

## Selection of Potential Bacterial Bioremediator for Tiger Grouper (*Epinephelus fuscoguttatus*) Juveniles Culture

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Different bacterial bioremediators were isolated from microalga *Chlorella vulgaris* and grouper culture water respectively. The total ammonia degradation test showed that all six strains degraded the total ammonia-nitrogen (TAN) *in-vitro*. One strain with the highest degradation activities, BP-GRP/2 was further tested *in-vivo* to tiger grouper, *Epinephelus fuscoguttatus* juveniles culture. No significant differences can be seen on TAN degradation and soluble reactive phosphorus (SRP) of the grouper culture water, except for the nitrite level was decreased in the presence of the strain. Higher survival of the fish in the BP-GRP/2 treatment was also observed compared to control. These results showed that certain bacterial strains from microalgae and grouper culture water can act as bioremediators and improve the survival of the fish host.

**Key words:** Bioremediator, bacteria, microalgae and tiger grouper.

There is an urgent need to develop effective solutions in improving aquaculture production. Marine aquaculture is increasingly becoming significant because of its economic benefits and one of the main marine aquaculture species is the grouper (*Epinephelus* sp.). Groupers are widely found in the coastal water of Malaysia and was first introduced in 1973 in net cages. The production for groupers are decreasing nowadays due to several stressors including poor water quality e.g., excess ammonia, salinity and

temperature fluctuation (Albert and Ransangan, 2013). Fishes exposed to high levels of ammonia over time are more susceptible to bacterial infections causing stress, gill damage, poor growth and low toleration to routine handling (Rama and Manjabhat, 2014). This can be prevented by introducing beneficial bacteria or probiotic into the host itself or the cultured water. Nowadays, probiotics are commonly used as a cure due to the demand for environmentally friendly approaches. Probiotic such as bacterial bioremediator is valuable in aquaculture because it is easy to manage, cost effective and harmless compared to the physical and chemical remedies (Mao *et al.*, 2014). Bioremediation is an ecologically sound practice which use natural biological processes to remove toxic contaminants. The process employs green

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plants, microorganisms or their enzymes to remediate contaminants reducing the toxicity of a pollutant (Prasad *et al.*, 2012). In other perspective, bioremediation uses relatively low-cost and low-technology techniques (Klinger and Naylor, 2012) which generally have a high public acceptance and can often be carried out on site. In aquaculture, bacterial bioremediators isolated from microalgae are promising as they are significantly utilized in aquaculture hatcheries and culture systems (González *et al.*, 2012, Natrah *et al.*, 2014). On the other hand, bacterial bioremediators such as *Bacillus* spp. have also been isolated from marine organism such as *Penaeus monodon* postlarvae which promoted better growth and survival of the animal (Devaraja *et al.*, 2013). The consortium might be the solution to improve low water quality in grouper culture due to excess ammonia from the grouper feeding and wastage. The objectives of this study were to isolate bacteria with bioremediation properties from microalgae and grouper culture water. The effects of the selected bacterial bioremediator on the survival and water quality of grouper culture was also determined.

## MATERIALS AND METHODS

### Enrichment of bacterial bioremediator from microalgae and grouper culture water

Samples for the enrichment of bacterial bioremediator was taken from grouper culture water in grouper experimental tanks at Institute Bioscience UPM. Meanwhile, water samples of four different microalgal species (*Nannochloropsis oculata*, *Nannochloropsis* sp. (Philippines), *Tetraselmis* sp. and *C. vulgaris*) were obtained from the Department of Fisheries Malaysia, Tanjung Demong, Terengganu. All samples were incubated with or without 20 parts per million (ppm) of ammonium sulphate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). Each solution were then spreaded evenly over the entire surface of Marine agar plate and incubated for 24 hours at 28 °C. The process was repeated until there is no bacterial growth in the control treatment (without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). The bacterial isolates which grew in the treatment with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were considered as potential bioremediators.

