Pertanika J. Trop. Agric. Sci. 39 (4): 495 - 505 (2016)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

The Effect of Cultivation Techniques on the Growth Rate of Marine Microalgae Species from the Coastal Waters of Kudat, Sabah, Malaysia

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ABSTRACT

This study evaluates the growth of five species of marine microalgae; *Chaetoceros muelleri, Isochrysis galbana, Pavlova lutheri, Nitzschia acicularis* and *Navicula sp.,* isolated from coastal waters of Kudat in Sabah, Malaysia, using different cultivation techniques for mass outdoor culture. The microalgae were locally isolated and identified based on their morphology. The growth of the microalgae was compared between carboy and polythene bag culture. Results showed that cell count for all species was significantly higher in the polythene bag culture compared to carboy culture ($p < 0.05$). The time to harvest was also shorter (2-3 days) compared to carboy culture. In particular, *Chaetoceros muelleri* produced the highest cell count of 87×10^6 cells/ml and shortest time of 2 - 3 days to harvest. There were significant differences ($p< 0.05$) in cell count between indoor and outdoor mass culture, and these suggest the feasibility to culture the microalgae outdoor, reduce hatchery operation cost and save time.

Keywords: Carboy, microalgae, mass culture, polythene bag

INTRODUCTION

Microalgae are the primary producer of the food chain and have become the main source of food and feed in many aquaculture industries. The usage of microalgae as food for rearing larvae and juveniles has been extensively reported (Laing, 1991; Martinez-Fernandez & Souchgate, 2007; Galley et al., 2009). The larval and juvenile stages signify the importance of nutrition to ensure proper growth, survival rate and hence, successful hatchery operations. High mortalities during larviculture of marine animals such as fish, shellfish and echinoderms remain a

ARTICLE INFO

Article history:
Received: 28 September 2015
Accepted: 27 May 2016

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ISSN: 1511-3701

major bottleneck in aquaculture that limits production (Dhert et al., 2001). Many factors contribute to the high mortality cases such as diseases, parasites and food availability. Good diet requirement has been proven as the key factor in overcoming these problems (Rainuzzo et al., 1997).

Culturing of live algae is considered necessary as feed in the hatcheries. However, the process can be costly especially for the indoor culture (Cotteau & Sorgeloos, 1992). Feeding mixed algal diet consisting of two or more algae culture has been shown to produce optimal growth and development of larvae and juveniles compared to monoculture (Ivy & Giraspy, 2006). However, many of the microalgae have been proven difficult to culture in sizeable quantities necessary for large scale hatchery operations due to contamination, genetic unstability, light limitation and weather, which subsequently compromise its suitability for commercial applications. It is important to identify the specific diet requirements to determine which microalgae species to be cultured in the hatchery. Commercial hatcheries have consistently used reliable species such as *Isochrysis galbana, Pavlova leutheri* and *Chaeteceros calcitrans*. These species have been extensively studied for their biochemical composition, as well as nutritional value.

Microalgae can be used as live feed or processed into artificial feed. So far, no completely satisfactory alternative has been found as compared to the use of live microalgae in aquaculture. In spite of all effort to replace the live algae by artificial feeds, marine scientists are still dependent on the production and use of microalgae as live food for commercial aquaculture industries (Albentosa et al., 2002; Enes & Borges, 2003; Bonaldo et al., 2005; Espinosa & Allam, 2006; Chomaco et al., 2007).

Most algae cultivation can be described as either open or closed system. Closed system generally consumes more energy and requires a large capital investment but delivers a relatively high biomass yield. In contrast, open algae growth ponds have lower energy requirement but produce less biomass for the same area. Open ponds can also be affected by contamination and evaporation losses (Lardon et al., 2009; Jorquera et al., 2010; Stephenson et al., 2010). Various physiological and technological approaches have been proposed and investigated for maximising productivity in mass algal culture system. Numerous, more or less sophisticated systems have also been developed for culturing some 40 algal species to feed larvae and zooplankton organisms. Many studies have proven that a large scale production of monospecific algae would be feasible (Chaumont, 1993).

Cultivation technique is crucial in determining the growth of algae culture. While popular indoor algae culture utilises carboy as the culture recipient, polythene bag also shows promising results with better growth rates and shorter duration (Sipauba-Tavares et al., 2011). Thus, there

is a significant difference in the cultivation technique during indoor algae cultivation between carboy and polythene bag culture.

