Thesis for the degree of Master of Fisheries Science

ELIMINATION OF WHITE SPOT SYNDROME VIRUS (WSSV) USING THE BIOFLOC SYSTEM

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BIOFLOC 시스템 하에서 흰 반점 바이러스의 감소에 대한 연구

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ELIMINATION OF WHITE SPOT SYNDROME VIRUS (WSSV) USING THE BIOFLOC SYSTEM

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Abstract

Intensified aquaculture farming requires more supplementary protein feed to increased production level. However, high level of protein in the feed also accompanies adversary effects such as nitrogenous wastes, which may affect on the growth performance and survival rates. Even though they are exposed to a little bit above the critical ammonia levels over time, fish are more susceptible to the various pathogens resulting in increased chance of disease outbreaks. Increased nitrite concentration has negative effects on the growth and survival of fishes as well as other aquatic organisms. Adding of carbonaceous materials increase C/N ration which favors the growth of

microbial protein by reducing the ammonia from the culture system. Nitrogen compound covalently bond with carbon and produce flocs of protein molecules, flocculated together and form a floating mass called bioflocs. These bioflocs may be used for water quality improvements, fish nutrients as well as antimicrobial agents. Considering the potential application of bioflocs, experiments have been conducted to verify the effect of bioflocs on reduction of White Spot Syndrome Virus (WSSV) particle. Bioflocs were prepared under laboratory condition in the rectangular tank with sea water. Commercial feed was given constantly as source of additional carbohydrates and water was vigorously aerated by aerator. After the preparation of bioflocs, hepatopancreas from infected shrimp (*Litopenaeus vannamei*) was inoculated. Water was taken from the tank on I^{st} and 3^{rd} day and copy number of WSSV/ng was measured by Real time PCR. The resulted show that copy number of WSSV in bioflocs tank decreases dramatically. After 1^{th} day, copy number of WSSV was 752,616 and decreased to 34,929 on 3^{rd} days. Findings of this experiments showed that bioflocs have effect on the reduction of WSSV particles from the culturetanks.

Introduction

Aquaculture is the rapidly growing sector and its production still needs to be increased 5 folds in next two decades to meet up the protein requirements for human nutrition (Fao, 2000). As consequences, increased aquaculture has negative effects on environments. Production process produces substantial amount of polluted effluents, containing uneaten feed and faces (Read & Fernandes, 2003). About 30% of the feed is excreted as a form of organic water (Brune *et al.*, 2003). The uneaten food and excreted faces causes the elevation of ammonia and nitrate in the water bodies which are harmful to fish and shell fish (Crab *et al.*, 2007). Use of biofilter and conventional photoautotrophic algae culture system was the way for the removal of excess ammonia but less competent to apply in the field appropriately (Crab *et al.*, 2007). Biofloc is the technique that can resolve this problem by adding carbohydrates into the culture system to increase the C/N ratio, favors the bacterial growth by using nitrogen from the water bodies and production of microbial protein take place (Avnimelech, 1999, Crab *et al.*, 2007). This aggregation of different kinds of microorganisms

called bioflocs and can be additional source of food for fish, Nile tilapia *(Oreochromis niloticus)* and other organisms (Azim & Little, 2008, Crab *et al.*, 2009, Kuhn *et a*l., 2009).

A devastating viral disease caused by the white spot syndrome virus (WSSV), which can reach mortality up to 100% within several days, particularly in cultured penaeid shrimp. This virus was first identified in Japan in 1993 (Inouye *et al.*, 1994). and extended many countries in the Asia, indopacific and western hemisphere and has a great impact in the shrimp farming industry in Southeast Asia (Flegel, 1997).

Outbreak of diseases causes serious losses to the shrimp industry in worldwide (Wongteerasupaya *et al.*). In Asia alone, the industry reported annual losses of about 4 billion USD. Most of these pandemic diseases are of viral origin and White Spot Syndrome Virus, WSSV is one of the two most lethal diseases (Flegel & Lio-Po, 2009). There is no treatment for WSSV and prevention is the best way to avoid diseases (Menasveta, 2002). Several studies have investigated the effect of disinfectants on WSSV (Chang *et al.*, 1998, Maeda *et al.*, 1998, Balasubramanian *et al.*, 2006). The use of vaccines and immune stimulants to control WSSV has also been explored (Citarasu *et al.*, 2006, Satoh *et al.*, 2008, Sajeevan *et al.*, 2009). In

discriminate use of antibiotics has resulted in the development and spread of (multiple) antibiotic resistance (Defoirdt *et al.*, 2007). As a consequence, there is an urgent need for alternative, more sustainable control techniques. Bioflocs could be effective tools for removing shell fish diseases as well as allowing eco-friendly shrimp culture practices.

