# Initial Study on the Growth of *Haematococcus pluvialis* for Astaxanthin Production



# Initial Study on the Growth of *Haematococus pluvialis* for Astaxanthin Production

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**Abstract.** This research emphasizes the identification of best nutrients among the four medium that will be used for a better growth rate of *Haematococcus pluvialis*. The growth rate of *H. pluvialis* is controlled or regulated by the physical and chemical parameters. Each of the parameters gives different yield productions to *H. pluvialis*. Different types of medium composed of different nutrients composition resulted in various maturation and growth rate of *H. pluvialis* monitored by the change in the optical density at 750 nm over time. The best medium was Rudic`s medium which gave OD750nm at 0.757 after 20-day culture. Then, Bold Basal medium, Basal medium and BG11 medium were followed as 0.677 at day 20, 0.358 at day 12 and 0.162 at day 15, respectively. Besides, this research also focuses on identifying the most suitable carbon source. Sodium acetate was found to be the best in the cultivation of *H. pluvialis* with the maximum absorbance of 0.99 in Rudic`s medium. The higher cell density cultures was achieved with 4 g/L concentration of sodium acetate. Carbon source also promoted better maturation process of *H. pluvialis* which will enhance yield of astaxanthin production in a shorter period of time. The primary composition that contribute to the high growth of *H. pluvialis* can be determined by adding 50% of concentration $CaCl_2$ in the medium with control flask contain 0.0585 g/L concentration of $CaCl_2$. The growth of *H. pluvialis* at day 9 was shown to increase by 27%. with absorbance of 0.301 if compared to control which gave absorbance 0.236. High concentration of $CaCl_2$ contribute to high growth of *H. pluvialis.*

## 1. Introduction

Astaxanthin is a member of the xanthophyll family of carotenoids and constitutes the highest value product derived by microalgae. As global population and consequently energy demand increase over time the introduction and commercialization of renewable sources of energy becomes a critical issue. Microalgal biomass as feedstock for bio-energy production is an attractive alternative to bioenergy derived from terrestrial plant utilization [1]. Microalgae, cultivated under specific stress conditions, can accumulate, along with the lipids and carbohydrates, a considerable number of secondary metabolites, whose industrial exploitation strongly enhances a bio-based economy [2].

There are a few microorganisms that can accumulate astaxanthin within their cells. The potential for astaxanthin production of each organism depends on their life cycle which has different physical and chemical properties. According to Md. Mahfuzur *et al*, *H. pluvialis* is believed to demonstrate the highest capacity to accumulate natural astaxanthin under environmental stress conditions [3]. However, one of the major difficulties of scaling up astaxathin production by *H. pluvialis* for commercial use is in the astaxanthin metabolism itself [4]. According to Ravi D *et al* the growth of microalgae is closely

related to the production of astaxanthin and its product, the high growth of microalgae will contribute to the high accumulation of astaxanthin [5].

Another study found that, the main problem of cultivation of *H. pluvialis* is the decrease in growth rate [6]. According to Harker *et al*, in commercial cultivation of *H. pluvialis* to produce astaxanthin has been limited to the growth of the alga in raceway pond [7]. The major problem encountered when operating this open pond system is the contamination of the ponds by bacteria, fungi, and other faster-growing algae, as well as protozoan predators, which had been reported to eliminate 90% of the algal biomass within 72 hours.

Even though it has some issues in the cultivation of *H. pluvialis*, this freshwater green microalgae *H. pluvialis* still is the best microbial source of astaxanthin. Therefore, the work reported in this study was conducted to evaluate the use of different types of media to promote the better of growth of *H. pluvialis* to produce a higher yield of astaxanthin. Also, to determine the most suitable carbon source which are sodium acetate and glucose in the cultivation of *H. pluvialis* for astaxanthin production. As well as to determine the primary medium composition such as $CaCl_2$ that responsible for the increasing cell density of *H. pluvialis* with time.

