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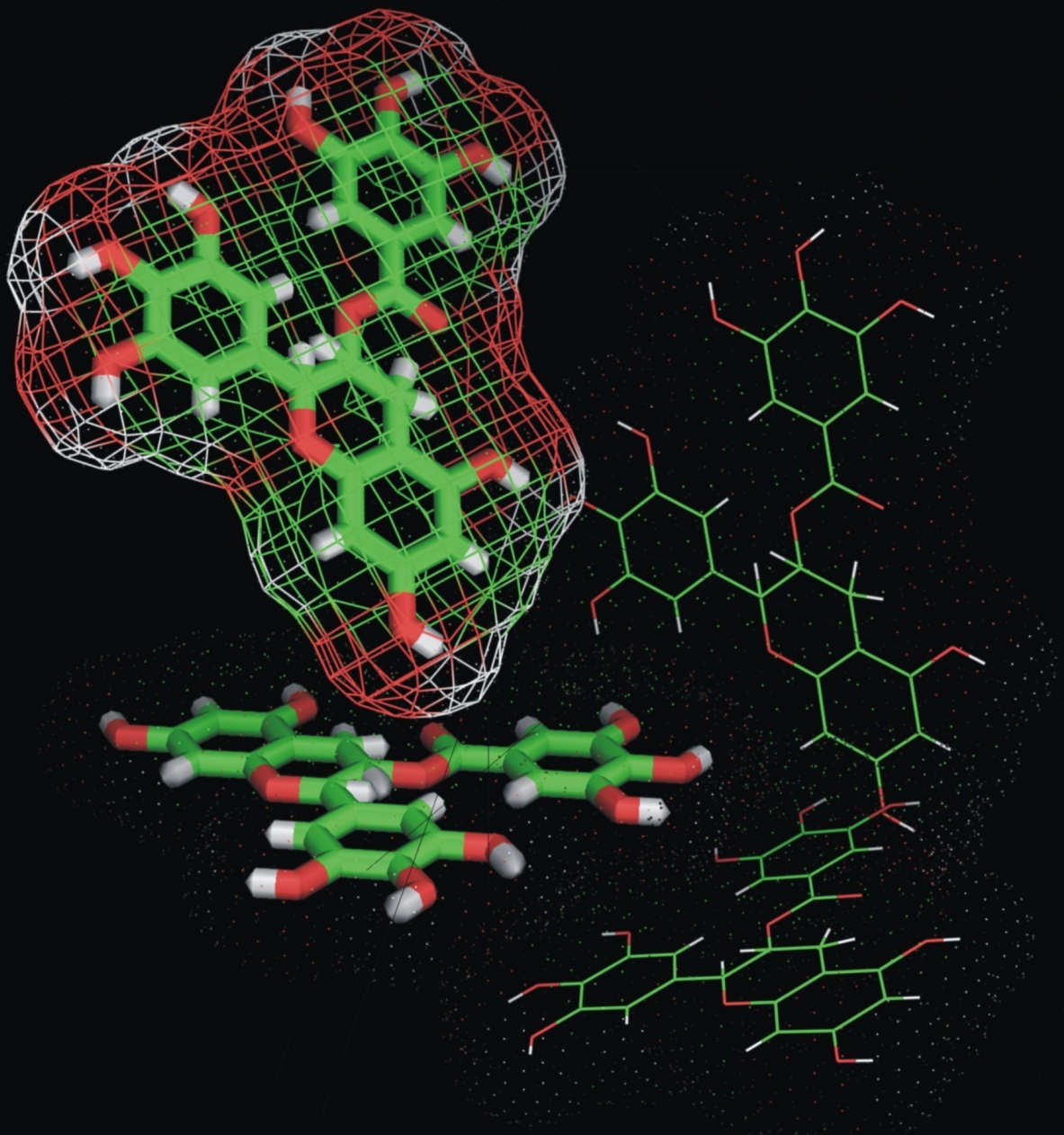

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Effect of Cell Density and *Benzyl Amino Purine* on the Growth of Somatic Embryo of Citrus Mandarin Batu 55 (*Citrus reticulata* Blanco.) in Liquid Culture

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Abstract

Citrus mandarin Batu 55 (*Citrus reticulata* Blanco.) is one of Indonesian fruits commodities that have high economic value and consumers demand. The propagation of citrus mandarin by plant tissue culture generally was carried out on solid medium. The liquid culture system could increase cell multiplication therefore it became alternative method of plant propagation through somatic embryogenesis. The effect of initial cell density and Benzyl Amino Purin (BAP) concentration in liquid media were investigated. The initial cells density and right concentration of BAP given in media can increase cell proliferation of somatic embryo in liquid culture. Globular somatic embryo were cultured on Murashige and Tucker media with initial cell density 4, 6, 8 and 10 mgL^{-1} and BAP 0, 0.25, 0.5, and 0.75 mgL^{-1}. Growth evaluation of somatic embryo were obtained by weighing fresh and dry weight every 2 weeks for 8 weeks for initial cell density treatment and 6 weeks of BAP treatment. The result of the research showed that cell density affect the growth of somatic embryo of citrus mandarin. Somatic embryo with low cell density showed slower growth compared than high cell density. Peak growth occured in 6th cultured with cell density 10 mgL^{-1}. In addition to cell density, the growth of somatic embryo in liquid culture was affected by BAP. The growth of somatic embryo on the media containing BAP showed better results than without BAP. The highest BAP concentration on media showed fresh and dry weight of somatic embryo increased. In this research, growth of somatic embryo is not optimal yet because fresh and dry weights of somatic embryo still increase with high concentration 0.75 mgL^{-1} of BAP.

