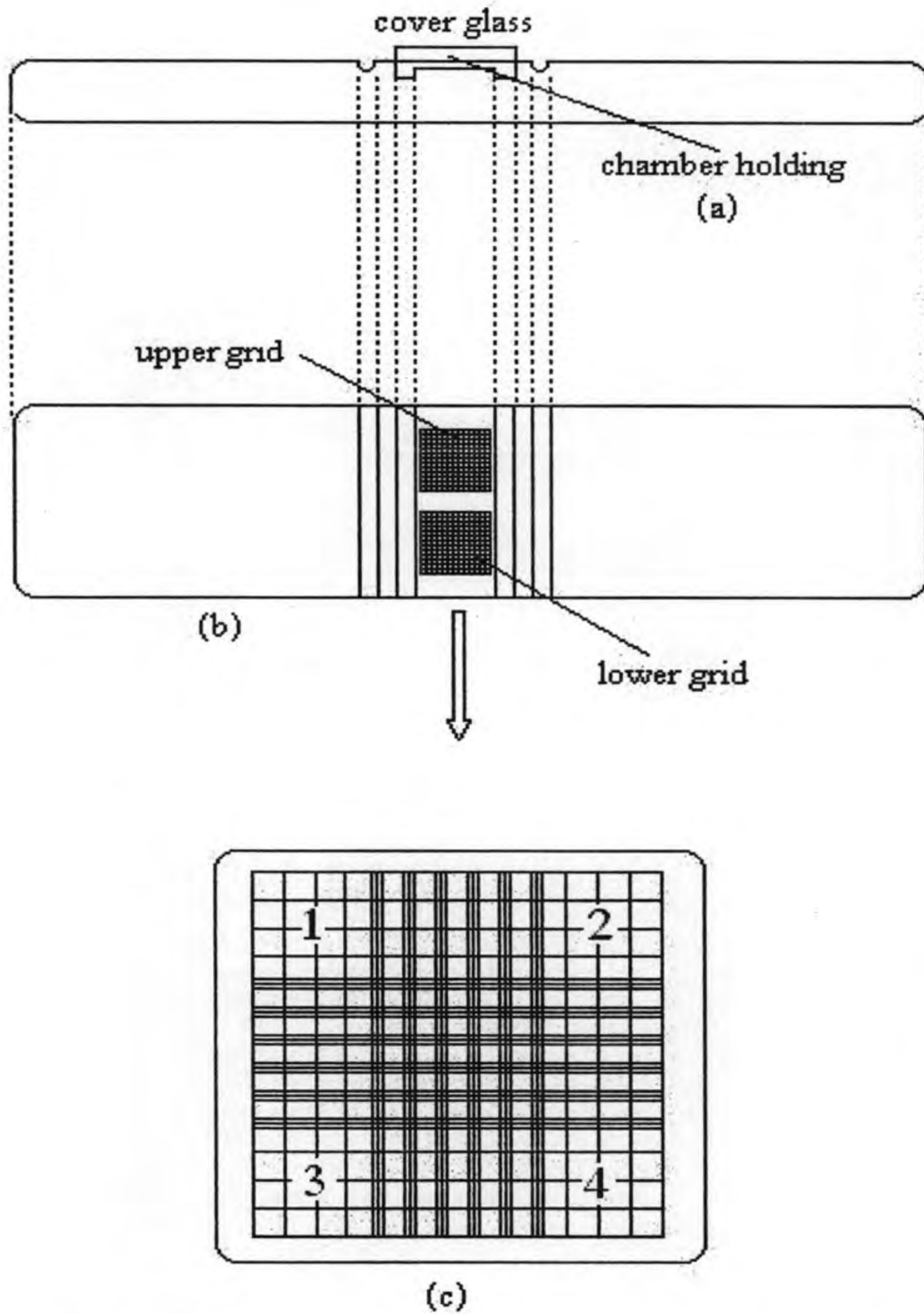

Figure 3.5 (a) Side view of the cell counting chamber showing the cover glass and the space beneath it that holds a microalgae suspension. (b) Top view of the chamber. The chamber has two grids located in the center of the side. (c) An enlarged view of the grid. The microalgae in the squares 1, 2, 3 and 4 are used for cell count.

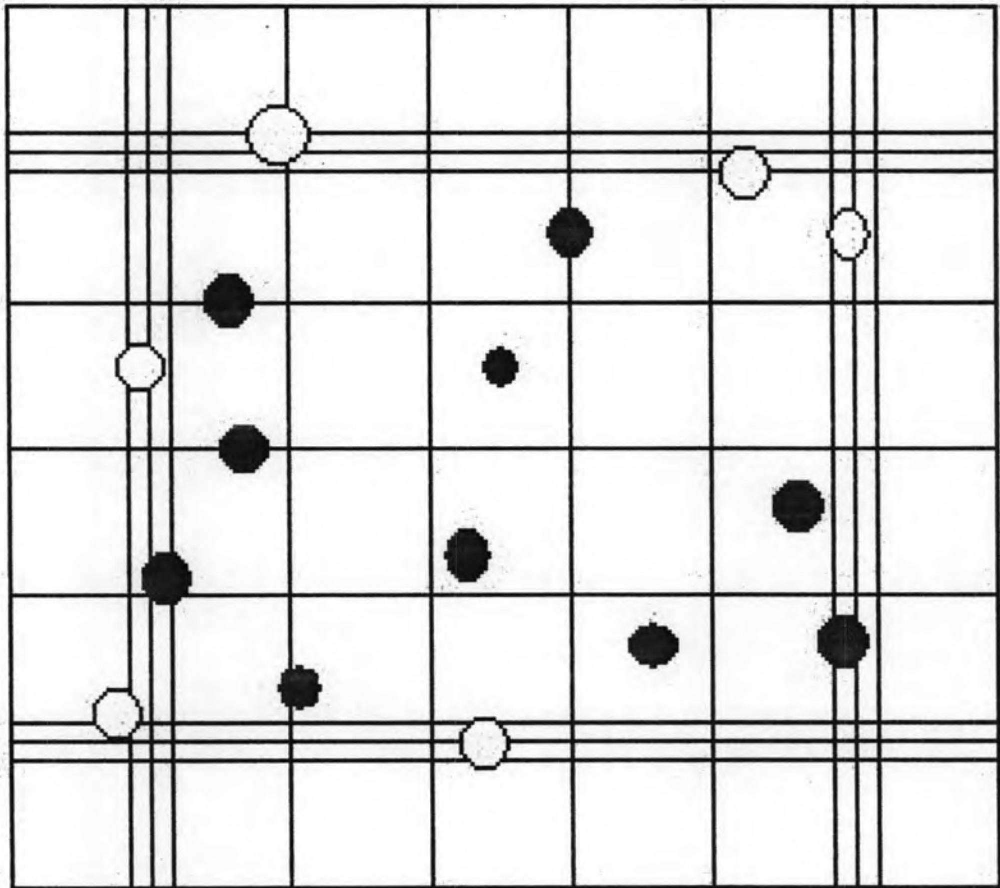

Figure 3.6 Counting cell density

Count the cells in the square and those which touch the top and left border (●), do not count the ones touching the right and lower border (○)