The importance of this biofloc technology are well reported mainly for shrimp culture (Burford *et al.*, 2004, Wasielesky, 2006) and to a lesser extent finfish culture (Milstein *et al.*, 2001, Serfling, 2006). To understand the antimicrobial and antipathogenic properties against the opportunistic pathogen *Vibrio harveyi* Glycerol-grown bioflocs were tested. However, bioflocs and biofloc supernatants decreased quorum sensing regulated bioluminescence of *V. harveyi*. Finding that the bioflocs had biocontrol activity against this pathogen (Crab *et al.*, 2010). Considering the importance of biofloc as bio-controlling agent, attempted has been made to reduce the infestation of WSSV from the culture tanks. Main purpose of this experiment was to observe any beneficial effects of bioflocs on the reduction of WSSV disease. We made the biofloc system and introduced infected shrimps juice containing white spot syndrome virus and control without bioflocs. Samples were taken after 24hours and 72 hours. It is found

that copy number of WSSV was downward trends in samples comparing controls. Large scale experiment may be needed for introducing this technology for commercial purpose. Findings of these experiments may be useful tools for reducing the infection of WSSV from the culture area as well as against many bacterial pathogens.

Table 1. Heterotrophic bacterial species available in bioflocs.

SL	Species
1	*Pseudoalteromonas sp*
2	*Vibrio parahaemolyticus strain*
3	*Paracoccus sp.II*
4	*Tenacibaculum sp.*
5	*Tenacibaculum sp.*
6	*Tenacibaculum sp.*
7	*Tenacibaculumlitoreum strain*
8	*Gaetbulibacter sp.*
9	*Gaetbulibacter sp.*
10	*Vibrio alginolyticus strain.*
11	*Tenacibaculumcrassostreae strain*
12	*Pseudoalteromonasnigrifaciens strain*
13	*Vibriobrasiliensis strain*
14	*Colwellia sp.*
15	*Uncultured bacterium*
16	*Flavobacterium sp.*
17	*Vibrio halioticoli*
18	*Vibrio harveyi*
19	*Unculturedbacterium*
20	*Vibrio sinaloensis*
21	*Pseudoalteromonas sp.*
22	*Colwellia sp.*
23	*Colwellia sp.*
24	*Colwellia sp.*

Table2. Composition of feed ingredients used as additional source of bioflocs.

Feed ingredients	Amount %
Protein	50
Fats	13
Fibers	3
Ash	15
Calcium	1.5
Others	2.7

Materials and methods

1. Identification of WSSV infected shrimps:

First, genomic DNA was isolated from the hepatopancreas of the suspected shrimps according to the protocol of the commercially available Kit in the market (Bioneer Inc. Korea). Concentration of genomic DNA was measured by the nano drop spectrophotometer.

2. Reverse transcription polymerase chain reaction:

Two step reverse transcription reaction was performed to identify the WSSV from the suspected shrimps. In first step, 30µl total PCR reaction mixture containing 1µl of DNA template, 3µl of dNTPs (2.5mm each), 10 x buffer (3µl), takara Ex Taq polymerase (0.2µl), primers 1µl (146F1, 5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3' and 146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'). The PCR condition was one cycle of 94°C for 4 minutes, 55°C for 1minute, and 72°C for 2minutes, followed by 39 cycles of 94°C for 1minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C.

The second step (nested PCR) PCR was carried out as same condition of step PCR except the primers (146F2 5'-GTA-ACTGCC- CCT-TCC-ATC-TCC-A-3' and 146R2 5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3'). The primers and other conditions for reverse transcription PCR were followed according to the Manual of Diagnostic Tests for Aquatic Animals 2009. PCR products were stained with 1.5% agarose gel electrophoresis and stained with ethidium bromide. PCR products were isolated from gel slices using gel extraction Kit (Bioneer Inc., Korea) and ligated into TA vector with the pGEM-T Easy Cloning Kit (Promega, USA), and transformed into XL1-blue competent cells. A 941bp DNA was sequenced with automated DNA sequence (ABI Biosystem, USA).

3. Analysis of deduced amino acid sequences:

Sequences were obtained with BLAST 2 SEQUENCES software (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi) and deduced amino acid sequence were obtained using an ORF finder program (http://blast.ncbi. nlm.nih.gov/bl2seq/wblast2.cgi).