### Gram staining

All strains of bacterial bioremediators were subjected to Gram staining following the

method by Beveridge (2001). The bacterial cell morphology was then observed under light microscope at 1000x of magnification (Leica, Germany).

### Ammonia degradation assay

The potential bacterial bioremediators that were successfully isolated from microalgae and grouper culture water were cultured in Marine broth in shaking incubator for 24 hours at 28 °C. The bacterial cultures were then washed with sterile seawater through centrifugation at 3000 rpm in 10 minutes. Each bacterium was tested for ammonia degradation assay at final concentration of 10<sup>7</sup> CFU/ml. Ammonia degradation assay was done according to Parson *et al.* (1984). A stock solution of ammonia was prepared by dissolving 0.09g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 100 mL deionized water. Each bacterial samples were added with 0.5 ppm (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. A series of standard solutions (0.03, 0.05, 0.07, 0.1, 0.3, 0.5, 0.7, and 1.0 ppm) were also prepared. The mixture was then measured using spectrophotometer (model UV-1601 Shimadzu, Kyoto, Japan) at 640 nm and the readings between the samples were compared.

### In vivo grouper culture water

The experiments were conducted in 50 liter tanks by stocking one tail of juvenile grouper per litre of seawater. A total of 30 tails of fish were stocked in each aquaria. No water change was done during the experimental time. Prior to the experiment, the fishes were acclimatized for one week. A total of eight tanks (four replicates) with continous aeration were set up. The BP-GRP/2 strain was chosen for the in vivo trial and was made resistant to 50 ppm rifampicin. The bacterial strain (10<sup>7</sup> CFU/ml) and control (without any strain addition) were then immersed in fish water culture at the first day of the experiment. The survival of the fish were observed daily and water quality parameters (pH, salinity, temperature, DO, ammonia, phosphorus, and nitrite) were recorded using Multi-Parameter Water Quality Meter (YSI Inc., Yellow Springs, OH) every two days. Chemical analyses of ammonia, soluble reactive phosphorus and nitrite were done according to Parson *et al.* (1984).

### Statistical analysis

Statistical analysis of TAN degradation assay was done using One Way ANOVA while the mean number of survival and all other chemical analyses for both treatments were compared

separately by independent sample t-test using SPSS Statistics 20.0 software. Each statistical analysis was tested at a 0.05 level of probability.

## RESULTS AND DISCUSSION

Enrichment of bacterial bioremediators were done using the culture water of four microalgal species (*Nannochloropsis oculata*, *Nannochloropsis* sp. (Philippines), *Tetraselmis* sp. and *Chlorella vulgaris*) with and without addition of  $(\text{NH}_4)_2\text{SO}_4$ . Experiment was stopped when no bacterial growth was observed in the control

(without addition of  $(\text{NH}_4)_2\text{SO}_4$ ). Based on Table 1, potential bacterial bioremediators were only found in *C. vulgaris* culture after the third cycle of enrichment with addition of  $(\text{NH}_4)_2\text{SO}_4$ . Meanwhile, no bacterial growth was observed in other algal species. Bacterial bioremediators were also enriched using culture water of tiger grouper (*E. fuscoguttatus*) juveniles from grouper experimental tanks (Table 2). In this experiment, another three potential bioremediators were isolated from tiger grouper culture water. All strains were then subjected to ammonia degradation assay. The results in Table 3 shows that all of the strains

**Table 1.** Enrichment of potential bacterial bioremediators from different microalgae

Species	$-(\text{NH}_4)_2\text{SO}_4$	$+(\text{NH}_4)_2\text{SO}_4$		
	Control	R1	R2	R3
<i>Nannochloropsis</i> sp. (Philippines)	-	-	-	-
<i>Nannochloropsis oculata</i>	-	-	-	-
<i>Tetraselmis</i> sp.	-	-	-	-
<i>Chlorella vulgaris</i>	-	+	+	+

‘-’ no bacteria colony appear on Marine agar plate; ‘+’ bacteria colony appear on Marine agar plate;  $-(\text{NH}_4)_2\text{SO}_4$ : without  $(\text{NH}_4)_2\text{SO}_4$  addition or control;  $+(\text{NH}_4)_2\text{SO}_4$ : with 20 ppm of  $(\text{NH}_4)_2\text{SO}_4$ , R1-R3 = Replicates.