The aim of the present study was to isolate the microalgae from the coastal waters of Kudat in Sabah, Malaysia, and subsequently determine the effects of cultivation techniques on the growth of marine microalgae.

METHODS

Isolation and Identification of Microalgae

Water and rock samples were collected using transparent bottle from the coastal waters of Kudat, Sabah, Malaysia (6°49'12.1"N, 116°51'37.5"E) on May 13, 2012 and July 15, 2012, respectively. The samples were sieved using 50 µm mesh net to eliminate coarse particles and species such as sand and zooplankton.

The mixed algal samples were cultured in a 500 ml shake flasks filled with 400 ml of a standard f/2 medium (Guillard, 1975), with an addition of meta-silicate for diatoms species. After 10 days, the microalgae were identified using stereomicroscope and isolated from other algal species using capillary tube method (Blanco et al., 2008; Debenest et al., 2009). The isolated microalgae were identified morphologically based on size, form and colour. Identification results were verified by comparing with three books, namely, 'The Diatoms: Biology and Morphology of the Genera', 'Marine Algae: Biodiversity, Taxonomy, Environmental Assessment, and Biotechnology' and 'Algae' (Round et al., 1990; Graham et al., 2008; Pereira et al., 2014). The species of *Chaetoceros muelleri, Isochrysis galbana, Pavlova lutheri, Nitszhia acicularis* and *Navicula sp.* were inoculated into fresh f/2 medium and used as inoculum for further studies. All the cultures were maintained at 25 ± 1 °C on a 12 h/12 h light/dark cycle with 1400 lux of light intensity.

Monoalgae Culture

The seawater used for culturing diatoms was filtered through sand filter and subsequently through 1 micron sediment filter cartridge and then treated with sodium hypochlorite for 12 h to kill microorganisms. The sodium hypochlorite in the seawater was neutralised with sodium thiosulphate before use. Non-axenic monocultures of the microalgae species were cultivated in f/2 medium (Guillard, 1975) with an addition of meta-silicate for diatom species in progressive volumes of 10 ml, 150 ml, 1 L. The size of inoculum was adjusted to 1.0×10^4 cells/ml for all the microalgae species.

Algal Culture in Polycarbonate Carboy and Polythene Bag

Monospecific diatom culture of 2 L was inoculated in either 20 L polycarbonate carboy or 20 L polythene bag filled with f/2 medium (Guillard, 1975), with an addition of meta-silicate for diatom species.

The culture from carboy of 20 L was inoculated in transparent fiberglass tank filled with 100 L f/2 medium and it was subsequently transferred into an outdoor tank filled with 1000 L f/2 medium.

The 20 L culture in the polythene bag, however, was combined with another 5 sets of similar polythene bag culture to make up 100 L culture and transferred directly into an outdoor tank filled with 1000 L medium. The differences of algal growth in both methods were recorded.

Continuous aeration was provided. Cell concentration was counted using a haemocytometer and expressed in cells/ml.

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA), SPSS version 17 (SPSS Inc., Chicago, IL, USA). Significant differences were determined at 0.05 level of probability.

RESULTS

Isolation and Identification of Microalgae

There were 5 species of microalgae isolated from the mixed culture sample collected from the coastal area of the sea cucumber hatchery. The species were identified based on their morphological characteristics.

Diatoms have a characteristic that they are enclosed within a cell wall made of silica. *Chaetoceros muelleri* had frustules with long, thin spines. *Navicula sp*. was characterised by valves with bilateral symmetry and a well-developed raphe system. *Nitzschia acicularis* had extremely long and narrow valves, which were lightly silicified. *Pavlova lutheri* was characterised based on the spherical shape, with two flagellae and golden brown colour of the cell. Their sizes were about 3-5 µm. Isochrysis galbana had the same morphology as *Pavlova lutheri* but the size was bigger than *Pavlova lutheri*, i.e. about 5-6 um length and 2-4 µm wide.

Mass Culture of Microalgae

Isolated microalgae were cultured in progressive volume of 10 ml, 150 ml and 1 L culture. All the cultures were kept indoor at the temperature of 25°C. After 5 to 7 days of incubation period in 1 L shake flask, the samples were collected and counted using a haemocytometer. Table 1 shows the cell count for the 5 species of isolated microalgae. The cell for *Isochrysis galbana* showed the highest count of 48×10^6 cells/ml, while Navicula sp. showed the lowest count with an average of 10×10^6 cells/ml compared to the other four species of microalgae. Meanwhile, *Pavlova lutheri*

Table 1
Cell count for 5 species of microalgae measured after 5 to 7 days of incubation period in 1 L culture.

