IMMOBILIZATION OF PURPLE NON-SULFUR BACTERIA TO REMOVE INORGANIC NUTRIENTS FROM AQUARIUM SYSTEM

LEE KAR CHUN

THIS DISSERTATION IS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE WITH HONOURS

AQUACULTURE PROGRAMME
FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH

2015

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

JUDUL: IMMOBILIZATION OF PURPLE NON-SULFUR BACTERIA TO REMOVE INORGANIC NUTRIENTS FROM AQUARIUM SYSTEM

IJAZAH: BACHELOR OF SCIENCE WITH HONORS (AQUACULTURE) (HS27)

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ACKNOWLEDGEMENT

I would like to take this opportunity to thank everyone that involved directly and indirectly in providing assistance and suggestion to me. First of all, a deepest gratitude will be given to my supervisor, Dr. Sujjat Al-Azad for his advices, guidance and encouragement all along the time.

I would like to thank to all lecturers, laboratory assistants and my fellow course mate who guided me in all sorts of work including field work and lab work. The lab assistants were Ms. Syafikah, Ms. Jojo, and Ms. Lulu.

Last but not least, I would like to thank my parents and friends who supported me mentally or financially during the completion of work. Their unconditional support serves as encouragement and motivation in helping me getting the study done within the given time frame.

To all whom I mentioned above, your mental and physical support is very much valued

Thank you.

ABSTRAK

Tujuan eksperimen ini ialah menentukan kesesuaian antara dua substrat iaitu substrat agar dan cebisan batu karang untuk diimobilisasi dengan *purple non sulfur bacteria (PNSB)* dan ia telah dikodkan sebagai FWS-KC1. Dua eksperimen telah dijalankan dimana eksperimen pertama iaitu pengasingan dan purifikasi PNSB diikuti dengan percubaan menggunakan akuarium dan menggunakan dua rawatan dimana PNSB diimobilisasi di permukaan substrat agar dan cebisan batu karang. Eksperimen yang pertama ialah pengasingan PNSB dari kolam takungan sisa buangan dan kolam semulajadi berhampiran Universiti Malaysia Sabah (UMS). Selepas pengasingan PNSB, ia diinkubasi di bawah keadaan anaerobik dimana kepekatan cahaya dikekalkan pada 2.5 klux dan suhu ialah 30$\pm$2 ^{0}C dan tempoh inkubasi ialah 1 minggu. Media kultur yang telah digunakan ialah media 112. Beberapa analisis telah dijalankan untuk menguji bakteria jenis ini (FWS-KC1) dalam terma yang tersebut berat kering sel dan pengeluaran yang tertinggi iaitu 1.9267 g/L (Hari 6), karrotenoid ialah 2.2603 mg/g (Hari 3) dan untuk klorofil bacteria, penyerapan yang tertinggi ialah 376, 379, 494, 524, 805, 864 nm. Justeru, ujian biokimia telah dijalankan seperti ujian anoxygenik dan oksigenik, ujian penggerakkan, ujian kemasinan, *gram staining* dan pandangan mikroskopik. Konklusi telah dibuat iaitu FWS-KC1 ialah *gram negative bacteria* yang berkemampuan untuk bergerak dan ia telah diklasifikasikan dalam kumpulan *Rhodopseudomonas* sp.. Untuk eksperimen kedua, percubaan menggunakan akuarium telah dijalankan untuk menentukan kepekatan ammonia, nitrit, nitrat and fosfat. Dua rawatan telah digunakan untuk menentukan kesesuaian untuk PNSB immobilisasi. Kesimpulan kedua ialah, imomobilisasi PNSB tidak sesuai dengan menggunakan substrat agar untuk menggurangkan kepekatan komposisi jenis nitrogen di dalam akuarium kerana kepekatan ammonia (2.4390 mg/L) ialah tertinggi (Hari 6) dan kepekatan fosfat (0.1398 mg/L) rendah di dalam akuarium.