4. Preparation of biofloc:

Bioflocs were prepared in the rectangular tank (35x30x50 cm) with sea water 20liter. Sea water (34ppt was maintained) was continuously flowed in the tank at a flowing rate 4l/minute. Temperature was maintained 25ºC. Water was vigorously aerated by aerator. Commercial feed was supply constantly as source of additional carbohydrates and components of feed given in Table 2. After the preparation of 20L of biolflocs it was divided into two tanks, each containing 5L of bioflocs and tanks containing sea water were used as controls.

5. Inoculation of WSSV to the biofloc tanks:

Previously identified WSSV infected shrimps were used as a source of virus particles. Whole shrimps were mixed with saline water and grind by homogenizer, 200µl was used for measuring total of WSSV copy number contained in the juice, and inoculated 10ml of juice into each tank.

6. Isolation of WSSV from the biofloc and control:

50ml of water was collected from each tank and passed through the filter paper (0.7µm) for the removal of unwanted debris from the water, and passes again through the 0.2µm size filter paper. The 0.2µm of filter paper was transferred it into the 2ml tube containing the 300µl of tissue lysis buffer and kept at 60ºC for whole night. Genomic DNA was isolated by the previously mentioned protocol.

7. *Measurement of WSSV copy number by Real time PCR:*

Quantitative PCR was carried out using the DNA Engine Opticon 2 Real-Time PCR Detection System (Bio-Rad, USA) to measure expression levels of WSSV between control and biofloc tank. Three samples of each group were analyzed individually. SYBER Green premix Ex TaqTM (Takara Bio Inc., Japan) was used with 3ng cDNA as template. Real-time PCR was carried out reaction (20µl) contained 5µl cDNA (3ng/µl), 2µl (4µm) sequence specific primers 10µl Syber mix (Takara Bio Inc., Japan. PCR conditions were 3min at 94 °C, followed by 30 cycles at 94 °C for 30 s,

62°C for 30 s, and 72 °C for 30 s and 40 cycles was performed. Standard curves were constructed to quantify copy numbers as described previously (Kim *et al.*, 2008) and confirmed the efficiency of each primer set. Calculated copy number from each sample was normalized by the copy number of 18S rRNA. Calculation of the relative copy numbers were as follows; the actual copy numbers of samples/the actual copy numbers of 18S rRNA.

Results

1. Identification and blast analysis of WSSV:

Traditional cloning strategy was performed for identifying partial WSSV sequences. A total of 941bp was cloned the PGEM T-easy vector. BLAST analysis showed 99% identities with wssv360 shrimp white spot syndrome virus sequence.

