Effective utilization of microalgae in fish larvae production
(種苗生産における微細藻類の有効利用)

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Roseobacter sp., a lineage of α-proteobacteria in *Nannochloropsis oculata*, tested against fish pathogenic bacteria in three different media. In VNSS, both *Roseobacter* sp. strain RO3 and *Vibrio anguillarum* showed normal growth and reached to the level of 10^9 CFU/ml. In contrast, in ESM, the co-existence of RO3 suppressed the growth of *V. anguillarum* after reaching stationary phase. Greater suppression of *V. anguillarum* could be observed with the co-existence of *Roseobacter* sp. and NCF. The co-existence of *Roseobacter* sp. alone and/or phytoplankton excreted substances come to be beneficial to larvae rearing in eliminating pathogenic bacteria

Aquaculture sector has large potential and could contribute to the country's total fish requirement in the future. In aquaculture industry, "green water", microalgae cultures are widely used for variety of applications but mainly for nutrition of aquatic animals. *Nannochloropsis oculata* is one of the most important green microalgae in aquaculture industry. The addition of "green water" into fish larvae tanks has proven to improve fish larvae survival rate, reduce transparency in rearing water and prevent disease outbreaks. Mostly "green water" is mass culture in the open air and expose to air-borne contamination such as bacteria and diatoms. Nakase and Eguchi [1] and Nakase et. al [2] reported that, in *Nannochloropsis oculata*, α-proteobacteria and *Cytophaga-Flavobacterium* cluster were predominant bacteria, which co-related with higher larval survival rate when microalgae were added into larvae tanks. Furthermore, 46% of total bacteria in *Nannochloropsis oculata* were active cells.

This study investigated the existence of *Roseobacter* sp., a lineage of α-proteobacteria in *Nannochloropsis oculata*, tested *Roseobacter* sp. inhibition effectiveness against fish pathogenic bacteria and presented possible mechanisms of the inhibition.

Materials and Method

N. oculata was taken from Susami Fish Nursery Center, Kinki University and had been continuously cultured since the past 5 years. Tilapia "green water" was semi-continuously cultured outdoor in 10 tonne tank in Hatchery of Borneo Marine Research Institute, Universiti Malaysia Sabah using 1000 tails of 3 inch size tilapia fry and was maintained at 10 ppt (brackish water) for further use as feed to rotifer cultures.

Bacterial community structures were analyzed, especially focusing on *Roseobacter* clade, using FISH method. *Roseobacter* clade affiliated (RCA) strains were isolated from *N. oculata* cultures. *Vibrio anguillarum* was originally isolated from diseased fish, *Plecoglossus altivelis* (Salmoniforms) in Lake Biwa, Japan.

RCA and *V. anguillarum* growth and/or inhibtion were compared in rich nutrient medium (VNSS), phytoplankton medium (ESM) and *N. oculata* culture filtrate (NCF). The preparations of media are as below. VNSS: complex rich bacteria culture medium [0.5 g yeast, 1 g trypticase peptone, 0.5 g glucose, 0.01 g $FeSO_4 \cdot 7H2O$, 0.01 g $Na_2HPO_4 \cdot H2O$, 1 L NSS (nine salts solution: 17.6 g NaCl, 1.47 g Na_2SO_4, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2 \cdot 6H2O$, 0.41 g $CaCl_2 \cdot 2H2O$, 0.01 g $SrCl_2 \cdot 6H2O$, 0.01 g H_3BO_3, 1 L Milli Q water)]. ESM media [12 mg $NaNO_3$, 0.5 mg K_2HPO_4, 0.1 μg Vitamin B_{12}, 0.1 μg Biotin, 10 μg Thiamine HCl, 25.9 μg Fe-EDTA, 33.2 μg Mn-EDTA, 100 mg Tris (hydroxymethyl) aminomethane, 2.5 ml soil extract (200 ml of soil from undisturbed deciduous woodland was added to 1000 ml distilled water and autoclaved for one hour at 105°C. It was autoclaved again for one hour at 105°C after cooling down to room temperature. After the second autoclaving, soil mixture was filtered through a GF/C filter. Lastly, distilled water was added to filtrate until reached 1000 ml and was sterilized by autoclaving for 15 min at 121°C) and 97.5 ml NSS (nine salts solution: 17.6 g NaCl, 1.47 g Na_2SO_4, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2 \cdot 6H2O$, 0.41 g $CaCl_2 \cdot 2H2O$, 0.01 g $SrCl_2 \cdot 6H2O$, 0.01 g H_3BO_3, 1 L Milli Q water) at pH 8.0. Lastly, NF: Indoor *N. oculata* was cultured for 7 days until reaching up to early stationary phase. 200 ml of the *N. oculata* culture was collected and transferred into centrifuge bottles, centrifuged at 5000xg for 15 min. After centrifugation, the supernatant was filtered through a series of filter starting with GF/F filter, then 0.2 μm pore size polycarbonate filter and followed by 0.1 μm pore size polycarbonate filter. The NF was sterilized by filtering method.