**Table 2.** Enrichment of potential bacterial bioremediators from grouper culture water

Treatment	$-(\text{NH}_4)_2\text{SO}_4$	$+(\text{NH}_4)_2\text{SO}_4$		
	Control	R1	R2	R3
Grouper culture water	-	+	+	+

‘-’ no bacteria colony appear on marine agar plate; ‘+’ bacteria colony appear on marine agar plate;  $-(\text{NH}_4)_2\text{SO}_4$ : Without  $(\text{NH}_4)_2\text{SO}_4$  addition or control;  $+(\text{NH}_4)_2\text{SO}_4$ : with 20 ppm of  $(\text{NH}_4)_2\text{SO}_4$ , R1-R3 = Replicates.

**Table 3.** Ammonia degradation by bacteria (10w CFU/ml) in each treatment and control

Strains	TAN concentration (ppm)
No bacteria	$0.57 \pm 0.05^a$
BP-MA/1	$0.10 \pm 0.06^b$
BP-MA/2	$0.14 \pm 0.04^b$
BP-MA/3	$0.15 \pm 0.00^b$
BP-GRP/1	$0.12 \pm 0.09^b$
BP-GRP/2	$0.10 \pm 0.05^b$
BP-GRP/3	$0.13 \pm 0.05^b$

Results are expressed as mean standard deviation; TAN: Total Ammonia-Nitrogen; ppm: parts per million

significantly degraded ( $p < 0.05$ ) 0.5 ppm  $(\text{NH}_4)_2\text{SO}_4$  after 48 hours of incubation. From the assay, the highest degradation was from BP-GRP/2 which significantly degraded 0.50 ppm to 0.10 ppm in 48 hours of incubation.

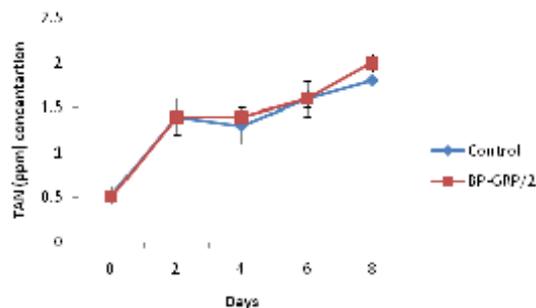
Based on the enrichment results, the isolation of potential bacterial bioremediators from microalgal culture water (*C. vulgaris*) resulted on three Gram-positive species of bacteria with rod shape. The selected bacteria were designated as BP-MA/1, BP-MA/2 and BP-MA/3. Meanwhile, the bacterial bioremediators isolated from grouper culture water resulted in Gram-negative bacteria

with rod shape. The strains were named as BP-GRP/1, BP-GRP/2 and BP-GRP/3, respectively. In the previous studies, Wang *et al.*, (2010) also found bacterial bioremediator from *C. vulgaris* which had high nutrient removal efficiency where in under certain conditions, can completely remove ammonia nitrogen, nitrate and total phosphorus. Ammonia-nitrogen is an important source of nutrients for bacteria since bacteria utilize nitrogen to make proteins and nucleic acids (Gregory *et al.*, 2012). *Chlorella vulgaris* alone is also able to remove nitrate in wastewater treatment (Kshirsagar, 2013). To our knowledge, this is the first report on bacterial bioremediator from grouper culture water. Previous studies by Devaraja *et al.* (2012) showed that bacterial bioremediator of *Bacillus pumilus*, *Bacillus licheniformis* and *Bacillus subtilis* can

be isolated from shrimp culture water and improved the growth and survival of the animal. Thus, there is a possibility that the microalgae *C. vulgaris* and the bacteria from grouper culture water could act as bioremediator in grouper culture.

