POTENTIALITY OF LOCALLY ISOLATED PURPLE NON-SULFUR BACTERIA AS AQUACULTURE FEED SUPPLEMENT IN FINFISH LARVICULTURE SYSTEM

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THESIS SUBMITTED IN FULFILLMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE

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BACTERIA AS AQUACULTURE FEED SUPPLEMENT IN FINFISH
LARVICULTURE

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ABSTRACT

POTENTIALITY OF LOCALLY ISOLATED PURPLE NON-SULFUR BACTERIA AS AQUACULTURE FEED SUPPLEMENT IN FINFISH LARVICULTURE SYSTEM

Six strains of purple non-sulfur bacteria (UMSSTR1, UMSSTR2, UMSSTR3, UMSSTR4, UMSSTR5 and UMSSTR6) were isolated from the different locations of UMS beach sediments with an aim to evaluate the potentiality as an aquaculture feed supplement. They were grown in 112 synthetic media under anaerobic condition at 2500 lux light illumination at 30±2°C temperature. Initial screening was done based on their growth characteristics in terms of dry cell biomass (g/L), production of total carotenoids (mg/g dry cell weight) and content of bacteriochlorophyll. The highest total carotenoid of 3.53±0.46 mg/g dry cell weight was obtained at 24hr culture in the strain UMSSTR1, but the highest dry cell weight of 5.67±0.42 g/L was obtained at 48hr culture in the strain UMSSTR2. No significant differences were observed in dry cell weight and the production of total carotenoid (p>0.05) among the six strains. Two strains (UMSSTR1 and UMSSTR2) were selected for identification based on 16S rRNA. They were identified as Rhodovulum sulphidophilum (Rhv. sulphidophilum). Mass cultivation of the highest produced biomass strain UMSSTR2, Rhv. sulphidophilum was selected for finfish feeding trials. Two feeding trials were conducted to observe the growth and survival of finfish larvae with traditional live feed incorporated with Rhv. sulphidophilum bacteria biomass. The first feeding trial was done in African catfish (Clarias gariepinus) larvae and the second feeding experiment was conducted in River catfish (Pangasius hypophthalmus) larvae. Six experimental feeds diets were prepared and used in both feeding trials. The experimental diets were: the fresh bacterial biomass of Rhv. sulphidophilum as diet 1, Nannochloropsis as diet 2, Artemia nauplii as diet 3, Rhv. sulphidophilum + Nannochloropsis as diet 4, Rhv. sulphidophilum + Artemia as diet 5 and Rhv. sulphidophilum + Artemia + Nannochloropsis as diet 6. In feeding trial with C. gariepinus larvae, the highest growth in length of 2.58±0.65cm, 23.15% specific growth rate and the highest survival of 90±4% obtained with diet 6. The results shows no significant differences (p>0.05) in the length and survival of the C. gariepinus larvae among six types of diets. The second feeding experiment was conducted with P. hypophtalmus larvae for the period of 20days. The highest growth of 2.73±0.2cm with 11.55% specific growth rate and the survival of 86±2% were observed with larvae fed with diet 6. Significant differences (p<0.05) were observed in length and survival of P. hypophtalmus larvae fed with six types of diets. Both the experiments showed increment in the growth and survival in larvae. The bacterium strain UMSSTR2, Rhv. sulphidophilum incorporated with the Artemia and Nannochloropsis act as feed supplement for larval diet and enhanced the growth and survival in C. gariepinus and P. hypophtalmus larvae. Thus, the locally isolated bacterium Rhv. sulphidophilum has the potentiality to be used as aquaculture feed additive in addition to other live feed during larval rearing period of C. gariepinus and P. hypophtalmus in larviculture system.
ABSTRAK