## 2. Methodology

### *2.1 Algal Culture*

The culture of *H. pluvialis* was obtained from National Institute Environmental Science (NIES-144), Japan. The culture was grown in four medium which are Rudic`s medium (RM), Basal medium (BM), Bold Basal medium (BBM) [8] and BG11 medium at pH below 8 [9]. The composition for each medium as shown in Table 1.

**Table 1.** The composition for each medium

| | **Composition** | **Concentration (g/L)** | | | |
|---|---|---|---|---|---|
| **No.** | **Medium** | **RM** | **BM** | **BBM** | **BG11** |
| 1. | $NaNO_3$ | 0.3 | - | 0.5 | 1.5 |
| 2. | $CaCl_2.2H_2O$ | 0.0585 | - | 0.025 | 0.0368 |
| 3. | $MgSO_4.7H_2O$ | 0.01 | 0.04 | 0.075 | 0.074 |
| 4. | $K_2HPO_4$ | 0.08 | - | 0.075 | - |
| 5. | $Na_2CO_3$ | - | - | - | 0.0202 |
| 6. | $KH_2PO_4$ | 0.02 | - | 0.175 | 0.0314 |
| 7. | NaCl | 0.02 | - | 0.025 | - |
| 8. | $FeSO_4$ | - | - | 0.00498 | - |
| 9. | $H_3BO_3$ | 0.0003 | - | 0.01142 | 0.00286 |
| 10. | $MnCl_2.4H_2O$ | - | 0.000108 | 0.00144 | 0.00181 |
| 11. | $ZnSO_4.7H_2O$ | 0.0001 | 0.0001 | 0.00882 | 0.00022 |
| 12. | $CuSO_4.5H_2O$ | 0.00008 | - | 0.00157 | 0.000079 |
| 13. | $Co(NO_3)_2.6H_2O$ | 0.00026 | - | 0.00049 | 0.000049 |
| 14. | $Na_2MoO_4.2H_2O$ | - | 0.0000075 | - | 0.00039 |
| 15. | KOH | - | - | 0.031 | - |
| 16. | $C_6H_8O_7$ | - | - | - | 0.00609 |
| 17. | $C_6H_8FeNO_7$ | - | - | - | 0.01015 |
| 18. | EDTA | 0.0075 | 0.00271 | 0.05 | 0.00112 |
| 19. | β-$Na_2$glycerophosphate | - | 0.05 | - | - |

| | | | | | |
|---|---|---|---|---|---|
| 20. | Vitamin $B_{12}$ | - | 0.0000001 | - | - |
| 21. | Biotin | - | 0.0000001 | - | - |
| 22. | Thiamine-HCl | - | 0.00001 | - | - |
| 23. | HEPES.NaOH | - | - | - | 0.11916 |
| 24. | $MnSO_4H_2O$ | 0.0015 | - | - | - |
| 25. | $FeCl_3.6H_2O$ | 0.017 | 0.00588 | - | - |
| 26. | $Ca(NO_3)_4.4H_2O$ | - | 0.15 | - | - |
| 27. | $KNO_3$ | - | 0.10 | - | - |
| 28. | $CoCl_2.6H_2O$ | - | 0.000012 | - | - |

*2.2 Media and Cultivation Conditions*

Cultivation was conducted in a 250 ml of conical flask. The conical flask was incubated in chamber equipment which controlled the temperature at 25 °C. Light intensity was maintained at 50 µmol $m^{-2}$ $s^{-1}$ with 18-watt daylight fluorescents and the light cycle was 24 h. Continuous bubbles of air were passed through a aeration pipe and filter [10]. The inoculum culture of *H. pluvialis* was grown under phototrophic conditions in a conical flask containing 180 ml of media described above. For experiments, glucose and sodium acetate were added in the medium. The concentration of glucose and sodium acetate were 1 g and 148 mg respectively [11]. The medium was sterilized by autoclaving at 120 °C for 2 hours. The culture was kept for 20 days at pH value below 8 [8].