ABSTRACT

The aim of this experiment is to determine the suitability between two substrates which were agar substrates and coral rubbles that immobilized with Purple Non-Sulfur bacteria (PNSB) and it was coded as FWS-KC1. Two experiments were conducted where the first experiment was isolation and purification of PNSB and followed by aquarium trial with two treatments which were immobilization of bacteria on agar substrates and coral rubbles. The first experiment which was isolation and purification of bacteria was started with isolation of bacteria from waste water ponds and natural pond around "Universiti Malaysia Sabah". After the isolation of PNSB, it was incubated under anaerobic condition where the light intensity was maintained at 2.5 klux and temperature was maintained at 30 ± 2 ^{0}C and the incubation period of PNSB was a week. The culture media that used was 112 media. Several analyses have been carried out to test on this strain of bacteria (FWS-KC1) which were in terms of dry cell weight and the highest production was 1.9267 g/L (day 6), carotenoid was 2.2603 mg/g (day 3) and for bacteriochlorophyll, the maxima absorption were 376, 379, 494, 524, 805, 864 nm. In addition, some simple biochemical tests were carried out such as anoxygenic and oxygenic test, motility test, salinity test, gram staining and microscopic viewing. Conclusion has been drawn out that FWS-KC1 was gram negative bacteria which were able to move around (motile) and it was classified in the group of *Rhodopseudomonas*. For second experiment, aquarium trial has been carried out to determine concentration of ammonia, nitrite, nitrate and phosphate. Two treatments were used to determine the suitability to immobilize PNSB. Another conclusion can be drawn out that, immobilized bacteria on agar substrates was not suitable to reduce nitrogenous compound in aquarium water because the concentration of ammonia (2.4390 mg/L) was the highest (day 6) and concentration of phosphate (0.1398 mg/L) was low.

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LIST OF ABBREVIATIONS

PNSB	Purple Non-Sulfur Bacteria
GM	Glutamate Malate
S	*Rhodobacter sphaeroides*
NR-3	*Rhodobacter sphaeroides*
P	*Rhodopseudomonas palustris*
COD	Chemical Oxygen Demand
FSNR	Faculty of Science and Natural Resources
UMS	Universiti Malaysia Sabah
DCR	Dead Corals Replicate
ASR	Agar Substrate Replicate
CR	Control Replicate

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CHAPTER 1

INTRODUCTION

1.1 Background

Aquarium is known as a confined area that consisting of at least one side of translucent mirror or glass. According to Collins Cobuild (2006), aquarium is a glass tank filled with water, in which people keep fish and other organisms. Therefore, aquarium system is considered as closed system. It can also be used to rear other organisms such as amphibians, turtles, marine mammals and aquatic plants as well.

In general, aquatic plants will be grown inside the aquarium and reared fish and other aquatic organisms. The combination of growing plants and rearing fish is known as aquascaping. In other words, aquascaping is planting of aquatic and wetland plants in a particular water bodies and aquarium system with other biotic components which are driftwood, rocks and cave designs (Sanaye & Tibile, 2009). The concept of this system was pioneered by China where they culture gold fish with aquatic plants in bowl (Skomal, 2007). This terminology which is aquascaping was continuing in Japan and started by Japanese named Takashi Amano (Sanaye & Tibile, 2009).

In nature, inorganic nutrients that mainly consist of nitrogenous compounds will be released from fish and other aquatic organisms such as snails and much more as excretory products. These nitrogenous compounds are unuseful in rearing of fish and other aquatic organisms and these nitrogenous compounds deteriorate water quality. Regarding to that, many things are used to reduce the level of nitrogenous compounds in aquarium system such as photosynthetic bacteria (Zhou *et al.*, 2014), aquatic plants (Corpron & Armstrong, 1983), and other filtering systems.

Aquarium system is an enclosed system that primarily consists of fish and other aquatic organisms, aquatic plants and filtering system for purifying water. This system can be sustained with a proper water filtering system. Purple Non-Sulfur Bacteria is a part of the water filter systems that can be immobilized on substrates and carrying out water purifying process (Honda *et al.*, 2006). In general, PNSB is able to survive in oxygen absence condition (Van Niel, 1944).