Enam strain bakteria ungu yang bukan penghasil sulfur (UMSSTR1, UMSSTR2, UMSSTR3, UMSSTR4, UMSSTR5 dan UMSSTR6) telah dipencilkan daripada lokasi yang berbeza di pantai UMS dengan tujuan untuk menilai potensi pengeluaran sebagai suplemen makanan akuakultur. Bakteria tersebut tumbuh dalam 112 Media sintetik dalam keadaan anaerobik di bawah 2500 lux pencahayaan pada suhu 30±2°C. Saringan awal telah dilakukan berdasarkan ciri-ciri pertumbuhan mereka dari segi biojisim sel kering (g/L), pengeluaran jumlah karotenoid (mg/g berat kering sel) dan kandungan bacteriochlorophyll. Jumlah tertinggi karotenoid 3.53 ± 0.46 mg/g berat kering sel didapati pada 24 jam daripada strain UMSSTR1, tetapi berat sel kering tertinggi 5.67 ± 0.42 g/L telah diperolehi pada 48 jam daripada strain UMSSTR2. Tiada perbezaan signifikan yang ketara telah diperhatikan dalam berat sele kering dan pengeluaran jumlah karotenoid (p>0.05) di kalangan enam strain. Dua strain (UMSSTR1 dan UMSSTR2) telah dipilih untuk mengenal pasti berdasarkan 16S rRNA. Mereka dikenalpasti sebagai bakteria Rhodovulum sulphidophilum (Rhv. sulphidophilum). Biojisim tertinggi dihasilkan oleh UMSSTR2 (Rhv. sulphidophilum) melalui pengkulturan skala besar telah dipilih untuk percubaan pemakanan ikan. Dua percubaan pemberian makanan telah dijalankan untuk memerhati pertumbuhan dan jangka hayat larva ikan dengan makanan hidup tradisional yang digabungkan dengan bakteria biomas Rhv. sulphidophilum. Ujian pemakanan yang pertama telah dilakukan dengan larva ikan Keli (Clarias gariepinus) dan ujian kedua telah dijalankan dengan larva Patin (Pangasius hypophthalmus). Enam diet eksperimen telah disediakan dan digunakan dalam kedua-dua percubaan pemakanan. Diet eksperimen adalah seperti berikut: biojisim segar bakteria Rhv. sulphidophilum sebagai diet 1, Nannochloropsis sebagai diet 2, Artemia nauplii sebagai diet 3, Rhv. sulphidophilum + Nannochloropsis sebagai diet 4, Rhv. sulphidophilum + Artemia sebagai diet 5 dan Rhv. sulphidophilum + Artemia + Nannochloropsis sebagai diet 6. Pertumbuhan tertinggi 2.58 ± 0.65cm, 23.15% kadar pertumbuhan spesifik dan jangka hayat yang tertinggi sebanyak 90 ± 4% diperolehi dengan diet 6 dalam ujian percubaan pemberian makanan kepada larva C. gariepinus. Keputusan menunjukkan tiada perbezaan signifikan (p>0.05) dalam panjang dan jangka hayat larva C. gariepinus antara enam jenis diet. Eksperimen pemakanan kedua dijalankan dengan larva ikan P. hypophthalmus bagi tempoh 20 hari. Pertumbuhan tertinggi 2.73±0.2 cm dengan kadar pertumbuhan 11.55% dan jangka hayat sebanyak 86±2% diperhatikan dari larva yang makan dengan diet 6. Perbezaan signifikan (p<0.05) telah diperhatikan dengan panjang dan jangka hayat larva P. hypophthalmus makan dengan enam jenis diet. Kedua-dua eksperimen menunjukkan peningkatan dalam pertumbuhan dan kelangsungan hidup dalam larva. Bakteria UMSSTR2, Rhv. sulphidophilum bekerjasama dengan Artemia dan Nannochloropsis sebagai suplemen makanan larva dan meningkatkan pertumbuhan dan kelangsungan hidup larva C. gariepinus dan P. hypophthalmus. Oleh itu, bakteria tempatan Rhv. sulphidophilum mempunyai potensi untuk digunakan sebagai makanan tambahan akuakultur dengan makanan hidup yang lain sepanjang tempoh pemeliharaan larva C. gariepinus dan P. hypophthalmus dalam sistem pengkulturan larva.
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SOC</td>
<td>Super Optimal Broth</td>
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<td>TAE</td>
<td>Tris-acetate-EDTA</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>FB</td>
<td>Fresh biomass</td>
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<tr>
<td>G</td>
<td>Guanine</td>
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<tr>
<td>C</td>
<td>Cytosine</td>
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<tr>
<td>X-Gal</td>
<td>Bromo-chloro-indolyl-galactopyranoside</td>
</tr>
<tr>
<td>LH</td>
<td>Light harvesting</td>
</tr>
<tr>
<td>GMM</td>
<td>Glutamate Malate Media</td>
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<tr>
<td>dAH</td>
<td>Day after hatch</td>
</tr>
<tr>
<td>PNSB</td>
<td>Purple Non Sulfur Bacteria</td>
</tr>
<tr>
<td>PSB</td>
<td>Purple Sulfur Bacteria</td>
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<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<td>HUFA</td>
<td>Highly Unsaturated Fatty Acid</td>
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HPLC  High Performance Liquid Chromatography
min  Minute
uv  Ultraviolet
Fig  Figure
TL  Total length
sp.  Species
ha  Hector
UMS  University Malaysia Sabah
UMSSTR1  University Malaysia Sabah Strain 1
UMSSTR2  University Malaysia Sabah Strain 2
UMSSTR3  University Malaysia Sabah Strain 3
UMSSTR4  University Malaysia Sabah Strain 4
UMSSTR5  University Malaysia Sabah Strain 5
UMSSTR6  University Malaysia Sabah Strain 6
D1  Diet 1 (*Rhodovulum sulphidophilum*)
D2  Diet 2 (*Nannochloropsis*)
D3  Diet 3 (*Artemia*)
D4  Diet 4 (*Rhodovulum sulphidophilum* + *Nannochloropsis*)
D5  Diet 5 (*Rhodovulum sulphidophilum* + *Artemia*)
D6  Diet 6 (*Rhodovulum sulphidophilum* + *Artemia* + *Nannochloropsis*)
# LIST OF SYMBOLS