*2.3 Growth Curve*

The growth of *H. pluvialis* cultures were determined by the change of Optical Density (OD) which were taken every three days using UV-vis spectrophotometer (Huch, USA) at 750 nm. Then, the OD was plotted to obtain the growth curve.

## 3. Results

The effect of different types of media and carbon sources on the growth *H. pluvialis* were simultaneously investigated for 20 days of cultivation period. The experiments were performed under the same growth conditions.

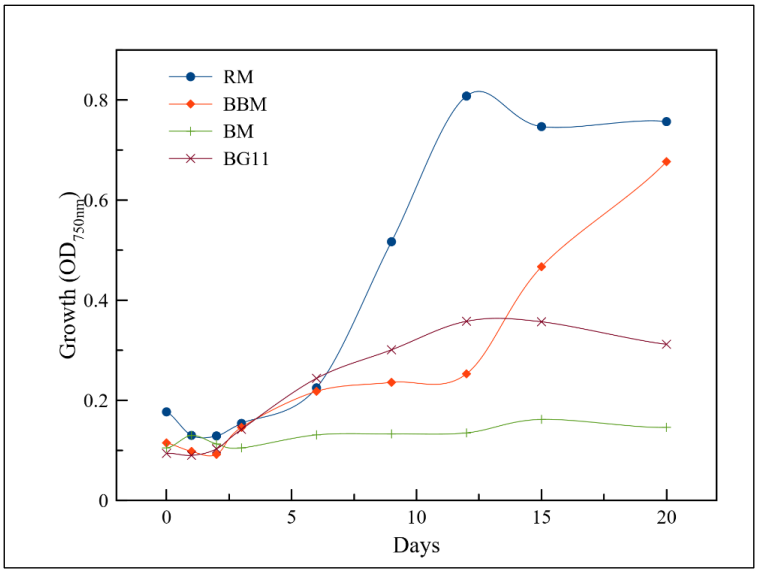


**Figure 1.** Growth of *H. pluvialis* in different medium without carbon source.

As shown in Figure 1, there were significant differences on the growth of cells beginning from 7 days of cultivation period in different culture media at the light intensity of 50 µmol photons $m^{-2}$ $s^{-1}$. The RM medium shown the fastest growth of *H. pluvialis* start from day 9 to day 20 with the maximum absorbance 0.757 $day^{-1}$. Besides that, BBM also shown the increase of growth *H. pluvialis* but it have small reading of absorbance than RM medium which is 0.677 $day^{-1}$. On the other hand, the growth of *H. pluvialis* in BG11 medium and BM medium increase linearly by day. The maximum absorbance for each medium are 0.358 $day^{-1}$ at day 12 and 0.162 $day^{-1}$ at day 15 respectively.