1.2 Research Objectives

i. To compare level of physico-chemicals which are total ammonia, nitrate-nitrite and phosphate-phosphorus in controlled condition aquarium with other two treatments by using freshwater type purple non-sulfur bacteria in aquarium system
ii. To compare the suitability of substrates (coral rubble and agar) for the immobilization of purple non-sulfur bacteria
iii. To determine the reduction rate of dissolved inorganic nutrients in aquarium system

1.3 Hypotheses

i. The substrates (coral rubble and agar) are suitable for the immobilization of Purple Non-Sulfur bacteria (PNSB).
ii. The substrates that contained immobilized bacteria are able to remove nitrogenous compounds in aquarium water efficiently.

1.4 Scope of Study

This project is focusing on the immobilization of purple non-sulfur bacteria (PNSB) on substrates. The substrates that are used in this experiment are coral rubbles and agar. The suitability of immobilization on substrates is evaluated. The best substrate will be selected and the substrates that attached with PNSB will be used on purifying water and reduce the level of nitrogenous compound in a particular water body which is aquarium system. The hypothetical result on the suitability of substrates is coral rubbles due to its high level of porosity.

Guppy; *Poecilia reticulata* will be used in this aquarium system. This species of fish will be used in aquarium system with the using of immobilized PNSB on substrates because it is a kind of live bearer. In other words, it can be bred in a short period and eggs will be hatched inside their parent's body. After a few weeks, offspring will be released out from the body of female guppy; *Poecilia reticulata* which is in the form of larvae. By using this species, the main advantage is where high number in larvae will excrete higher level of nitrogenous compounds into the water in the aquarium system. Apart from that, guppy; *Poecilia reticulata* is a hardy species and thus it can adapt in a high level of nitrogenous compounds of water in aquarium system. Therefore, reduction rate of dissolved inorganic nutrients in aquarium system can be analyzed.

CHAPTER 2

LITERATURE REVIEW

2.1 Purple Non-Sulfur Bacteria (PNSB)

In general, PNSB can be found mainly in stagnant water bodies which are pond, fresh water marsh (Oda *et al.*, 2003), paddy field (Imhoff, 1992) and stratified fresh water lakes (Eichler & Pfennig, 1988). Inside the lakes and ponds, PNSB is located at the boundary layer of the oxidative and reductive zones under the several conditions which are presence of H_2S and the light intensity is lower than 10% of the surface value (Takahashi & Ichimura, 1968). Regarding to the distribution of PNSB, the water layer at the surface is normally milky green or pink in colour due to the presence of *Thiorhodaceae* or *Chlorobacteriaceae* which is green sulfur bacteria.

Purple non-sulfur bacteria (PNSB) are categorized under anoxygenic phototrophic bacteria and it performs anoxygenic photosynthesis (Imhoff, 1995). The term "anoxygenic photosynthesis" means the process of photosynthesis that is without air and without producing oxygen. Anoxygenic bacteria comprised of green sulfur bacteria (*Chlorobiaceae*) and purple bacteria. Under purple bacteria, there are three families which are (1) *Chromatiaceae*, (2) *Ectothiorhodospiraceae* and (3) Purple Non-Sulfur Bactera (PNSB) (Imhoff, 1995). However, according to Bryant & Frigaard, (2006), purple non-sulfur bacteria can be categorized into five bacterial phyla which are (1) *Chlorobi*, (2) Cyanobacteria, (3) *Chloroflexi* (filamentous anoxygenic phototrophs), (4) *Firmicutes* (heliobacteria) and (5) Proteobacteria (purple sulfur and purple non-sulfur bacteria).

The products from anoxygenic photosynthesis that performed by PNSB are bacteriochlorophyll a and b (Feng *et al.*, 2014), organic acid and carotenoid as pigment (Hubas *et al.*, 2011). However, there are some essential substances for this process which are carbon dioxide, pH value and cell density at the time of switching from aerobic to anaerobic conditions (Rudolf & Grammel, 2012). Absorption of red light and transformation to chemical energy are the function of bacteriochlorophyll a (Hubas *et al.*, 2011). Therefore, inference can be carried out that the red to purple colour biofilm attached on substrate underwater is one of the species of PNSB.

