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PRIMARY RESEARCH

Isolation and selection of purple non-sulfur bacteria for phosphate removal in rearing water from shrimp cultivation

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Index Terms
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***Abstract*—** Therefore, this study aimed to isolate and screen PNSB from shrimp ponds with their ability to reduce phosphate in water from shrimp cultivation. A total of 83 PNSB strains were isolated from water and sediment samples collected from various 15 shrimp ponds located in Phang-nga and Songkhla provinces. For primary screening, there were 42 strains (51%) that grew well (OD_{660}> 1.0) in glutamate-acetate broth supplemented with 1.5% (w/v) NaCl, under conditions of microaerobic-light and aerobic-dark. However, in secondary screening, only two strains (W12 and W48) could grow in sterile rearing water collected from shrimp ponds. They were selected for tertiary screening to investigate their ability to remove phosphate in sterile rearing water under both incubating conditions. Both PNSB strains produced no significant differences for phosphate removal efficiency (> 50%) with the exception under microaerobic-light conditions as strain W12 roughly reduced 46% phosphate. Of these, 2 strains could be used as inoculants to remove phosphate from rearing water in shrimp ponds. One of the key environmental concerns about shrimp cultivation is the discharge of rearing water with high levels of nutrients, especially phosphate, into waterways, resulting in eutrophication. To solve this problem, biological treatment is well-recognized, and the use of purple non-sulfur bacteria (PNSB) is one of the attractive alternative choices because of their high removal efficiency in wastewater treatment with various metabolic growth conditions.

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I. INTRODUCTION

Over the past 40 years, shrimp has become the most-traded product due to higher-value product, although shrimp cultivation is faced with many serious problems. Shrimp ponds are mainly located in the Asia-Pacific region, including Thailand, China and Viet Nam, which are the major global exporting countries. A total world production of shrimp product was over 3 million tonnes in 2008 [1]. However, occurring of shrimp diseases in 2012 caused a decline of exports in some countries. Recent reports state that global shrimp production has been increased to 3.5 million tonnes with prediction to continue growing in the next decade [2].

It has long been known that shrimp cultivation is one of the most important aquaculture productions due to giving high income and employment. However, it is often responsible for severe economic and environmental problems. Several studies have reported the impacts of shrimp cultivation on ecosystems such as [3, 4, 5, 6, 7, 8]. One of the major environmental problems from shrimp cultivation is the discharge of large quantities of rearing water without any treatments into waterways. Since there are 84% of phosphorus and 71% of total nitrogen from uneaten feeds, fertilizers inputs and faeces remaining in rearing water [9, 10]; therefore, high levels of nutrients, especially phosphate can lead to severe eutrophication problems [11, 12, 10].

Moreover, rearing water discharge from farms causes an adverse impact on shrimp production since it is able to contaminate the water supplies used by other farms [13]. To solve these problems, the rearing shrimp water must be treated before releasing into waterways in order to remove (or reduce) these nutrients including phos-

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phate. One of the methods of treating wastewater is the use of photosynthetic bacteria [14, 15, 16]. A number of previous studies used purple non-sulfur bacteria (PNSB), a type of phototrophic bacteria to treat industrial and domestic wastewaters, and it was confirmed that they are highly effective in reduction of organic compounds in various wastewaters [17, 14].

In addition, they are capable of taking up phosphate into their cells and accumulating in the form of polyphosphate (Poly-P) granule for energy source. Thus, they have been considered as one of polyphosphate-accumulating organisms [15, 16, 18]. Nevertheless, there are a few researches focusing on using PNSB for treatment of rearing shrimp water from shrimp cultivation. Hence, objectives of this study were to isolate and select PNSB from shrimp water and sediment, and to evaluate the efficiency of phosphate removal in water from shrimp cultivation by using selected PNSB strains.