Fifty ml of ESM and VNSS media were prepared, respectively in 100 ml flask for each bacterium and sterilized by autoclaving at 121 ℃ for 15 min. Fifty ml of NF was transferred into pre-sterilized 100 ml flasks for each different bacterium. All media were prepared in duplicate.

RCA strain RO3 was challenged with *V. anguillarum* M93 with initial inoculation at 1 x 10^5 cell/ml in NF, ESM and VNSS media, respectively. RO3 and *V. anguillarum* M93 individually were also inoculated at the same amount in NF, ESM and VNSS media, respectively. They are treated as controls. Everyday, 1 ml sub-samples were taken and went through a serial of 10-fold dilution. 0.01 ml of diluted samples were dropped (5 drops per dilution) onto VNSS agar plate (Herigstad *et al.*, 2001) and incubated in dark at 20 ℃ for 5 days. Sub-samples were taken at day 1 until day 8.

Result

Roseobacter clade affiliated (RCA), isolated from *N. oculata* culture inhibitory effect were tested against pathogenic bacteria, *Vibrio anguillarum*. In the rich medium, both *Roseobacter* and *V. anguillarum* showed normal growth from exponential to stationary phase and reached to the level of 10^9 cfu / ml. Even in the co-culture, they do not influence each other. In contrast, in the poor medium (ESM), the co-existence of RO3 suppressed the growth of M93 after reaching stationary phase. Greater suppression of M93 could be observed with the co-existence of *Roseobacter* sp. and phytoplankton substances (in NF). Neither the existence of *V. anguillarum* (in ESM) nor phytoplankton substances (in NF) inhibit the growth of RCA.

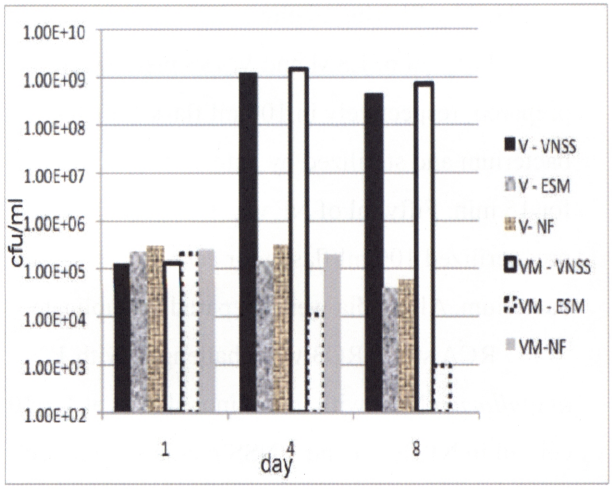

Fig. 2. Growth of *Vibrio anguillarum* M93 in 3 different media, VNSS, ESm and NF. V-VNSS, independent *V. anguillarum* in VNSS medium, V-ESM, independent *V. anguillarum* in ESM medium, V-NF, independent *V. anguillarum* in NF medium and VM-VNSS, *V. anguillarum* (mix with RCA) in VNSS medium, VM-ESM, *V. anguillarum* (mix with RCA) in ESM medium, VM-NF, *V. anguillarum* (mix with RCA) in NF medium.

Discussion

Roseobacter clade are often most abundant in bacterial communities associated with marine algae, including natural phytoplankton blooms and algae cultures.[3] *Roseobacter* clade abundance in the coastal seawater were correlated with high amount of dimethylsulfoniopropionate (DMSP) released by phytoplankton, *Emiliania huxleyi*.[4] *N. oculata* may produce excreted substances which were preferable to *Roseobacter* clade and enhanced its growth in "green water".

VNSS was used as a medium in this experiment as it contained high organic concentration of nutrients, approximately 800 mgC/l.[5] In rich medium such as VNSS, most marine bacteria including pathogenic bacteria grew well and reached high growth yield. This indicated that selected bacteria showed no specific selectivity and no competition on nutrient compounds in VNSS, therefore they grew well independently and could co-exist.

Alonso and Pernthaler[6] reported *Roseobacter* sp. was the main glucose consumers at low glucose concentration. This indicated that RCA has a higher ability in utilizing nutrient under an oligotrophic nutritional condition, such as ESM medium which do not contain any nutrient supplements from phytoplankton (NF). Under such a low nutrient condition, RCA, which posses an effective uptake system, can be a winner of nutrient competition.

Greater suppression of *Vibrio anguillarum* M93 could be observed with the co-existence of RCA and phytoplankton excreted substances in NF media. Bruhn *et al.* [7] reported that *Roseobacter* 27-4 inhibited fish pathogenic bacteria by a sulfur-containing antibacterial compound. NF may not only produce bacterial growth promoting substances, which enhanced RO3 growth but also enhanced the production of killing factors by RCA.

Reference

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