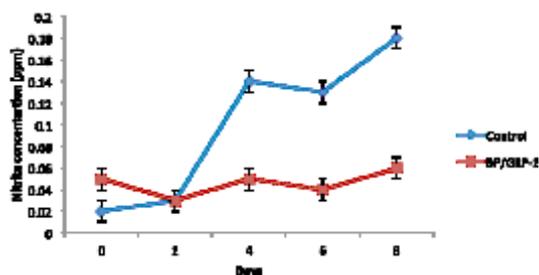
**Table 4.** Gram staining and morphology of bacterial bioremediators

Bacterial bioremediators	Gram reaction	Cellular morphology
BP-MA/1	Positive	Rods
BP-MA/2	Positive	Rods
BP-MA/3	Positive	Rods
BP-GRP/1	Negative	Rods
BP-GRP/2	Negative	Rods
BP-GRP/3	Negative	Rods



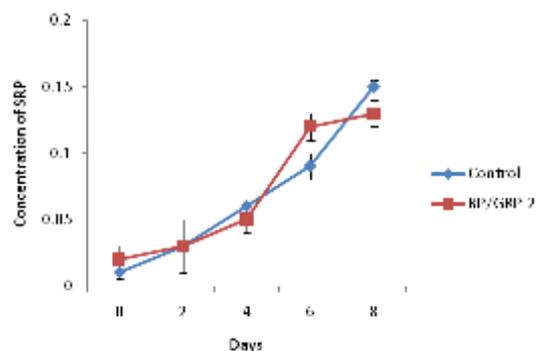
Results are expressed as mean  $\pm$  standard deviation. Control: without bacterial bioremediator, BP/GRP-2 : bacterial bioremediator.

**Fig. 1.** Concentration of TAN (ppm) during eight days of culture period (days)



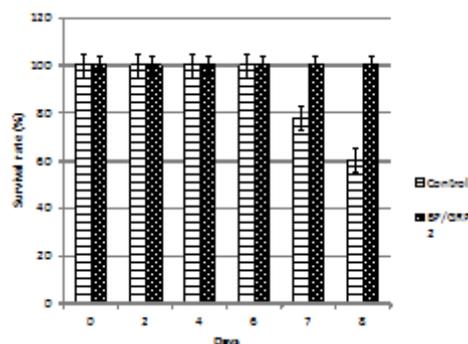
Results are expressed as mean  $\pm$  standard deviation. Control : without bacterial bioremediator, BP/GRP-2 : bacterial bioremediator

**Fig. 2.** Concentration of nitrite (ppm) during eight days of culture period (days)



Results are expressed as mean  $\pm$  standard deviation. Control : bacterial bioremediator, BP/GRP-2: bacterial bioremediator.

**Fig. 3.** Concentration of SRP (ppm) during eight days of culture period (days)



Results are expressed as mean  $\pm$  standard deviation. Control : without bacterial bioremediator, BP/GRP-2 : bacterial bioremediator.

**Fig. 4.** Percentages of fish survival rates of *Epinephelus fuscoguttatus* (%) during eight days of culture period (days)

Next, the rifampicin resistant strain of BP-GRP/2 was then tested on grouper juveniles without any water changes. The dissolved oxygen (DO), pH, temperature and salinity during the eight days of culture periods were not significantly different from day 0 to day 8. Meanwhile, total ammonia nitrogen concentration (TAN) (Figure 1) was not significantly different between the control and BP-GRP/2 treatment after eight days of culture. The TAN concentration in the control tank was observed to gradually increase from day 0 ( $0.61 \pm 0.12$  ppm) until day 8 ( $1.90 \pm 0.12$  ppm). The TAN also increased with BP-GRP/2, addition with  $0.56 \pm 0.04$  ppm on day 0 and  $2.04 \pm 0.02$  ppm on day 8. Generally for juvenile grouper, the ideal TAN is  $<0.02$  ppm (Ismi *et al.*, 2012). However in this experiment, the groupers in all the treatments were capable to tolerate and survived in high TAN concentration.