II. LITERATURE REVIEW

Shrimp cultivation has developed from traditional, small businesses into global industries which offer high employment opportunities in many countries including Thailand. Nowadays, there is a variety of different types of shrimp products such as raw, cooked, canned, pickled, and breaded found in global markets [1, 2, 5]. To increase carrying capacity of shrimp pond, fertilizers and mineral nutrients are pre-used to activate the growth of the phytoplankton for forming food chain. During cultivation, shrimp needs to be fed four to five times daily and more than 70% of them become waste [9, 7]. Along with uneaten feeds, faeces and phytoplankton remaining in rearing water likewise increase level of nutrients [3, 9, 5, 7]. Thus, discharge of rearing water from shrimp ponds can dramatically lead to the eutrophication wherever they are located. Eutrophication is the state of waterways which exceed in the nutrients including nitrate, organic compounds and phosphate which is a limiting factor for causing eutrophication [4, 19, 5, 7]. This problem causes many adverse effects on aquatic ecosystem such as increasing of the macrophyte growth, the phytoplankton blooms, the turbid state, but decreasing of oxygen at night time with finally decreasing of biodiversity [11, 5, 7]. Purple Non-Sulfur Bacteria (PNSB) is a type of phototrophic Gram-negative bacteria that naturally inhabit in fresh water, marine water, sediment and wastewater. These PNSB are capable of various metabolic growth modes (photoorganotroph under anaerobic and microaerobic-light conditions, and chemoorganotroph under aerobic-dark conditions) [15,17, 14]. Several previous studies have selected PNSB to treat industrial and domestic wastewaters, and results showed that they have great efficiency to remove organic compounds from wastewaters [15, 17, 14, 16]. Besides the high efficiency in wastewater treatment, some PNSB have ability to detoxify mercury in shrimp pond by volatilization process [20]. Moreover, PNSB have high protein in their biomass which can be used as Single Cell Protein (SCP) for animal feed [17, 14]. Recently, PNSB have been investigated for their ability to control shrimp pathogens (vibrios) and their use as probiotics for shrimp cultivation and results found that PNSB have great potential to control vibrios for sustainable shrimp cultivation [21]. According to above information, there is not much research work to use PNSB for removal of phosphate from rearing shrimp water. Hence, this research aimed to reduce phosphate level in shrimp ponds by PNSB for sustainable shrimp cultivation as the use of environment-friendly tool.

III. MATERIAL AND METHODS

A. Collecting of Water and Sediment Samples

With the aim to use PNSB for treating rearing water in shrimp cultivation, both sediment and water samples were collected from shrimp ponds that are located in Phang-nga and Songkhla provinces, Thailand. To obtain the representative samples, both types of sample were sampling for a total of 13 subsamples in each pond. Roughly amount of 100 g of sediment was collected at a 10 cm depth and a 100 mL of water at 50 cm depth from the surface water. All samples were kept in an ice box during transportation to a laboratory. All samples were rapidly used to isolate PNSB and then determined parameters as follows: phosphate, pH, Electrical Conductivity (EC) and salinity.

B. Isolation of Purple Non-Sulfur Bacteria from Shrimp Ponds

Glutamate-Acetate (GA) medium added with 1.5% NaCl (w/v) was used as an isolation medium (20) as follows: 3.8 g sodium L-glutamate, 5.4 g sodium acetate, 2.0 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.8 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4.7H_2O$, 0.053 g $CaCl_2.2H_2O$, 0.001 g nicotinic acid, 0.01 g biotin, 0.012 g $MnSO_4.5H_2O$, 0.025 g ferric citrate, 0.001 g thiamine hydrochloride, and 0.95 g $CoCl_2.6H_2O$ and added up with distilled water to

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1000 mL and adjusted pH to 6.8. For water samples, 10 mL of each subsample was filled into a screw cap test tube (150 × 15 mm: 20 mL) containing 10 mL of double strength GA medium and applied with its screw cap lid [17]. Whilst, 1 g of each sediment subsamples was transferred into a screw cap test tube (150 × 15 mm: 20 mL) containing 10 mL of normal strength GA medium and covered with liquid paraffin and applied with its screw cap lid.

All tubes were incubated under microaerobic-light conditions by setting with tungsten bulbs and adjusted light intensity to 3,500±200 lux at 30ºC for 5-7 days. For obtaining pure PNSB strains, culture broths displaying in pink to brown shade were re-streaked on GA agar medium plates and incubated in an anaerobic jar under light condition as previously described. A light microscope was used to check their purity after Gram staining, and each pure culture was kept in 20% glycerol at -80ºC in a freezer.

