Vol. 7
No. 1
January
2015

ISSN: 2075-4124
E-ISSN: 2075-7107
An international journal

BEYNƏLXALQ ELMİ ARAŞDIRMALAR JURNALI

INTERNATIONAL JOURNAL OF ACADEMIC RESEARCH

PART A

APPLIED
AND NATURAL
SCIENCES

PROQRES
BAKU, AZERBAIJAN

The nation's future success lies with science and education!

Heydar Aliyev
National Leader of Azerbaijan

INTERNATIONAL JOURNAL OF ACADEMIC RESEARCH

Vol. 7. No. 1
January, 2015

PART A.
APPLIED AND NATURAL SCIENCES

Member of crossref

DOI: 10.7813/2075-4124.2015

Daxil edildiyi elmi bazalar:
Indexed by:

Master Journal List (ISI-Thomson Reuters, USA)
CAB Abstracts (ISI-Thomson Reuters, USA)
Zoological Records (ISI-Thomson Reuters, USA)
Norwegian Social Science Data Services (Norway)
ZentralBlatt MATH (Springer-Verlag, European Math. Society, Germany)
IndexCopernicus International (Poland)
EBSCO-Academic Search Complete (USA)
SCIRUS (Elsevier, Netherlands)
ULRICH's Web (USA)
JournalSeek (USA)

"PROGRESS" IPS
Baku, Azerbaijan, 2015

INTERNATIONAL JOURNAL *of* ACADEMIC RESEARCH

Vol. 7, No. 1, January, 2015, Part A

DOI for issue: 10.7813/2075-4124.2015/7-1

Publishing bimonthly
Print **ISSN: 2075-4124**
Online **ISSN: 2075-7107**
National reg. No. **2996**

Editorial office:

97/2 I.Gutgashinli str.,
Baku, Azerbaijan
E-mail: **subijar@gmail.com**
Tel.: (+994 12) **510 02 80**

URL: www.eu-print.org

Beynəlxalq Elmi Araşdırmalar Jurnalı (BEAJ)

2009-cu il, Milli Mətbuat Günündə Azərbaycan Respublikası Ədliyyə Nazirliyi tərəfindən rəsmi Dövlət Qeydiyyatına alınıb **(№ 2996)**. BEAJ Beynəlxalq ISSN Mərkəzində (Paris, Fransa) qeydiyyatdan keçərək mətbu orqan kimi **ISSN 2075-4124**, elektron jurnal kimi **E-ISSN 2075-7107** nömrələri ilə beynəlxalq nəşr statusu qazanıb.

Jurnal dünyanın 83 ölkəsinə (universitet və kitabxanalar) paylanır. Jurnalın təsisçisi "Proqres" İnternet və Poliqrafiya Xidmətləri MMC-dir. BEAJ ildə 6 dəfə - **Yanvar, Mart, May, İyul, Sentyabr və Noyabr** aylarında dərc olunur. Redaksiyanın yazılı icazəsi olmadan materialların təkrar nəşri, tərcümə edilərək yayılması qadağandır. Məqalələr bir qayda olaraq Beynəlxalq Redaksiya Heyətinin yekun qərarı ilə dərc olunur. Məqalələr unikal DOI ilə nömrələnir.

Növbəti buraxılış tarixi: 30.03.2015
Materiallar toplanıb: 15.11.2014 - 15.01.2015. Çapa imzalanıb: 05.02.2015.
Format: 60x84 $^{1}/_{8}$. Şrift: Arial. Səhifələrin sayı: 240. F.ç.v. 30. Tiraj: 300
Jurnal "Proqres İPX" tərəfindən nəşrə hazırlanıb və çap olunub.