Species	Temperature (°C)	Cell Count (cells/ml)	Incubation period (day)	Volume (L)
Isochrysis galbana	25.0 ± 1.0	$48 \times 10^6 \pm 43 \times 10^5$	5 - 7	1
Pavlova lutheri	25.0 ± 1.0	$34 \times 10^6 \pm 33 \times 10^5$	5 - 7	1
Chaetoceros muelleri	25.0 ± 1.0	$33 \times 10^6 \pm 54 \times 10^5$	5 - 7	1
Navicula sp.	25.0 ± 1.0	$10 \times 10^6 \pm 12 \times 10^5$	5 - 7	1
Nitzschia acicularis	25.0 ± 1.0	$16 \times 10^6 \pm 12 \times 10^5$	5 - 7	1

and *Chaetoceros muelleri* had an average count of 34 × 10^6 and 33 × 10^6 cells/ml, respectively. Low cell count was also recorded on Nitzschia acicularis with 16 × 10^6 cell/ml.

Two litres of monoalgal cultures from the shake flask were used as the starter to inoculate 20 L carboy or polythene bag culture. The cultures were left to grow for 3 to 5 days indoor with temperature ranging from 24 – 26°C before they were used as starter culture for the mass outdoor culture. Table 2 and Table 3 show the cell count of the 5 species of microalgae in carboy or polythene bag culture respectively during the day of harvest.

Isochrysis galbana recorded the highest cell count in carboy culture with the average of 55 × 10^6 cells/ml, while *Nitzschia acicularis* recorded the lowest cell count, with the average of 14 × 10^6 cells/ml. *Isochrysis galbana* was significantly higher than other species ($p < 0.05$) in the carboy culture. However, in the polythene bag culture, *Chaetoceros muelleri* had the highest cell count of average 87 × 10^6 cells/ml [$F(4,10) = 99.6$, $p = 0.000$)]) compared to other culture. Meanwhile, *Nitzschia acicularis* showed the lowest cell count in the polythene bag culture as well, with an average of 25 × 10^6 cells/ml. There were also increases in the cell count of *Nitzschia acicularis* and *Navicula sp*., although not as much as *Chaetoceros muelleri* and *Isochrysis galbana*. The highest cell count for *Nitzschia acicularis* was in the polythene

Table 2
Cell count for 5 species of microalgae measured after 3 to 5 days of incubation period in 20 L carboy culture.

Species	Temperature (°C)	Cell Count (cells/ml)	Incubation period (day)	Volume (L)
Isochrysis galbana	25.0 ± 1.0	55 × 10^6 ± 12 × 10^5	3 - 4	20
Pavlova lutheri	25.0 ± 1.0	37 × 10^6 ± 22 × 10^5	3 - 4	20
Chaetoceros muelleri	25.0 ± 1.0	42 × 10^6 ± 21 × 10^5	3 - 4	20
Navicula sp.	25.0 ± 1.0	21 × 10^6 ± 37 × 10^5	3 - 5	20
Nitzschia acicularis	25.0 ± 1.0	14 × 10^6 ± 16 × 10^5	3 - 5	20

Table 3
Cell count for 5 species of microalgae measured after 2 to 3 days of incubation period in 20 L polythene bag culture.

Species	Temperature (°C)	Cell Count (cells/ml)	Incubation period (day)	Volume (L)
Isochrysis galbana	25.0 ± 1.0	80 × 10^6 ± 16 × 10^5	2 - 3*	20
Pavlova lutheri	25.0 ± 1.0	47 × 10^6 ± 29 × 10^5	2 - 3*	20
Chaetoceros muelleri	25.0 ± 1.0	87 × 10^6 ± 24 × 10^5	2 - 3*	20
Navicula sp.	25.0 ± 1.0	33 × 10^6 ± 24 × 10^5	2 - 3*	20
Nitzschia acicularis	25.0 ± 1.0	25 × 10^6 ± 21 × 10^5	2 - 3*	20

*All cultures show a significant difference at $p < 0.05$ when compared to carboy culture

culture with average cell of 25 × 10^6 cells/ml while *Navicula sp*. recorded an average of 33 × 10^6 cells/ml. Meanwhile, *Pavlova lutheri* recorded an average of 37 × 10^6 cells/ml in the carboy culture and 47 × 10^6 cells/ml in polythene bag culture. All the cultures showed a significant ($p < 0.05$) increase in their cell count when cultured in polythene bag.