C. Inoculum Preparation

Each pure culture was loaded into a screw cap test tube (150 × 15 mm: 20 mL) of 18 mL GA containing 1.5% NaCl and incubated under microaerobic light conditions as previously described in the isolation step for 48 h. After twice subcultures, each bacterial culture was adjusted to an optical density at 660 nm (OD_{660}) of 1.0 by using a spectrophotometer. GA medium containing 1.5% NaCl was used as diluents and blank.

D. Selection of PNSB Strains with Potential of Phosphate Removal

Shrimp water preparation

Water samples from shrimp ponds were selected for this study as they were rich in nutrients before were flushed out of ponds.

Rearing waters were collected from various shrimp ponds to obtain a representative sample and fully filled in a 25 L black plastic tank to avoid aerobic light conditions and kept under 4±2 ºC in a cold room until use. Before testing, the rearing water was filtered through cheese cloth and then autoclaved to get sterile rearing shrimp water.

Primary screening

To select PNSB that could grow well in rearing water from shrimp farm, all isolated PNSB were screened in GA broth supplemented with 1.5% NaCl under both microaerobic-light and aerobic-dark conditions. The PNSB strains that grew well under both incubating conditions were selected for secondary screening.

Aerobic-dark conditions (Chemoorganotroph): 1 mL of each inoculum was transferred into 9 mL GA broth containing 1.5% NaCl in a screw cap test tube (150 x 25 mm: 50 mL) and incubated under dark-covered shaker at 150 rpm, 30ºC for 48 h. Bacterial growth was measured by a spectrophotometer as previously described with using the uninoculated medium as blank and control.

Microaerobic-light conditions (Photoheterotroph): 2 mL of each inoculum was added to a screw cap test tube (150 x 15 mm: 20 mL) containing 18 mL GA with 1.5% NaCl to provide no headspace and incubated under light using tungsten lamps for 48 h. Bacterial growth was measured by a spectrophotometer as previously described.

Secondary screening

To select the potential PNSB to grow in shrimp water, selected PNSB obtained from primary screening were grown in sterile rearing shrimp water. To avoid nutrients from GA broth, each PNSB strain was centrifuged and washed twice with 0.85% NaCl before setting inoculums at OD_{660}=1.0 using 0.85% NaCl. Tested PNSB were incubated under both aerobic-dark and microaerobic-light conditions as previously described for 7 days. Any PNSB strains that produced maximal growth under both incubation conditions were chosen to exam in their phosphate removal capacity.

Tertiary screening

To select the potential PNSB with ability to reduce phosphate, the PNSB strains were cultured as described in secondary screening. Before analysis, all samples were centrifuged at 6,000 rpm for 15 min to obtain culture supernatants for phosphate and pH measurements at days 0 and 7.

The percentage of removal was calculated as below formula. For cell pellets, methylene blue staining was applied to determine poly-P accumulation [15, 22] and then analyzed with a bright field microscope.

$$(\%)Phosphate\ \ removal = \frac{[phosphateconcentrationatday0]-[phosphateconcentrationatday7]}{phosphateconcentrationatday0} \times 100$$

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E. Water and Sediment Analysis

All samples of water and sediment collected from shrimp ponds were used to determine the following parameters: pH and Electrical Conductivity (EC) by a pH-conductivity meter (Seven Multi, Mettler Toledo, USA), phosphate by phosphate test (Merck, 1.14842.0001, APHA 4500-P C) and salinity (%) by a salinometer.

F. Statistical Analysis

All experiments in this study were performed in triplicate. Statistical analysis using SPSS program version 11.5 (Lead Technologies, Armonk, NY, USA), one way ANOVA to analyze statistical differences at a p-value <0.05 and compared means were conducted by the Duncan's multiple test.

IV. RESULTS

A. Characterization of Water and Sediment from Shrimp Ponds

Physicochemical properties of water and sediment samples collected from 15 shrimp ponds in southern Thailand, Phang-nga and Songkhla are shown in Table 1. The average phosphate contained in rearing water was 3.99 ± 0.92 mg/l with a high variation. Average pH value of water samples was slightly alkaline (7.24 ± 0.20); however, pH in sediments was nearly neutral (6.76 ± 1.06) with a wide range. Values of EC measured from water and sediment were much different.