2.2 Role of Purple Non-Sulfur Bacteria (PNSB) in Aquaculture

Purple Non-Sulfur Bacteria (PNSB) possesses many roles in different sectors or fields. This group of bacteria is also considered as probiotics since PNSB can provide various sectors and fields with beneficial functions. PNSB can be used internally and externally where it can be ingested as food and purify water respectively.

In aquaculture, PNSB are used vitally in variety of systems and cultures. For example, it can be used in aquarium system, pond culture and tank culture. The most important role that PNSB plays is in purifying water or known as water stabilization (Veenstra *et al.*, 1995) and it has a capability of degrading organic material (Qi *et al.*, 2009). Apart from that, PNSB are used as food additive for stimulating the growth of fish and shrimp (Zhang *et al.*, 1988), enhance survival rate of larvae and improve the production of scallop seed (Huang *et al.*, 1990). Furthermore, PNSB are used as the live food to increase the population growth rate of rotifer; *Brachionus plicatilis* (Xu *et al.*, 1992). In addition, PNSB is a primary producer where it is capable to carry out photosynthesis without the presence of oxygen (Takahashi & Ichimura, 1968). In other words, PNSB does not require CO_2 as the component of photosynthesis and yet it is able to produce primary products which are mainly of O_2.

2.3 Immobilization of Bacteria using Substrates

There are many species of bacteria that can reduce the concentration of inorganic substances that present in aquarium system. By using these bacteria, level of nitrogenous products and organic waste can be reduced and degraded respectively, and able to produce clear water for efficient anoxygenic photosynthesis.

According to Lozinsky *et al.* (1997), the bacteria that used in their research were *Citrobacter intermedius, Zymomonas mobilis,* and *Pseudomonas* sp. in reducing the level of nitrogenous compounds. Freeze-thawing method is used to immobilise these bacteria. All these types of bacteria have their own cultivation method and method of harvesting. In addition, the dry weight contents for any particular species of bacteria are different in terms of percentage. The dry weight percentage for *C. intermedius* was 17%, *Z. mobilis* was 20%, *Pseudomonas* sp. was 39% and *S. cerevisiae* was 32%. Before the bacteria are harvested, centrifugation of the cells need to be carried out with different speeds in terms of revolution per minutes (rpm) by using centrifuge machine where the *C. intermedius* was centrifuged with the speed of 3500 rpm for 15 minutes and *S. cerevisiae* was 6000 rpm for 25 minutes.

Other than that, *Rhodovulum* sp. with self-flocculated cells is another type of photosynthetic bacteria that cultured in high density and high density of photosynthetic bacteria are used for waste water treatment processes (Watanabe *et al.*, 1998). This type of photosynthetic bacteria is grown on an acetate NaCl basal minimal medium.

2.4 Immobilization of Purple Non-Sulfur Bacteria (PNSB) using Substrates

In general, immobilization of Purple Non-Sulfur Bacteria (PNSB) on substrates is to develop the colony of bacteria. The proliferation of the colony of bacteria is to intensify the function of the bacteria itself. Immobilization can be defined as prevent something from moving (Online Oxford Dictionary) or simply known as movement prevention. This immobilization process is necessary because when a single strain of bacteria is immobilized on substrate, this specific strain of bacteria will grow into a larger colony.

PNSB can be immobilized onto Na-alginate (Wako Chemical Co. Ltd., Osaka in Nagadomi *et al.*, 1999), polyvinyl alcohol cryogels (PVA, Kuraray PVA-HC, Kuraray Co, Ltd., Osaka in Nagadomi *et al.*, 1999), porous ceramics (Nagadomi *et al.*, 2000). Immobilization on substrates is important in which the substrates will hold the PNSB and it will be more sustainable than in water column. Substrate is mandatory for PNSB due to its nature behavior where PNSB can be found in stagnant water.