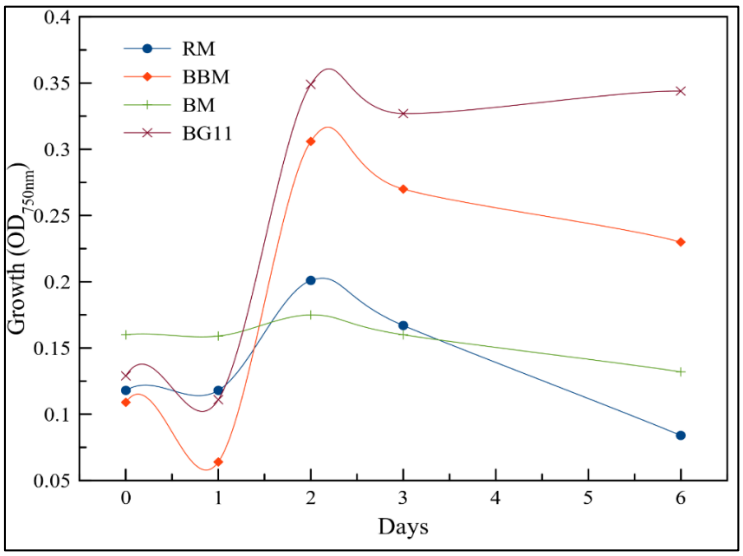


**Figure 2.** Growth of *H. pluvialis* in different medium with glucose

In microalgae cultivation, generally parameters such carbon source play crucial role in determining the growth of the culture. In this experiment, glucose solution and sodium acetate were use as carbon source in four different types of medium, which are BBM medium, BG11 medium, RM medium and BM medium. The absorbance value for each medium at the wavelength of 750 nm have been measure from day 0 to day 20. However, the culture with glucose can only be measured until day 6 as shown in Figure 2 as all the medium was then shown to be fully contaminated with fungus and bacteria. Adding of antifungal reagent, which is Nystatin before inoculation, was identified not to be a good solution to this problem.

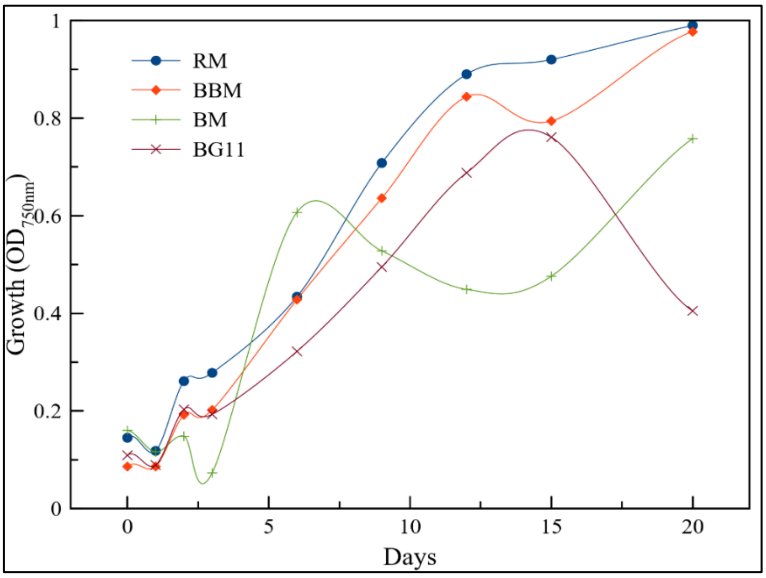


**Figure 3.** Growth of *H. pluvialis* in different medium with sodium acetate.

Another carbon source used in this experiment was sodium acetate. From Figure 1, the best medium was RM medium, thus by adding sodium acetate it had enhanced the growth of *H. pluvialis*. From Figure 3, the maximum growth of *H. pluvialis* can be identified in RM medium followed by BM medium with the absorbance of 0.99 $day^{-1}$ and 0.977 $day^{-1}$ respectively. The growth of *H. pluvialis* inside BM medium and BG11 medium have highest absorbance 0.758 $day^{-1}$ at day 20 and 0.761 $day^{-1}$ at day 15 respectively.