The EC value that represents the ion of nutrient contained in solution was 27.99 ± 11.06 dS/m for the water samples, and 19.33 ± 14.51 dS/m for the sediment samples. Average salinity in water samples was 2.2 ± 1.1%. Value is mean of three determinations and its standard deviation (SD), nd = not determined.

TABLE 1
PHYSICOCHEMICAL PROPERTY OF SAMPLES COLLECTED FROM 15 SHRIMP PONDS IN PHANG-NGA AND SONGKHLA PROVINCES, THAILAND

	Water		Sediment	
Parameters	Mean± SD	(Range)	Mean± SD	(Range)
Phosphate (mg/l)	3.99 ± 0.92	(<0.5 - 5.3)	nd	nd
pH	7.24 ± 0.20	(6.31 - 7.60)	6.76 ± 1.06	(3.79 - 8.11)
Electrical conductivity (dS/m)	27.99 ± 11.06	(10.13 - 46.20)	19.33 ± 14.51	(0.82 - 79.32)
Salinity (%)	2.2 ± 1.1	(1.0 - 4.2)	nd	nd

B. Isolation of PNSB from Shrimp Ponds

With the use of GA medium supplemented with 1.5% NaCl (w/v), the PNSB that grew in the test tubes from both water and sediment samples were isolated to obtain single colonies and re-streaked for their purification. A total of 83 pure cultures were obtained from 15 shrimp ponds. There were 68 isolates (81.93%) from 45 water samples (roughly 3 isolates/2 samples) and 15 isolates from 45 sediment samples (roughly 3 isolates/10 samples) as shown in Table 2.

TABLE 2
NUMBERS OF PNSB STRAINS ISOLATED FROM SHRIMP PONDS IN PHANG-NGA AND SONGKHLA PROVINCES

	Number of PNSB Isolates	
Sample	Phang-nga (2 districts)	Songkhla (2 districts)
Water (n = 45)	47	21
Sediment (n = 45)	13	2
Total (n = 90)	60	23

n = number of samples

C. Selection of Potential PNSB for Phosphate Removal from Rearing Shrimp Water
Primary screening

A total of 83 PNSB strains isolated from both water and sediment samples collected from 15 shrimp ponds were screened by GA broth medium supplemented with 1.5% NaCl under 2 incubating conditions of microaerobic-light and aerobic-dark.

Most of the PNSB isolates grew well under aerobic-dark condition which showed values of OD $_{660}$ > 1.0 for 51 isolates (61.45%). However, there were only 42 isolates (50.60%) that similarly presented high OD values under the light condition (Figure 1). Then, all PNSB isolates were chosen for further test on their potential growth in sterile shrimp water.

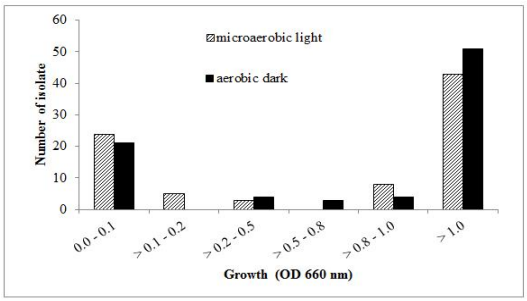

Fig. 1 . Number of PNSB strains grown in GA broth containing 1.5% NaCl under microaerobic-light and aerobic-dark conditions for 48 h

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Secondary screening

All 42 PNSB isolates obtained from primary screening were objected to test for their ability to grow in sterile rearing shrimp water. Figure 2 shows all of them presented OD $_{660}$ < 0.3 in both incubating conditions as only strains W12 and W48 showed the highest growth (OD $_{660}$ ≈ 0.2). Hence, both PNSB strains were further tested for their ability to remove phosphate in sterile rearing shrimp water.