The time taken to harvest the cultures was also faster in polythene bag compared to the carboy. It took about 3 to 4 days to harvest *Isochrysis galbana, Pavlova lutheri* and *Chaetoceros muelleri* in carboy culture, whereby *Navicula sp.* and *Nitzschia acicularis* required longer period of 3 to 5 days to harvest. Meanwhile in the polythene bag culture, it took an average of 2 to 3 days for all the microalgal species to be harvested.

One hundred litres of culture from carboy or polythene bag was used as inoculum for outdoor mass culture with volume of 1 m^3 and temperature ranging from 30 to 32°C. Cell count was taken on the day of harvest. All microalgae culture showed a slightly lower cell count compared to indoor culture, this may be due to changes in environment such as temperature and light. Table 4 and Table 5 present the cell count for the 5 species of microalgae using inoculum from carboy and polythene bag, respectively.

Nitzschia acicularis had the lowest cell count, while *Chaetoceros muelleri* grew well in outdoor mass culture. The *Chaetoceros muelleri* sample from outdoor mass culture tank using carboy culture as inoculum recorded an average cell count of 49 × 10^6 cells/ml, which was significantly higher [$F(4,10) = 117.7$, $p = 0.000$)] than the other microalgae culture and indoor culture as well. Following that, *Isochrysis galbana* showed a high cell count of an average of 47 × 10^6 cells/ml. *Navicula sp*. and *Nitzschia acicularis* recorded a low cell count with an average of 20 × 10^6 and 12 × 10^6 cells/ml, respectively. However, the amount was considered as feasible for an outdoor mass culture. There were no significant differences between the outdoor and indoor cultures, except for *Isochysis galbana* ($p < 0.05$).

Table 4
Mean temperature (°C) and cell count (cells/ml) for 5 species of microalgae measured at the harvest time of the outdoor algae culture using microalgae cultured in carboy as inoculum.

Species	Temperature °(C)	Cell Count (cells/ml)	Time taken to harvest (h)	Volume (m^3)
Isochrysis galbana	31.5 ± 1.5	47 × 10^6 ± 21 × 10^5	60 - 72	1
Pavlova lutheri	31.3 ± 1.3	34 × 10^6 ± 17 × 10^5	60 - 72	1
Chaetoceros muelleri	31.2 ± 0.6	49 × 10^6 ± 29 × 10^5	60 -72	1
Navicula sp.	31.7 ± 0.5	20 × 10^6 ± 24 × 10^5	60 - 72	1
Nitzschia acicularis	31.7 ± 0.5	12 × 10^6 ± 12 × 10^5	60 - 72	1

Table 5
Mean temperature (°C) and cell count (cells/ml) for 5 species of microalgae measured at the harvest time of the outdoor algae culture using microalgae cultured in polythene bag as inoculum.

Species	Temperature (°C)	Cell Count (cells/ml)	Time taken to harvest (h)	Volume (m^3)
Isochrysis galbana	31.3 ± 1.3	$73 \times 10^6 \pm 27 \times 10^5$*	60 - 72	1
Pavlova lutheri	31.7 ± 0.5	$40 \times 10^6 \pm 12 \times 10^5$	60 - 72	1
Chaetoceros muelleri	31.3 ± 1.3	$89 \times 10^6 \pm 42 \times 10^5$*	60 - 72	1
Navicula sp.	31.7 ± 0.5	$26 \times 10^6 \pm 22 \times 10^5$	60 - 72	1
Nitzschia acicularis	31.5 ± 1.5	$15 \times 10^6 \pm 12 \times 10^5$	60 - 72	1

*Cultures show significant difference ($p<0.05$) compared to culture using carboy as inoculum.