Even though the TAN does not show any significant changes in both treatments, the concentration of  $\text{NO}_2\text{-N}$  decreased significantly in the BP-GRP/2 treatment. The concentration of  $\text{NO}_2\text{-N}$  seems to decrease in the treatment with BP-GRP/2 from the fourth day onwards until the end of experiment. The nitrite decreased along the addition of BP-GRP/2 at  $0.06 \pm 0.01$  ppm on day 8 compared to the control tank ( $0.23 \pm 0.15$  ppm). It is observed that nitrite level was influenced by the presence of the bacterial strains where the value decreased with the addition of BP-GRP/2. Generally for juvenile grouper, the amount of nitrite that the fish can handle is 1 ppm (Ismi *et al.*, 2012). High nitrite level leads to low survival of fish due to ammonia toxification (Silva *et al.*, 2013). Nitrite is the intermediate product in the process of nitrification of ammonia to nitrate and it is toxic for the fish because it affects the blood haemoglobin's ability to carry oxygen (Timmons *et al.* 2002).

On the other hand, no significant differences ( $p>0.05$ ) can be observed between both treatments for Soluble Reactive Phosphorus (SRP). Figure 3 shows that the SRP concentration in the control increased from day 0 ( $0.02 \pm 0.01$  ppm) until day 8 ( $0.15 \pm 0.05$  ppm). The SRP also increased in BP-GRP/2 treatment with  $0.01 \pm 0.01$  ppm on day 0 and  $0.13 \pm 0.02$  ppm on day 8. The SRP concentration is still in the ideal range as fish can accept 0.2-3ppm SRP. Phosphorus may cause algae growth in large quantities which cause oxygen

depletion problems and subsequently causing fish mortality. For this reason, phosphorus level control is an essential role of a good bioremediator (Akpors and Muchie, 2010).

From the results in Figure 4, the fish from the treatment of bacterial strain BP-GRP/2 was more tolerant and survived better than the control even without water changes. Higher survival can be seen in the treatment with bacterial bioremediator in day 7 and day 8 compared to control which was probably due to the better water quality. During the experiment, the water in the bioremediator treatment tank was also observed to be clearer than the control tank. There are several studies on the effects of bacterial bioremediator in culture water for aquaculture. For example, *Bacillus* sp. used as bacterial bioremediators in shrimp culture break down large organic compounds to reduce water turbidity in tank (Zhou *et al.*, 2009). Other benefits of bioremediation include maintaining a diverse and stable fish pond community (Moriarty and Decamp, 2012).

## CONCLUSION

This study showed that different bacterial bioremediators isolated from grouper culture water and microalga, *C. vulgaris* have the potential to degrade ammonia *in-vitro*. A strain from grouper culture water was later found to be able to degrade nitrite concentration *in-vivo* in grouper culture water and increased the fish survival compared to control treatment. The identification of the bacterial bioremediators isolated from both grouper culture water and microalgae will be further elucidated.