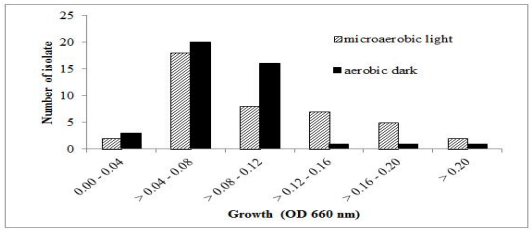

Fig. 2 . Number of isolated PNSB grown in sterile rearing shrimp water under microaerobic-light and aerobic-dark conditions for 7 days

Tertiary screening

Removal of phosphate from sterile rearing shrimp water under both incubating conditions for 7 days by both selected PNSB strains (W12 and W48) is presented in Figure 3. Strain W12 showed the highest percentage of phosphate removal (56.69 ± 0.17%) under dark condition, followed by strain W48 (55.00 ± 0.04%) under microaerobic-light and (52.14 ± 0.40%) aerobic-dark conditions although these were not significantly different (p> 0.05). In contrast, strain W12 was the least effective to remove phosphate (45.67 ± 1.06%) under microaerobic-light conditions. The loss of phosphate was also found in abiotic control sets (2.54 ± 0.06% and 3.28 ± 0.06% under microaerobic-light and aerobic-dark conditions, respectively). In addition, pH value dramatically increased from 6.90 to 8.22 at the end of the treatment including in the abiotic control sets.

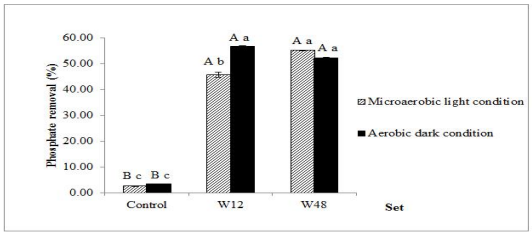

Fig. 3 . Efficiency of phosphate removal by selected PNSB strains in sterile rearing shrimp water under conditions of aerobic-dark and microaerobic-light for 7 days.

The different upper case letters on each bar indicate significant differences among 3 sets (control, W12 and W48); whereas different lowercase letters in each bar indicate significant differences between 2 incubating conditions of each set (p< 0.05).

Control is an abiotic control as without addition of inoculants. Moreover, the cell pellets of both selected strains after treatment were objected to detect intracellular poly-P granules by staining with methylene blue. The methylene blue is specific for poly-P granules and the signal is relatively strong. Poly-P granules appeared to be pink-violet on a pale blue cell background (Figure 4).

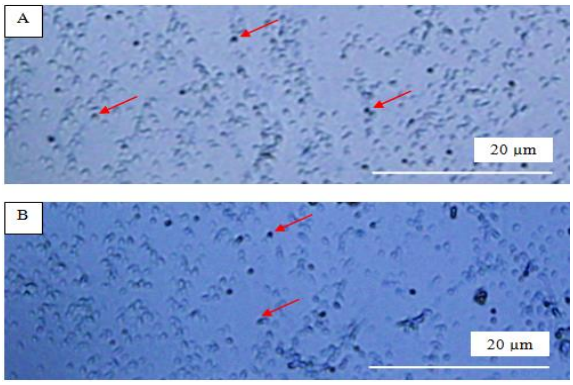

Fig. 4 . Polyphosphate detection by methylene blue straining of PNSB strains (A) W12 and (B) W48. The arrows showed intercellular poly-P granules

V. DISCUSSION

A. Physicochemical Property of Water and Sediments Collected from Shrimp Ponds

Parameters measured in water from shrimp ponds such as pH and salinity were in ranges of standard water quality (Table 1) that were suitable for marine shrimp farming production of Thailand (TAS-7401-2009) and the recommendation for water quality in shrimp farming in the study of [8]. The pH values required for shrimp cultivation are in a range of 7.0 - 8.3 and the salinity is between 0.05 and 3.50%.

However, our measurements indicated that phosphate level was extremely higher than the standard level that should be less than 0.1 mg/l [8]. This was also higher than the exceeding-phosphate value measured in effluent water of shrimp farm in Gila Bend, AZ [19] who reported a high level of phosphate at 0.33 mg/l (ranging between 0.06

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and 0.78 mg/l). These may be due to the overload of pellet feed use and fertilizer use for the first water input, the using reused-water and the remaining of high nutrients [3].