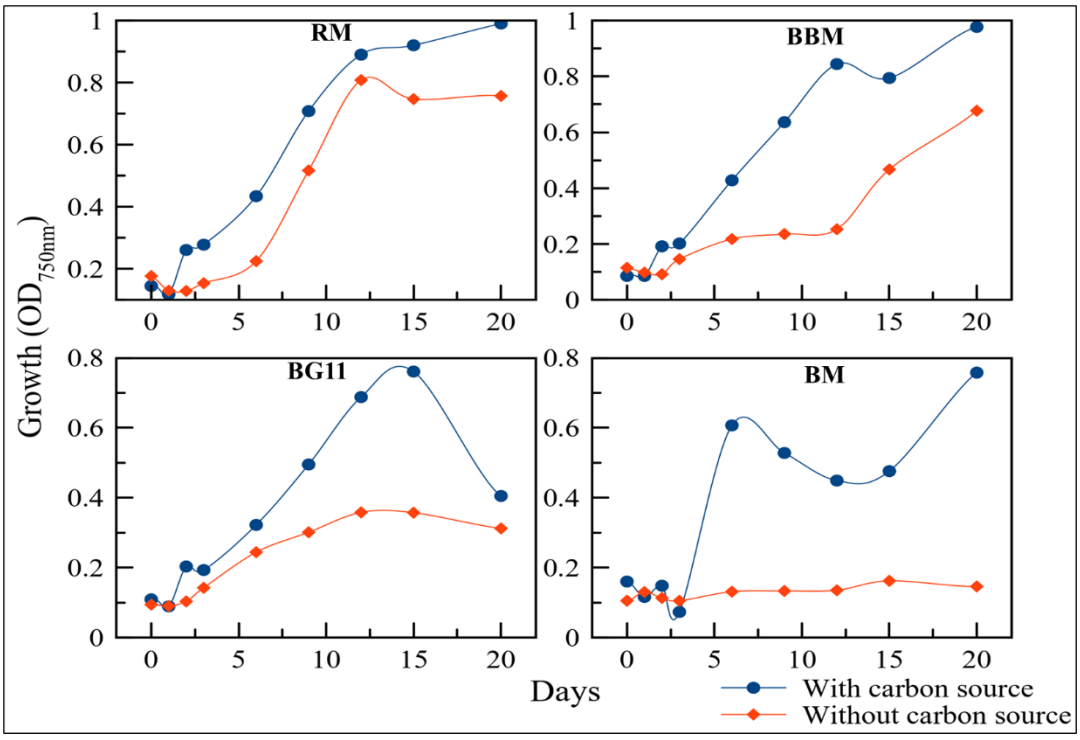


**Figure 4.** Comparison of growth of H. pluvialis with and without carbon source.

In this experiment, the carbon source efficiency for higher density and better growth rate was investigated by the addition of sodium acetate from day 0 to 20. As shown in Figure 4; the absorbance value gave much higher re value confirming higher biomass *of H. pluvialis* compared to the control flask which do not contain any carbon source. From Figure 4, it shows highest absorbance of 0.99 $day^{-1}$ at day 20 with the present of sodium acetate in RM medium. The highest absorbance, which is 0.977 $day^{-1}$ at day 20, was obtained in flask that contain sodium acetate in BBM medium. While the highest absorbance was 0.761 $day^{-1}$ at day 15 in BG11 medium with the present of sodium acetate. Lastly, from the BM medium have highest absorbance in the present of sodium acetate compared to control with absorbance of 0.758 $day^{-1}$.