2. Nucleotide sequence white spot syndrome virus (WSSV)

(Forward primer) 146 F2

GTAACTGCCCCTTCCATCTCCACCACACTTTTACTCCCTCAGATAACGAGCATCTGGTATCCTCTTTCGCATTCGC

CCGCCCAGAAGTCTCCATGGAAGAAATTAGAGCCACACCCTATCAGGCCAACAAGCTTATTAGTGACAAACATTAC

GTGATGAACATGTCCAAGATCGATTCTAGAGTAACAGGATCTTCCCTCCTTAAGAAGGTTAGCGAATGGACTGAAA

TGAGAATGAACTCCAACTTTAATGGAACATTTGAACCATCAAGACTCGCCCTCTCCAACTCTGGCATGACAACGGC

AGGAGTCAACCTCGACGTTATTGTCAAACCAAATAATGCAAGAAGTGTACTAGGAATATTGGAATGTCATCGCCAG

CACGTGTGCACCGCCGACGCCAAGGGAACTGTCGCTTCAGCCATGCCAGCCGTCTTCCAGGCAACCGATGGAAACG

GTAACGAATCTGAACTGATCCAGAATGCTCTGCCAAGGAACAGATACATCCAAAAGAGCACAATGAACGCTCAAAC

TGTCGTGTTTGCTAATGTTTTGGAACAACTTATCGCCGATCTTGGAAAGGTTATCGTGAACGAACTGGCCGGCACC

ATCGCTGAATCTGTACCAGGAAGCGTATATGAAAACACCAAGGAAATGATTGATAGACTAGGCTCTGACGACCTCT

TCAAATCTAATAATAATGGAGGAGTAGAATCAATGGATTATGAAGATAGCGAAACAACATCCAACAATGGTCCCGT

CCTCATCTCAGAAGCCATGAAGAATGCCGTCTATCACACACTAATTTCCGGCAAGGCAGCTCGCCCGGAAAATGTA

CCATTCGCCTCATGCGCCAGCGGCCCTCTCGCCTTTGATTTCCTTCTGTCAAAGGGAGATACATTCGAAGAAAAGA

ACGCCGA***ACAAGGTGCAGCAGCTGCCGTA***

(Reverse primer) 146 R

3. Protein sequence of white spot syndrome virus

```
NCPFHLHHTFTPSDNEHLVSSFAFARPEVSMEEIRATPYQANKLISDKHYVMNMSKIDSRV
TGSSLLKKVSEWTEMRMNSNFNGTFEPSRLALSNSGMTTAGVNLDVIVKPNNARSVLGILE
CHRQHVCTADAKGTVASAMPAVFQATDGNGNESELIQNALPRNRYIQKSTMNAQTVVFANV
LEQLIADLGKVIVNELAGTIAESVPGSVYENTKEMIDRLGSDDLFKSNNNGGVESMDYEDS
ETTSNNGPVLISEAMKNAVYHTLISGKAARPENVPFASCASGPLAFDFLLSKGDTFEEKNA
EQGAAAAV
```

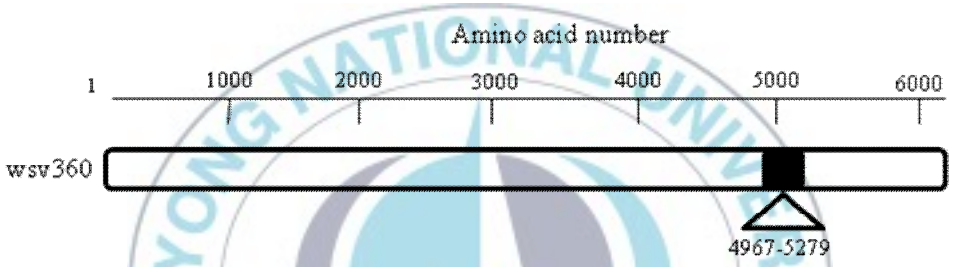

Fig.1. Position of WSSV amino acid sequence which was identified from *L. vannamei comparing with* wsv360 (Shrimp white spot syndrome virus, Gen bank accession number NP_477882.

4. Comparison of WSSV copy umber from control and samples:

Expression analysis of WSSV was performed by using real time PCR. Hepatopancreas from infected shrimp was chopped and grind by homogenizer and the total solution was made into 30ml. Equal volume of solution (10ml) was poured into each of the tank. After 1st day and 3rd days, samples were collected and relative copy numbers were made by the procedure discussed above. Copy number after 1st and 3rd days has been given in a table.3. Results showed that WSSV copy number were decrease sharply compared to control. After one day, relative copy number was 752,616 which became 34,929 after 3rd days.

Table 3. Relative copy number of WSSV on different days

Days	Samples (average)	Control (average)
1st day	752,616	266,981,734
3rd day	34,929	885,795