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PART A. APPLED AND NATURAL SCIENCES

Library of Congress Classification: S1-(972), SH1-691

ORAL VACCINE WITH ALGINATE MICROENCAPSULATION TO OVERCOME VIBRIOSIS IN GROUPER SEED

Sumaryam[1], Dwirini Kartikasari[2], Hari Suprapto[3]*

[1,2]Department of Fisheries, Agricultural Faculty, Universitas Dr. Soetomo, Semolowaru 84, Surabaya,
[3]Faculty of Fisheries and Marine, Universitas Airlangga, Mulyorejo, Surabaya **(INDONESIA)**
***Corresponding author:** supraptohari@yahoo.com

DOI: 10.7813/2075-4124.2015/7-1/A.35

Received: 02 Nov, 2014
Accepted: 14 Jan, 2015

ABSTRACT

The purpose of this research is creating *Vibrio* sp Formalin Killed Cells (FKC) microcapsules vaccine which is mixed in the feed of the grouper *(Chromyleptes altivelis)* seed as one effort to overcome vibriosis diseases. The method used to make microcapsules is spraying FKC suspension which is mixed with alginate into hardener solution ($CaCl_2$). The vaccine then tested orally in the grouper seed with mixing it in the feed, and it is proven to increase the survival rate of the seeds of the challenge test with *Vibrio* sp.

Key words: *Vibrio* sp, Formalin Killed Cells (FKC), *Chromyleptes altivelis*

1. INTRODUCTION

The grouper *(Chromyleptes altivelis)* is the only reef fish which can live and grow rapidly in the tropics area. Grouper demand increased mainly in the export markets, such as Hong Kong, Singapore, Taiwan, China and Japan. Department of Marine and Fisheries notes, the most living grouper market in the world is the East Asian markets, especially Hong Kong, with total imports 30,000 tons over two decades. Throughout the first nine months of 2005, the export of living grouper elevate 5.7% which total is 9434.3 tons or 508.945 billion rupiahs and if compared to the same period in 2004, the total is 8295.5 tons or about 481.5 billion rupiahs [1].

Indonesia is the largest producer of grouper with 13.94% of produce and 90% of it derived from the nature. Given this reality, Indonesia should be able to do seeding with high SR, so that continuity of fish delivery can be maintained.

The grouper farming nowadays is still faced with many obstacles. The main obstacle is the death of the large amount of grouper seed in the *hatchery*, that is due to the presence of a pathogen infection caused by viruses, bacteria, parasites and fungi. One of it is vibriosis disease caused by bacteria from the *Vibrio* genus which caused up to 80-90% death of seeds [2].

Clinical symptoms of fish that infected vibriosis include the weakness of fish, often swim to the surface, loss of appetite, circling behavior *(whirling)*, body color becomes dark, the swelling of the skin that resulting an injury, bleeding in the abdominal wall and the surface of the heart, and when the surgery done we can see swelling and damaged kidneys, liver and spleen [3]. Fish that have been infected with vibriosis is difficult to treat and become quickly death.

The effort to overcome the death of grouper seed is widely practiced the prevention through the use of chemicals and antibiotics, but if it's done continuously, it will be resulting the emergence resistance of bacteria, the occurrence of drug residues in fish body and also polluting the waters that can cause degradation of water quality [4]. One way that can be choosen is the vaccination method that can improve fish immunity [5].

Vaccination can be done by injection, immersion, or per oral. The appropriate vaccination for grouper seed is orally through feed, because the injections and immersion method can easily cause stress for the fish seed which can lead to death. But the oral vaccination has constraints that can be damaged when entering the digestive system due to the low of pH, and also water soluble so that antigens contained in it did not get into the body of fish [6]. Because of that, it required a coating method which can protect the antigen for passing the digestive system.

One of coating method that can be applied is microencapsulation, which is a technology that coat the core with a polymer layer walls and become microscopic small particles that protected from external environment [7]. Alginate gel can shrink in acidic conditions, and eroded under alkaline conditions so that it can protect the antigen when exposured by the acid in the fish stomach, then release of antigen in the fish digestion that have more alkaline condition. Alginate gel is also mucoadhesive that can be attached to the intestinal mucosa so that the antigen is not immediately removed from the fish body through the feces. Microencapsulation of FKC *Vibrio* sp in the intestine will release the antigen step by step so that can stimulate the immune system of the grouper and can cause the fish's natural immunity against vibriosis. This study aim is to make an *Vibrio* sp FKC microcapsules oral vaccine to overcome vibriosis disease in grouper seed.