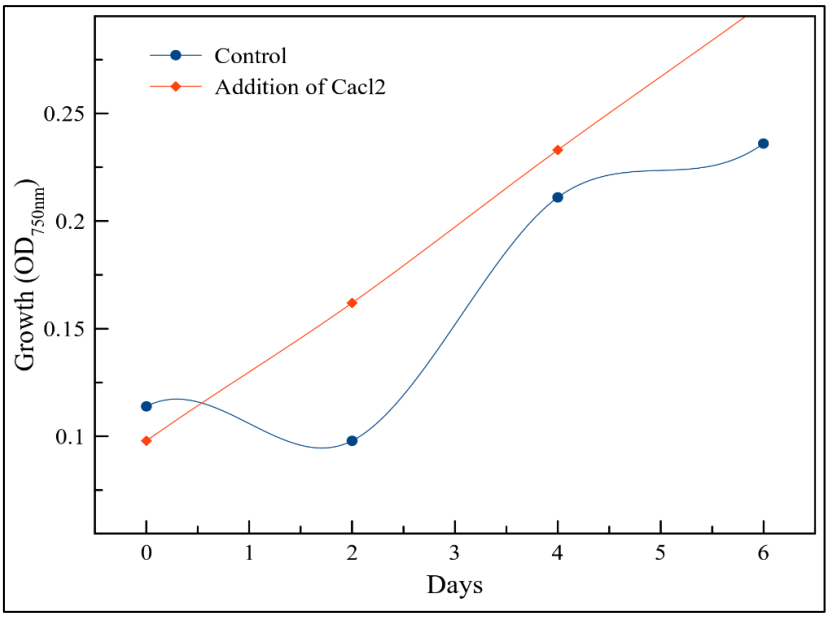


**Figure 5.** Comparison of growth of *H. pluvialis* in BBM medium with control and adding 50% of $CaCl_2$.

The initial study regarding the component of medium was conducted after obtaining the result of growth of *H. pluvialis*. The medium for cultivation was randomly chosen and *H. pluvialis* was cultured in BBM medium. The reading of absorbance was taking every three days to observe the growth of *H. pluvialis*. The growth of *H. pluvialis* inside the flask that contain high amount of concentration of $CaCl_2$ increase gradually from day 0 to day 9. The maximum absorbance that can be obtain from day 9 was 0.301 $day^{-1}$. While in control flask, it only contains normal concentration of $CaCl_2$ which have low growth curve compare to the flask that contain high concentration of $CaCl_2$. From day 9, the highest absorbance in control flask was 0.236 $day^{-1}$.

## 4. Discussion

### *4.1 Growth of H. pluvialis in different medium without carbon source*

In this experiment, the RM medium have high growth compare to BBM medium, this result can be supported by previous study from [8] which they obtained the result for maximal growth of *H. pluvialis*, 0.195 $day^{-1}$ in RM medium under continuous illumination (40 µmol photons $m^{-2}$ $s^{-1}$) at 25°C during 12 days of growth period.

The choice of medium used depends on several factors: the growth requirements of the algae, how the constituents of the medium may affect final product quality, and cost [12]. BM medium contain three vitamins vitamin B1 (thiamine HCl), vitamin $B_{12}$ (cyanocobalamin), and vitamin H (biotin) which are

usually expensive, whereas BG11 culture medium contain none. Among vitamins, thiamine was established as a growth factor for this microalga, while $B_{12}$ stimulated growth but was not essential [13]. Other authors have found that biotin, thiamine and $B_{12}$ have no significant effect on growth rate and final dry weight [10]. Nitrogen is a very important element for life, as a main constituent of protein and genetic material and is the most abundant element in microalgae (after carbon, oxygen and hydrogen) without mineralized wall. Therefore, as cells grow and divide they require a supply of nitrogen [14]. Based on Table 1, BG11 medium have high concentration of nitrate element compare to BM medium. It may cause the growth of *H. pluvialis* in BG11 medium was higher than BM medium as shown in Figure 1. In addition, BG11 medium is more economical than BM medium since it required less cost of component compare to BM medium.

*4.2 Growth of H. pluvialis in different medium with glucose*

The cultivation process for all four mediums in glucose gave negative results from day 6, as contamination was observed in the culture medium as shown in Figure 6. The contamination has been viewed under microscope and showed presence of bacteria as shown in Figure 7. Therefore, the culture had been discharged at day 6. Excessive presence of glucose in any culture medium may lead to residual sugars in the culture after the consumption of H. pluvialis which then lead to growth of other microorganism causing contamination.. Although, in previous study stated the efficiency of using glucose as carbon source instead of acetate can increase the growth of microalgae [15] but in this research, sodium acetate was chosen as the best carbon source since the medium do not easily contribute towards growth of other contaminants.