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## REFERENCES

1. Akpor, O. B., Muchie, M. Bioremediation of polluted wastewater influent: phosphorus and

- nitrogen removal. *Scientific Research and Essays*, 2010; **5**(21): 3222-3230.
2. Albert, V., Ransangan, J. Effect of water temperature on susceptibility of culture marine fish species to vibriosis. *International Journal of Research in Pure and Applied Microbiology*, 2013; **3**(3):48-52.
  3. Beveridge, T. J. Use of the Gram stain in microbiology. *Biotechnic & Histochemistry*, 2001; **76**(3): 111-118.
  4. Devaraja, T., Banerjee, S., Yusoff, F., Shariff, M., Khatoon, H. A holistic approach for selection of *Bacillus* spp. as a bioremediator for shrimp postlarvae culture. *Turkish Journal of Biology*, 2013; **37**(1): 92-99.
  5. González, R., García-Balboa, C., Rouco, M., Lopez-Rodas, V., Costas, E.. Adaptation of microalgae to lindane: A new approach for bioremediation. *Aquatic Toxicology*, 2012; **109**: 25-32.
  6. Gregory, S. P., Dyson, P. J., Fletcher, D., Gatland, P., Shields, R. J. Nitrogen removal and changes to microbial communities in model flood/drain and submerged biofilters treating aquaculture wastewater. *Aquacultural Engineering*, 2012; **50**: 37-45.
  7. Ismi, S., Sutarmat, T., Giri, N. A., Rimmer, M. A., Knuckey, R. M., Berding, A. C., Sugama, K. *Nursery management of grouper: a best-practice manual*. Australian Centre for International Agricultural Research (ACIAR). 2012; 201-208.
  8. Klinger, D., Naylor, R. Searching for solutions in aquaculture: charting a sustainable course. *Annual Review of Environment and Resources*, 2012; **37**: 247-276.
  9. Kshirsagar, A. D. Bioremediation of wastewater by using microalgae: an experimental study. *International Journal of Life Sciences Biotechnology and Pharma Research*, 2013; **2**(3): 340-346.
  10. Lu, L., Tan, H., Luo, G., Liang, W. The effects of *Bacillus subtilis* on nitrogen recycling from aquaculture solid waste using heterotrophic nitrogen assimilation in sequencing batch reactors. *Bioresource technology*, 2012; **124**: 180-185.
  11. Maita, Y., Parsons, T. R., Lalli, C. M. In *A manual of chemical and biological methods for seawater analysis*. Pergamon press. 1984.
  12. Mao, X. Y., Shao, X. H., Mao, J. Q., Yin, C., Wang, L., Sun, H. B., Chang, T. T. Environment Research with Progress of Bioremediations for Aquaculture Effluent. *Advanced Materials Research*, 2014; **977**:264-269.
  13. Moriarty, D. J. W., Decamp, O. Microbial remediation of pollution in tropical coastal environments. *Aquatic Ecosystem Health and Management*, 2012; **15**(3): 253-259.
  14. Natrah, F.M.I, Bossier, P., Sorgeloos, P., Yusoff, F.M., & Defoirdt, T. Significance of microalgal-bacterial interactions for aquaculture. *Reviews in Aquaculture*, 2014; **6**(1), 48-61.
  15. Prasad, M., Garg, A., Maheshwari, R. Decontamination of Polluted Water Employing Bioremediation Processes: A Review. *International Journal of Life Sciences Biotechnology and Pharma Research*, 2012; **1**(3): 11-21.
  16. Rama, S., Manjabhat, S. N. Protective effect of shrimp carotenoids against ammonia stress in common carp, *Cyprinus carpio*. *Ecotoxicology and environmental safety*, 2014; **107**: 207-213.
  17. Silva F. J.R., Santos, F. R., Vale, D. A., Carmo, M. V. High levels of total ammonia nitrogen as NH<sub>4</sub><sup>+</sup> are stressful and harmful to the growth of Nile tilapia juveniles *Acta Scientiarum. Biological Sciences*, 2013; **35**(4): 475-48.
  18. Svobodova, Z., Machova, J., Poleszczuk, G., Hoda, J., Hamáková, J., Kroupova, H. Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems. *Acta Veterinaria Brno*, 2005; **74**(1): 129-137.
  19. Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J. *Recirculating Aquaculture Systems*, 2nd edn. New York. 2002; pp800-819.
  20. Wang, L., Wang, Y., Chen, P., Ruan, R. Semi-continuous cultivation of *Chlorella vulgaris* for treating undigested and digested dairy manures. *Applied Biochemistry Biotechnology*, 2010; **162**: 2324-233.
  21. Zhou, Q., Li, K., Jun, X., Bo, L. Role and functions of beneficial microorganisms in sustainable aquaculture. *Bioresource technology*, 2009; **100**(16), 3780-3786