From the tank of outdoor mass culture using polythene bag culture as inoculum, the cell count for *Chaetoceros muelleri* was 89×10^6 cells/ml, which was significantly higher [$F(1,4) = 124.1$, $p = 0.000$)] than the sample from the tank of outdoor mass culture with carboy as inoculum and its indoor culture. All the cultures were generally higher in cell count compared to the cultures grown in the outdoor mass tank using carboy culture as inoculum. *Nitzschia acicularis* had the lowest cell count of an average of 15×10^6 cells/ml. There were significant differences ($p < 0.05$) in the cell growth for *Isochrysis galbana* [$F(1,4) = 108.6$, $p = 0.000$)] and *Chaetoceros muelleri* [$F(1,4) = 124.1$, $p = 0.000$)] between the indoor and outdoor cultures, while *Pavlova leutheri*, *Navicula sp*. and *Nitzschia acicularis* showed no significant difference between the indoor and outdoor cultures ($p >0.05$).

The time taken to harvest the outdoor mass cultures was about 60 – 70 hours after inoculation, depending on the cell count and demand of the species cultured such as the sea cucumber larvae and juvenile.

DISCUSSION

The advantageous effects of dietary algae are their contributions to minerals, dietary fibre, carotenoids, chemical feeding attractants, vitamins and synergistic effects with vitamins and antioxidants. Microalgae are widely used in aquaculture due to their easy culture and fast growth. They have also been used as sources of essential fatty acids. There were 5 species of commonly used microalgae in the aquaculture industries isolated and identified from a sea cucumber hatchery in Kudat, Sabah, namely, *Isochrysis galbana, Pavlova lutheri, Chaetoceros muelleri, Navicula sp.* and *Nitzschia acicularis*.

Isochrysis galbana is generally considered the best food for bivalves larvae. *Isochrysis galbana* is small, naked, free-living motile flagellates, which are easily ingestible by small (larval) invertebrate (Martines-Fernandez et al., 2004). *Pavlova lutheri* has the same morphological features with *Isochrysis galbana*, but they differ in terms of size, whereby *Pavlova lutheri* cells are bigger than those of *Isochrysis galbana*.

Based on the results the monoalgal cultures were successfully cultured indoor and outdoor. The algal cultures were species specific. Each species carries specific characteristics and requirement for their growth (Cobelas et al., 1998). There were significant differences between the indoor and outdoor cultures for *Pavlova lutheri, Navicula sp*. and *Nitzschia acicularis* from the polythene bag cultures, as well as *Isochysis galbana* from carboy cultured. However, there were no differences in the rest of the species cultures. These results showed that the mass outdoor cultures for these species were feasible.

There were slight decreases in the cell number of a few species of microalgae in this study when they were being transferred outdoor. Numerous studies have shown that the biochemical composition and physiological status of microalgae cells are altered by different environmental factors (Alonso et al., 2000; Sanchez et al., 2000; Renaud et al., 2002; Berges et al., 2002; Tzovenis et al., 2003). Optimum temperatures for diatoms range from 19°C to 27°C; thus, the high temperature outdoor may have killed the cells or delay the growth. However, there were also reports indicating that most algae can tolerate high temperature up to 36°C. This further explains why a few species of microalgae grew so well in outdoor culture. Furthermore, the presence of contaminants is unavoidable in outdoor culture. Culturing microalgae in outdoor environment can achieve higher yields, leading to a reduction of operating cost compared to the cultures cultivated indoors (Elias et al., 2005), however in this study, slightly lower yields were observed in the outdoor culture compared to indoor culture.

All the microalgae cultures in the polythene bag have a higher cell count compared to the carboy culture. Lin et al. (2007) reported that light is the main factor that interferes with the growth of microalgae. Polythene bag has better light penetration compared to carboy. Furthermore, the surface area is wider in polythene bag compared to carboy which has round surface. All the five species of microalgae showed the same growth trend when the carboy and polythene bag were compared. The usage of polythene bag as culture recipient has proven to be efficient with good growth rates and shorter duration (Sipauba-Tavares et al., 2011). Furthermore, it incurs low cost and requires smaller space in the laboratory.

CONCLUSION

This study showed that the mass outdoor cultures of five species of microalgae isolated from Kudat in Sabah, Malaysia are feasible. Furthermore, there were significant differences between the indoor and outdoor cultures ($p < 0.05$). All the microalgae cultures in polythene bag showed significant differences ($p < 0.05$) compared to those of the carboy culture, suggesting that culturing using polythene bag is more efficient, resulting in a shorter harvest period. Future studies need to optimise the growth parameter of the mass outdoor culture further by studying other factors such as light, temperature, genetic stability and weather.

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