**Figure 6.** Detection of fungus inside flask in BG11 medium with glucose

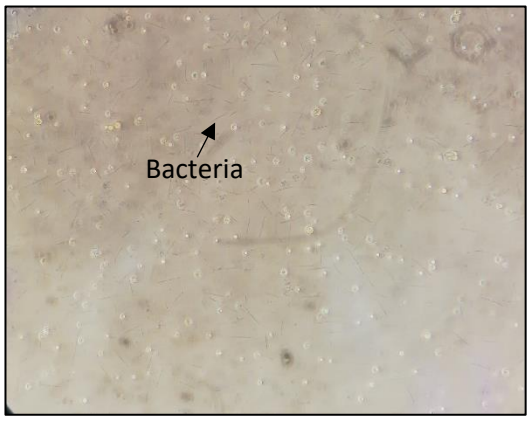


**Figure 7.** Observation under microscope with 100x magnificient in RM medium with glucose.

Furthermore, sodium acetate offers prefabricated chemical energy which has been known to be stored as lipid droplets [15] due to the several biochemical effects exerted by acetate. Steinbrenner *et al* 2001 reported enhanced expression of phytoene synthase and carotenoid hydroxylase, which essential enzymes involved in astaxanthin biosynthesis in cultures supplemented with acetate [17].

*4.3 Growth of H. pluvialis in different medium with sodium acetate*

Growth curves for *H. pluvialis* in medium with sodium acetate demonstrated uniform increase in cell density and healthy cells. According to the absorbance value for sodium acetate that plotted from day 0 to 20, the reading increasing rapidly in RM medium followed by BBM medium. The growth decreased from day 9 to 12 due to contamination from rotifer. Rotifer is a well-developed cuticle, which may be thick and rigid, giving the animal a box-like shape, or flexible, giving the animal a worm-like shape. These create a current that sweeps food into the mouth, where it is chewed up by a characteristic pharynx (mastax) containing tiny jaws [18].

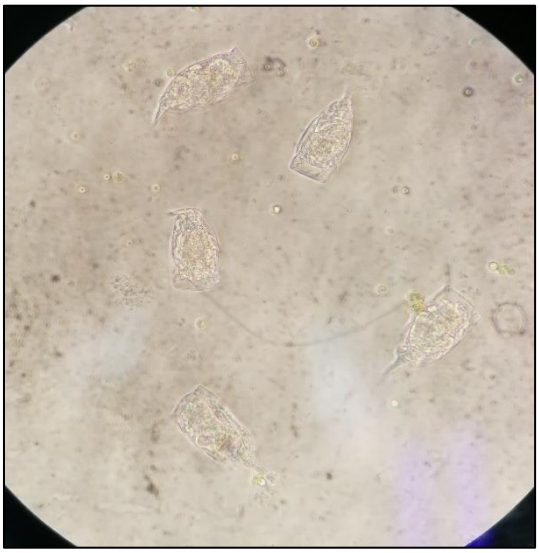

**Figure 8.** Observation of rotifer under light microscope.

The structure of rotifer was shown in Figure 8, this contamination can affect the growth of *H. pluvialis* by engulf the cell of *H. pluvialis* cause the *H. pluvialis* cell decreased. This problem was overcome by increase the salinity of the culture as the rotifer cell burst in high salt concentration. While increasing the salinity, the cell of *H. pluvialis* was was observed under microscope to ensure it is not affected. The growth of *H. pluvialis* inside the BG11 medium from day 15 to day 20 decrease because of lack of nutrient.

*4.4 Growth of H. pluvialis in Addition of $CaCl_2$*

In this research, the $CaCl_2$ have been studied as the chemical composition that need to be added in the medium due to the high concentration of the $CaCl_2$ is higher in RM medium compare to BBM medium. Since there is a limitation of time, the study has been carried out only until day 6. The results in Figure 5 shows high content of $CaCl_2$ contribute to high growth of *H. pluvialis* but more study need to be carried out to determine the best concentration of $CaCl_2$ in algae culture.

## 5. Conclusion

In conclusion, best medium with optimum nutrient composition can be identified for the better growth and maturation of *H. pluvialis*. In this experiment, the Rudic`s medium is the best medium to use for cultivation of *H. pluvialis* with higher biomass showed by absorbance value at 750nm. which is 0.757 at day 20. For the source of carbon, sodium acetate was shown to facilitate growth without contamination and gave higher biomass rate for *H. pluvialis* with absorbance value of 0.99 compared to glucose.

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