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<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>%</td>
<td>percentage</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>&gt;</td>
<td>More</td>
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<tr>
<td>&lt;</td>
<td>Less</td>
</tr>
<tr>
<td>±</td>
<td>Plus or minus</td>
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<tr>
<td>=</td>
<td>Equal</td>
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<tr>
<td>+</td>
<td>Plus</td>
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<tr>
<td>μl</td>
<td>Microliter</td>
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<tr>
<td>CFU/ml</td>
<td>Colony forming unit/milliliter</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td><em>et al.,</em></td>
<td>And others</td>
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<tr>
<td>g/L</td>
<td>Gram/liter</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mg/g</td>
<td>Milligram per gram</td>
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<tr>
<td>mg/L</td>
<td>Milligram per liter</td>
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<tr>
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<td>Nannogram</td>
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<td>kb</td>
<td>Kilobase</td>
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<td>Nannometer</td>
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<tr>
<td>rpm</td>
<td>Revolution per minute</td>
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<td>v/v</td>
<td>volume/volume</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>sd</td>
<td>Standard deviation</td>
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<td>e.g</td>
<td>For example</td>
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<tr>
<td>etc</td>
<td>And so on</td>
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<tr>
<td>ml</td>
<td>Milli liter</td>
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<td>hr</td>
<td>Hour</td>
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<td>Appendix</td>
<td>Description</td>
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<td>Appendix A</td>
<td>Gram Stain</td>
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<td>Motility Test</td>
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<td>Reagent for Cloning</td>
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<td>Appendix M</td>
<td>Preparation of LB Agar</td>
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<td>Appendix N</td>
<td>Characteristics of bacteriochlorophyll</td>
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<td>Appendix O</td>
<td>Dry cell weight (g/L)</td>
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<td>Appendix P</td>
<td>Carotenoid (mg/g dry cell weight)</td>
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<td>Appendix Q</td>
<td>Length, body Weight, and survival of African catfish larvae (Clarias gariepinus) Larvae</td>
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<tr>
<td>Appendix R</td>
<td>Length, body Weight, and survival of river catfish larvae (Pangasius hypophthalmus) larvae</td>
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CHAPTER 1