B. PNSB Isolation from Shrimp Ponds

Purple non-sulfur bacteria normally distribute in various habitats such as fresh and marine water, sediments, and wastewaters [16, 20, 23]. According to the isolation of PNSB strains from the shrimp ponds as shown in Table 2, it was indicated that most of them were isolated from water as a greater number of isolates (81.93%). These results are in accordance with a previous study of [20] this is due to the effect of the turbidity in the water column, and some distance between sediment and water surface could block sunlight from passing through the sediment.

C. Selection of PNSB for Phosphate Removal in Shrimp Ponds

Our isolated PNSB strains are able to grow in rearing shrimp water although their growth abilities were not high (Fig. 2). This might be low organic matter in rearing water; however, they have the possibility to be used in shrimp ponds.

Two PNSB strains showed quite high efficiency to remove phosphate from rearing shrimp water without optimization (Fig. 3). Their removal seemed to be higher when incubated under aerobic-dark conditions than under microaerobic-light conditions. This indicates that they preferred chemoorganotroph by using organic compounds as sources of energy and carbon, and O_2 as a final electron acceptor [17, 16, 24].

Nonetheless, there is a limited report on relevant literature concerning the use of PNSB for treating phosphorus in rearing water from shrimp farm, then this study was comparable with others related to treatments by PNSB. Our PNSB cultures in sterile rearing shrimp water without optimization could remove phosphate up to 57% which was higher than a study of [21] who reported the ability of Rhodobacter sphaeroides IL106 that could reduce only 10% phosphorus from an oyster farm waste after 7 days of incubation.

However, after 24 h PNSB could remove roughly 88% phosphate from industrial and domestic wastewaters under their optimal conditions and stimulation using infrared irradiation [15].

A lower efficiency to remove phosphate by our PNSB strains is due to a lower level of nutrients in rearing shrimp water compared to other wastewaters and no adjustment of the optimal conditions. Interestingly, the selected culture had a quite high capacity to remove phosphate without the exceeding growth. This was because cells were led to grow in starvation stage since there was lower nutrient in rearing shrimp water than that in enrichment GA medium, thereby they responded by reducing growth and increasing the accumulation of inorganic poly-P granules for an energy source inside the cells [25]. These results indicated that our PNSB strains could remove phosphate from environment and then continuously accumulate them in the form of poly-P [Fig. 4; 15, 16]. However, poly-P detection by staining with methylene blue could not present clear intracellular poly-P granules due to the effect of organic matter in rearing shrimp water as shown in Figure 4. After the end of the experiment, the pH increased from 7.0 to 8.2 roughly, which were in the optimal range for shrimp cultivation.

The increase of pH values in wastewater plants being likely due to the removal of soluble organic matter and the uptake of CO_2 by PNSB could lead to raise up the pH values [26, 14]. However, the possible reason in our study was due to the ammonification occurred during the treatment [17] by releasing of ammonium ion from organic nitrogen. In addition, previous studies demonstrated that high pH values of 7.5 and 8.0 could be advantage for increasing the rate of phosphorus uptake and biomass growth of polyphosphate accumulating organisms than a low pH [27, 28, 29]. A high pH level also provides the condition for precipitation of phosphate to insoluble calcium or magnesium phosphate [26], as one of the chemical processes to reduce phosphate in natural water ways.

VI. CONCLUSION

Our results concluded that phosphate level was dramatically higher than the standard level for discharge. Therefore, the rearing water from shrimp cultivation must be treated before discharge to avoid ecological problems.

As PNSB is normally found in rearing water and sediment of shrimp ponds, however, there are a few PNSB strains which have high efficiency of phosphate removal. Our results proved that two promising PNSB strains (W12 and W48) have a great potential to be used for reducing phosphate in shrimp cultivation since they displayed quite high ability of phosphate removal from rearing shrimp water without optimization. Therefore, their ability to reduce phosphate in non-sterile rearing shrimp water under opti-

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mization is being investigated in our current work, in order to verify an actual use in real rearing water.

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