2.5 Immobilization of Purple Non-Sulfur Bacteria (PNSB) and Advantages of Immobilization

In nature, PNSB is attached on substrate primarily hot spring, sulfide-rich water, mud and sediment. Therefore, solid substrates are used for immobilization of PNSB. After that, this colony of bacteria will proliferate. The following are the literature reviews attempt to demonstrate and support the hypothesis.

According to Nagadomi *et al.* (1999), photosynthetic bacteria are used to denitrify aquarium water and the bacteria were immobilized in polyvinyl alcohol beads. The species used in that experiment was *Rhodobacter sphaeroides* (S). This specific bacterium was cultured in glutamate-malate medium (GM medium). The main ingredient of this GM medium was 5g/l of KNO_3. The condition for this specific bacterium to grow in GM medium was at 30°C under static-light conditions using two tungsten bulbs (5 klux). After cultivation, cells were harvested by centrifugation (10,000Xg, 20 min) and washed twice with deionized water and immobilized it immediately. The substrates for the cells immobilization were Na-alginate and polyvinyl alcohol beads. PVA gel beads serve a medium for *R. sphaeroides* and active in COD reduction but this medium is not good in denitrification.

According to another research by Hisashi Nagadomi *et al.* (2000), photosynthetic bacteria is immobilized in porous ceramic and it is used to remove chemical oxygen demand (COD), phosphate, nitrate and H_2S simultaneously in the synthetic sewage wastewater. This research is supporting the hypothesis which it can be applied in an aquarium system. Three types of purple non-sulfur bacteria are used in this experiment which is *Rhodobacter sphaeroides* (S), *Rhodobacter sphaeroides* (NR-3) and *Rhodopseudomonas palustris* (P). The medium for cultivating these three different species is glutamate-malate (GM) medium. These three species of bacteria were cultured at 30°C under microaerophillic conditions for 2 days (S and NR-3 strain) and 3 days (P strain). The cells were then harvested in the early stationary phase by centrifugation. Lastly, the cells were washed with deionized water and immobilized it immediately. The substrate used to immobilize the bacteria was porous ceramic (40g) with air space (80%).

According to Takeno (2005), immobilized photosynthetic bacteria are used in treating oil-containing wastewater. The microorganisms used in the experiment were purple non-sulfur bacteria that scientifically known as *Rhodobacter sphaeroides* (S), *Rhodobacter sphaeroides* (NR-3) and *Rhodopseudomonas palustris* (P). These three types of bacteria have its own function and the most suitable for aquarium system is *Rhodobacter sphaeroides* (NR-3). It is because this bacterium is capable to reduce relatively high COD and remove phosphate simultaneously from the wastewater. On the other hand, *Rhodobacter sphaeroides* (S) bacteria were capable to remove oil from waste water effectively compared with the other NR-3 and P strain of bacteria. According to the results, agar is better than Na-alginate where it can be maintained for 2-3 months.

Glutamate-malate (GM) medium is the common medium that used in culturing photosynthetic bacteria primarily of PNSB. This medium is essential for growth and proliferation of PNSB. Besides, a small amount of yeast extract is needed for further growth (Blankenship *et al.*, 2004) for these colonies of bacteria. Large colonies or many colonies will enhance the purifying power on waste water.

2.6 Bacteriochlorophyll Profile of Freshwater Purple Non-Sulfur Bacteria (PNSB)

Generally, bacteriochlorophyll profile can be used as a marker or control in determining and identifying species of bacteria. Absorption maxima are the unit that used to measure wavelength of bacteriochlorophyll and by using absorption maxima, photosynthetic pigment and bacterial group of specific bacteria can be determined. The table below shows the absorption maxima with the specific pigments and bacterial group.

Table 2.1 Absorption maxima of different types of bacteriochlorophyll (Source: C.B. Van Niel, 1932)

Pigment	Bacterial Group	*In vivo* infrared absorption maximum (nm)
Bacteriochlorophyll a	Purple bacteria, Heliobacteria, Green Sulfur bacteria, Chloroflexi, *Choracidobacterium thermophilum*	805, 830 – 890
Bacteriochlorophyll b	Purple bacteria	835 – 850, 1020 – 1040

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