INTRODUCTION

1.1 Purple Non Sulfur Bacteria

The d-Proteobacteria is purple non sulfur bacteria which is able to perform anoxygenic photosynthesis (Imhoff, 2006). Representatives of the purple non sulfur bacteria are widely distributed in nature and are found in all kinds of stagnant water bodies, in lakes, waste water ponds, coastal lagoons, and other aquatic habitats, sediments, moist soils, and paddy fields (Imhoff and Truper 1991).

Typically, phototrophic d-Proteobacteria grows under anoxic conditions in the presence of light. Their phototrophic growth; photosynthetic pigment synthesis and internal membrane formation are inhibited by oxygen (Imhoff, 2006). Due to the presence of photosynthetic pigments, cell suspensions appear in various colors from beige, olive green, peach-brown, brown, brown-red, red or pink and able to absorb the light spectrum. Photosynthetic pigments bacteriochlorophyll a or b and various types of carotenoids are located in the cytoplasmic membrane and internal membrane systems (vesicles, lamellae or membrane stacks) (Imhoff, 2006).

The purple non sulfur bacteria grow fast in the anaerobic light condition than in aerobic dark condition. The cells yields in aerobic dark condition are lower than in the anaerobic light condition (Azad et al., 2001). They also have the ability to grow in synthetic media and as well as in waste water (Azad et al., 2003).

Research was conducted to study the application of purple non sulfur bacteria in aquaculture sector. The application of live microbial feed supplement plays an important role in aquaculture feed industry and has contributed to the commercial success of finfish larvae culture system (Qi et al., 2009). However, successful larviculture still highly depends on live feeds (Liao et al., 1990). Phototrophic bacteria have the potential to be used as an aquaculture feed
supplement since they have been found to be nutritious and non-toxic (Getha et al., 1998a). They contain biological cofactors that improve the survival and growth of the fries of Oreochromis niloticus (Banerjee et al., 2000), shrimps (Azad et al., 2002; Cui et al., 1991) and fish larvae (Kobayashi and Kobayashi 1995). The biotechnological application of purple non sulfur bacteria as an aquaculture feed supplement could improve the future production of aquaculture.

1.2 Challenges in Malaysian Aquaculture Industry in seeds production

Over the past 30 years, increase in population, over fishing and water pollution have resulted in the declining of many wild fish populations in the Southeast Asia. Fish farming is helping to bridge the declining catches to increase the demand of the fish (Petkam and Moodie, 2001). Similar to other nations, fish and fish based products are playing an important role in daily diet of people in Malaysia. Majority still depend on seafood as the main source of animal protein.

Aquaculture is a new and developing industry in Malaysia. The aquaculture industries are facing many challenges in producing high quality fish seed for commercial scale. The production of fish larvae is still a major constraint in the development of freshwater and marine fish culture in Malaysia (Othman, 2010). Large amount of fish larvae are still being imported from neighborhood countries such as Indonesia, Thailand, Singapore and as far as from Taiwan.

Freshwater fishes are the main aquaculture species in Malaysia in addition to brackish and seawater fish. Freshwater aquaculture was predominated by pond culture covering an area of 4769ha with a production of 49 951 tonnes. In 2003 freshwater aquaculture constituted about 30 percent of the total aquaculture production (Anon, 2004). More than 80 percent of the freshwater aquaculture production comprised of freshwater species such as red hybrid tilapia, hybrid walking catfish and climbing perch. Floating net cage culture of red tilapia and river catfish, Pangasius and the Mystus, are practiced in lakes, reservoirs and ex-mining pools, occupying an area of 2734 ha (Anon, 2004). A small percentage of about 10 percent of the freshwater pond area is used for the polyculture of the Chinese
carps, Javanese carp and common carp, for river mahseer, snake head, marble goby, arowana and giant freshwater prawns (Anon, 2004).