2. MATERIALS AND METHODS

This study used the *Vibrio* sp certified isolates which is obtained from BBAP Jepara, TSB medium, NaCl, PBS, formaldehyde, alcohol, Sodium Alginate and $CaCl_2$ solution, with equipment of centriguge tube, Erlenmeyer, Beaker glass, Petri dishes, Eppendorf, Ose, diluter, Bunsen, vortex, microscope, micropipette, thermometer, pH meter, DO meter, spray gun and compressor [6, 8].

Bacterial Culture of Vibrio sp

Bacterial isolates were cultured in *Erlenmeyer* with TSB media. To make 500 ml culture medium needed 15 g TSB and 10 g NaCl that mixed into 500 ml of distilled water and stirred by shaking to dissolve completely. When the solution is completely dissolved, *Erlenmeyer* is covered with *aluminum foil* and put in the *autoclave* at 121° C, in the 1 atm pressure up to 15 minutes to make the medium sterile from other pathogens.

After the media stored at room temperature for 24 hours and no contaminants are growing, 1 ml of *Vibrio* sp isolate is put in the media, then Erlenmeyer is covered with *aluminum foil* and incubated for 24-48 hours in water shaker at 37°C. Based on the calculation of the density of bacteria in the Central Quarantine Fish Juanda, the amount of bacteria that grow as much as 9.2 x 10^7 CFU / ml [6].

Vibrio sp Formalin Killed Cell Making Process

Formalin Killed Cells making processed were done according to Suprapto (2005). After 24-48 hours the media TSB will look muddy, it indicates bacteria growing and ready for harvesting. The harvesting is done by centrifuge the TSB media at 4000 rpm of speed for 10 minutes. Supernatant is discarded and the sediment is added with 40 ml of PBS and then centrifuged at the same speed and time. Supernatant disposal process and the addition of PBS is done 3 times to obtaine precipitate clean bacteria from the TSB media. This process is also called bacterial washing.

Bacteria that have been washed is added 25 ml PBS and inactivated for 18-24 hours with 0,5 ml of 0.3% formaldehyde addition. Formalin is killing the bacteria so that it no longer virulent and preserve it. Bacteria that have been formalined stored at 4°C for 72 h, and then washed once again with centrifugation at 3000 rpm for 15 minutes. The results of this process is called *formalin killed cells* (FKC), and stored in the *refrigerator* for unused time [6].

FKC Bacteria Microcapsules Making Process

The last stage of making a vaccine is FKC bacteria microcapsules making, by spraying the FKC suspension with alginate into a hardener solution ($CaCl_2$). The comparison of FKC and alginate used is 1 : 6 v/v. Spraying is done by using a *water gun* and *compressor* at 7 bar of pressure [8].

The microcapsules that are formed in $CaCl_2$ solution then washed using distilled water with sentrifugation at 4000 rpm of speed for 10 minutes. Supernatant formed accommodated and then washed again with the same speed and time. The washing process was done three times until the microcapsules free from $CaCl_2$ solution. After the washing process, dried microcapsules with aerated at room temperature ± 3-7 days. The dried microcapsules then crushed and sieved using a 400 mesh filter, and stored in sterile bottles.

Measurements of vaccinated grouper seed survival in challenge with Vibrio sp infection

To determine the performance of microcapsules FKC vaccine against *Vibrio* sp to the grouper seed survival, conducted a preliminary test by mixing microcapsules vaccine in feed at a dose of 0.0125 and 0.025 mg/fish/day. This dose refers to the study [9] that at a dose of 10 mg/fish/day can increase antibody titers and significantly different from the control without vaccination. Vaccinated feed then given to the grouper seed which age is 3-4 months and the size is 8-11 cm for 7 days and then tested challenged by immersed in water that mixed with 6-9 x 10^4 CFU/ml suspension *of Vibrio* sp [6]. Immersing is done for about 24 hours, then observed during the seven-day maintenance to monitor water quality, knowing mortality and calculating the seed's survival rate with the formula of [10]:

$$SR = \frac{Nt}{N0} \times 100\%$$

Explanation:

SR = Survival grouper seed = *Survival Rate* (%)
N0 = number of fish seed at the beginning of the study
Nt = number of fish fry at the end of the study

3. RESULTS AND DISCUSSION

After FKC *Vibrio* sp microencapsulation process using alginate, the result is white powder vaccine. And after observed using a microscope with 100x magnification, the following result is like figure bellow:

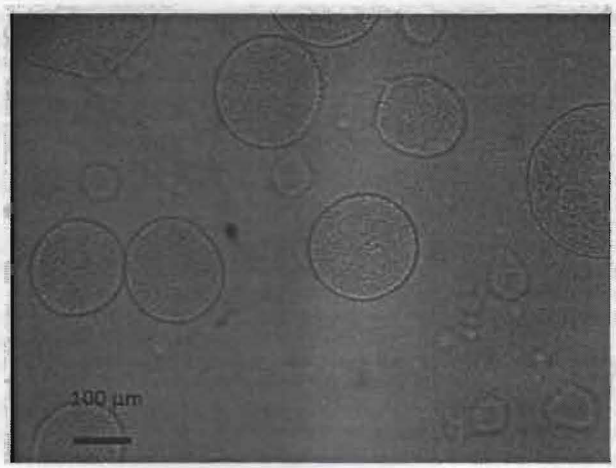

Fig. 1. *Observation microcapsules with a microscope*

The presence of alginate coat will protect FKC *Vibrio* sp vaccine from external environment in the form of microcapsules. The microcapsules can be a single particle or aggregate form [8]. Microcapsules vaccine in this study measuring ± 50-100 µm.

Observations of mortality and survival rate of grouper seed is done for 7 days after challenge test with a bacterial infection of *Vibrio* sp. Data percentage of mortality and survival rate of the grouper seed which is given a treatment and control during maintenance, can be seen in Table 1 below.

Table 1. Data percentage of mortality and survival rate of grouper seed for 7 days after challenge test

Treatment	Mortality (%)	Survival (%)
Dose of 0.025 mg / fish / day	28	72
Dose of 0.0125 mg / fish / day	30	70
Control without vaccine	74	26

Calculation of grouper seed survival rate show that the survival rate of treated seed at a dose of 0.025 and 0.0125 mg/fish/day is different from the control seeds, and can be seen from the increase in the average percentage of survival. The grouper seed survival in treated seed is in good level. This is due to oral microcapsules FKC *Vibrio* sp vaccination which is given to the grouper seed can increase the production of antibodies, so that the vaccinated grouper seed had more antibodies than the control grouper seed. Fish seed which has more antibodies have capability to neutralize antigens that enter the body, so that mortality can be reduced. Vaccination is considered successful if it has a value of Survival average above 60% which shows that the effect of vaccine protection against pathogen infection is very large [11].

The data of water quality parameters during the 21-day maintenance period can be seen in Table 2 below.

Table 2. Measurement of grouper water quality

Parameter	Unit	Value
The water temperature	° C	29-31
Dissolved oxygen	mg / L	5-6
pH		7
Ammonia	mg / L	0
Salinity	ppt	33-34

Water quality measured during the maintenance period is a good range of water quality for the maintenance of grouper seed, the temperature between 28-31°C, dissolved oxygen > 4 ppm, pH between 7-8, amoniac levels < 1 mg/l and salinity 30-35 ppt [2].

4. CONCLUSION

From this study, it can be concluded that the oral vaccine from *Vibrio* sp can be produced by *Vibrio* sp FKC microencapsulation method using alginate, and administration of *Vibrio* sp FKC microcapsules oral vaccine on grouper seed can trigger the production of grouper seed antibodies which impact on the increase Grouper seed survival against vibriosis infection.

ACKNOWLEDGEMENTS

We would like to thank KOPERTIS Region VII East Java, the Directorate General of Higher Education, Ministry of Education and Culture on competitive grant funding in 2013 (Letter Research Program Implementation Agreement number 094 / SP2H / PDSTRL / K7 / KL / III / 2013, dated March 8 2013), and Head of Brackish Water Aquaculture Situbondo for permission in the conduct of the study.

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