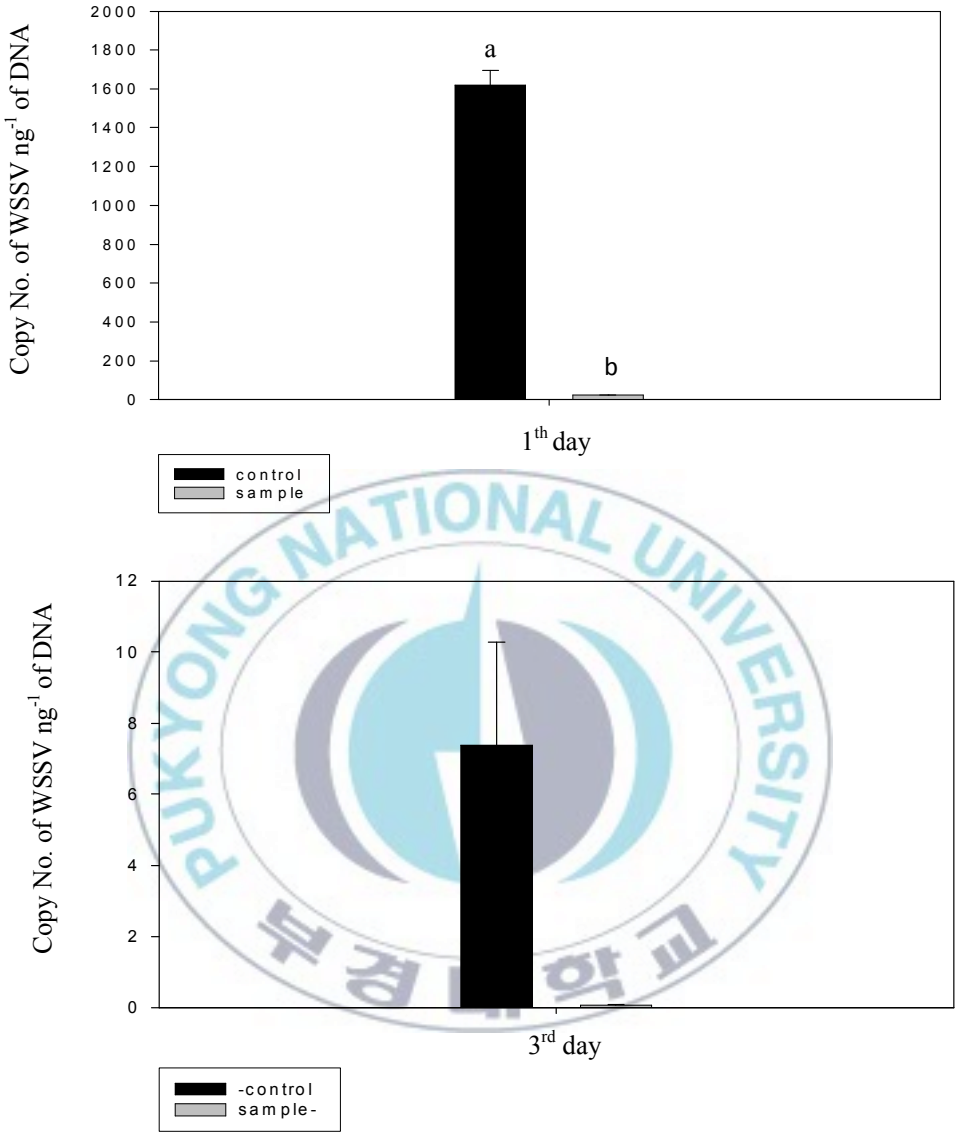

Fig.2. The relative copy no. of WSSV/ng of DNA (30 ng) after 1st day and 3rd days of introducing WSSV particles into the bioflocs (n=2) Statistical significance accepted only when $P<0.05$.

Discussion

Bioflocs is zero-exchange aquaculture system which can reduce negative impact on environment and is also a sustainable technology for removing ammonia-nitrogen from aquaculture system as well as effectively utilize of microbial protein as a source of food for fish and shrimp. Polyhydroxyalkanoate (PHA) is biopolymer of bacteria granules, these synthesized by bacteria (bacteria species available in bioflocs is shown in table1.) and occur when the lack of essential nutrient like nitrogen limited in the presence of an excess carbon source, these process can break down naturally and biodegradable (Salehizadeh & Van Loosdrecht, 2004, Avnimelech, 1999). These PHA are the polymer of β-hydroxyl short chain fatty acids and could have antibacterial activity similar to short chain fatty acids (SCFAs) or organic acids (Yu *et al.*, 2005). And SCFAs also shown to down regulated the virulence factor expression and positively influence the gut health of animals, depending on the physiological condition of the host (Teitelbaum & Walker, 2002, Ricke, 2003). SCFAs have been seen to be effective for anti-microbial a long times, reasons these compound have been found used in the commercial diets for the terrestrial animals to control the

pathogen such as *E.coli* and *Salmonella* (Van Immerseel *et al.*, 2005). It is indicated that, bioflocs can be a novel strategy for disease managements for long term basis. These experiments have been conducted to protect virus particles by using bioflocs system, our findings indicates that copy number of WSSV have been decreased dramatically in third days after inoculating WSSV virus particles into the thanks containing bioflocs compare with survival of WSSV virus particles have been found viable for at least 30days at 30°C in seawater under laboratory conditions and viable in dry condition ponds for at least 3–4 days (Momoyama *et al.*, 1998, Nakano *et al.*, 1998). So we consent that bioflocs can be effectively used for the reduction of White Spot Syndrome Virus from aquaculture tank. But how reduction of WSSV virus particle in bioflocs system is unknown; the working mechanism of polyhydroxyalkanoate may interfere with cells structure of virus and bacteria that may consume the viral particles as protein sources. However, the diagnostic of WSSV virus particle from water is not exactly correction, such as used the 0.7µm filter paper to removed debris and bioflocs clumps before collected virus particle for isolation, which may virus particle adhesives with those debris and biofloc clumps were remove out from the water sample.

Conclusion

Bioflocs could be an effective tool for improving fish and shellfish farming in extensive and semi-intensive culture system. In our experiments, it is shown that copy numbers of WSSV were decreased dramatically as the time passes away. It is concluded that bioflocs have positive effects on the reduction of WSSV virus particle form the culture tank. This may have happened due to the presence of bacteria may use those virus particles as source of protein. We shall address the questions by analyzing the bacterial composition grown in the bioflocs, and next trial should compare with different condition of bioflocs such as heterotrophic bacteria species, salinity, temperatures, pH and re-infection after vanish of virus particle in the bioflocs system.

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