At the present, local seed production centers are still too small to supply the demand especially when dealing with multi species of fish production. More over most do not always meet the requirement of good quality seed for sustainable grow out farms (Othman, 2010). Nutrition and feeds, disease outbreaks, predators and water quality are the well known major problems in fish larviculture system.

In spite of artificial feeds, fish larvae culture system during the primary nursing phase still depends heavily on natural food. Live feeds including rotifers, Artemia and other microorganisms are often the first food in aquaculture (Stickney, 2000). Larval nutrition and live feed culture like Artemia and rotifers are one of the most important obligatory matters for successful fish culture. Feeding with live prey for fish larvae is most essential because during first few days of their life they do not have complete developed system, especially their digestive tracts (Stickney, 2000).

There are many researches were done applying probiotic bacteria in aquaculture. Microbial feeds produced through biotechnological processes have been actively investigated as alternative or unconventional feed supplement as well as probiotic resources for aquaculture and aquaculture systems (Banerjee et al., 2000). Thus, the use of probiotics in aquaculture has received a great deal of attention. Some common strains are used as probiotics products including Lactobacillus acidophilus, L. rhamnosus, L. plantarum, Streptococcus lactis and Carnobacterium sp. (Watson et al., 2008).

1.3 Purple non sulfur bacteria as growth promoting substances in aquaculture
Purple non sulfur bacteria have the potential to be used as aquaculture feed supplement. The presence of these bacteria enhances the growth and survival of the fish. The previous studies show that purple non sulfur bacteria are rich in nutrient and it could be applied in larvae production by advanced mass production
In aquaculture (Azad et al., 2002). Phototrophic bacteria contain biological cofactors that improve the survival and growth of the fish larvae and enhance the skin development of the carps and prawns (Qi et al., 2009). The potential biotechnological applications of phototrophic bacteria include: production of single cell protein (SCP) (Noparatnaraporn and Nagai 1986a), enzymes (Buranakarl et al., 1988), carotenoid, vitamins, ubiquinones (Q10) (Sasaki and Nagai 1979) and uptake of heavy metal from polluted water (Vatsala, 1987).

The applications of phototrophic bacteria provide positive results in aquaculture. *Rhodopseudomonas palustris* strain B1 which is an isolate from starch noodle processing wastewater was used as a complete diet in *Artemia* culture system. Experimental feeding trials on brine shrimp (*Artemia*) larvae showed the bacteria enhanced *Artemia* larval growth and survival rate (Getha et al., 1998a). Further, fry of loach, goldfish, carp, ark shell, and sweet fish are direct predators of phototrophic bacteria soon after hatching, resulting in an increase in weight and survival rate more than two fold within 2 to 4 weeks after hatching (nearly no death was noted in some experiments) (Kobayashi et al., 2001).

The purple non sulfur bacteria are incorporated in other live feeds to induce the growth of fish and shrimp larvae. The addition of *Rhodovulum sulfidophilum* biomass and *Skeletonema* increases the growth and survival of the *P. monodon* larvae (Azad et al., 2002). Study was done with sea bass (*Lates calcarifer*) larvae at Borneo Marine Research Institute of UMS, showed that the highest growth and survival of seabass larvae was observed with diet enriched with phototrophic bacterium, *Artemia* and *Nannochloropsis* (Azad et al., 2008).

The previous study at University Malaysia Sabah was conducted to monitor the growth and survival of seabass (*Lates calcarifer*) larvae with *Artemia* enriched phototrophic bacterium. The mix feed of purple non sulfur bacteria, *Nannochloropsis* and *Artemia* increased the survival and growth of the seabass larvae (Azad et al., 2008). Normally farmers apply commercial microbes in aquaculture, especially for marine fish and shrimps to overcome disease outbreaks, improve water quality and to increase the survival rate of the cultured species.
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