Keywords: Benzyl Amino Purine, cell density, citrus mandarin, liquid medium, somatic embryo.

INTRODUCTION

Citrus mandarin (*Citrus reticulata* B.) is one of Indonesian fruit commodities that has high economic value and consumers demand [1]. Citrus mandarin high vitamin and sweet taste [2]. But, some problems faced in cultivation of citrus mandarin found are limited land provision, low seedlings available, and a difficulty to get well seeds for high quality crops. Propagation of citrus mandarin has been developed by plant tissue technology through somatic embryogenesis. Generally, this technique using solid medium which has several disadvantage that are the low absorption of nutrients and easily accumulated toxic compounds [3].

Culture in liquid medium has several benefits including high multiplication of cells, the entire cell surface is in direct contact with the medium, better aeration, and there is no gradient nutrients and gas in the medium [4]. Liquid culture system have the potency for bioreactor culture, alternative method of micropropagation of citrus mandarin [5].

Initial cell density and growth regulators on media are important factors that affect the growth and development of cells in liquid medium [7]. Cell density during early inoculation effects the growth of somatic embryo in liquid culture [8]. In general, low cell density causes low proliferation, but high cell density inhibit cell growth because of accelerate accumulation of toxic compound in liquid culture, resulted of an imbalance between medium and cell density in the medium [8]. The highest multiplication of somatic embryo of *Citrus suhuinensis* and *Citrus kalamondin* were obtained at cell denisty 4 - 6 mgL^{-1} and 2 - 6 mgL^{-1}, respectively [2].

Benzyl Amino Purine (BAP) is one of the cytokinin compounds to induce and stimulate the growth of citrus somatic embryos. The previous report showed that 1.5 mgL^{-1} BAP increased multiplication of somatic embryo of *C. aurantifolia* on solid media whereas 0.1 mgL^{-1} BAP increased the multiplication of somatic embryo *C. suhuinensis* [4]. The objective of the research was to

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study the effect of initial cell density and BAP on the growth of somatic embryo in liquid culture.

MATERIALS AND METHODS

Induction and Multiplication of Somatic Embryo

Somatic embryo were induced from nucellus explants cultured on MT (Murashige and Tucker) medium +50 gL^{-1} sucrose +3 mgL^{-1} BAP +50 mgL^{-1} malt extract. The culture was incubated at room temperature 24°C for two months [8]. Formed somatic embryo was subcultured on MT medium + 30 gL^{-1} sucrose every 6 weeks for 2-3 times.

Treatment of cell density and BAP concentration

Globuler stage of somatic embryo were subcultured on liquid media MT + 30 gL^{-1} sucrose with treated cell density of 4, 6, 8, and 10 mgL^{-1}. The cell density that resulted best growth of somatic embryo is used as basis for further experiment treatment.

BAP treatment was done with the globular stage somatic embryo subculture (best cell density) in liquid media MT + 30 gL^{-1} sucrose + 0, 0.25, 0.5, and 0.75 mgL^{-1} BAP. Culture was incubated on temperature 24°C and homogenated by using shaker on 230 rpm. Growth was evaluated by weighing fresh and dry weight of somatic embryo every 2 weeks for 8 weeks for cell density treatment and 6 weeks for BAP treatment.

Data Analysis

This study used a randomized complete block design with repetition as a group. Factors used were initial cell density and BAP concentration. Data were analyzed using ANOVA and Duncan advanced test using significance α <0.05.

RESULT AND DISCUSSION

Somatic embryo in liquid culture showed a friable texture and white color (Fig. 1). Initial cell density of somatic embryo affected cell growth and proliferation of somatic embryo in liquid culture. The growth of somatic embryo at high initial cell density was better than low initial cell density (Fig. 1).

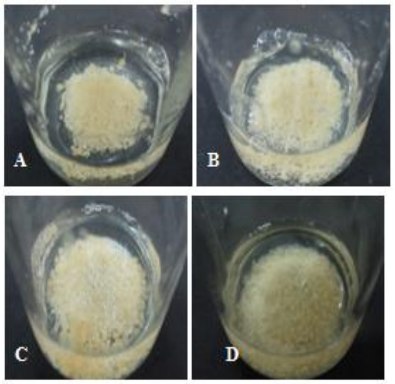

Figure 1. The growth of somatic embryo with different initial cell density at 6 weeks of culture in liquid media; A. 4 mgL^{-1};B. 6 mgL^{-1}; C. 8 mgL^{-1}; D. 10 mgL^{-1}

The inoculation of culture with different initial cell density affected fresh and dry weight of somatic embryo in liquid culture. The growth of somatic embryo at low initial cell density was slow, while at high initial cell density it was faster. The optimum fresh weight of somatic embryo was at 6 weeks of culture and decreased after 6 weeks of culture (Fig. 2A), but the dry weight of somatic embryo still continue to increase until 8 weeks culture (Fig.2B).

The multiplication of somatic embryo with 10 mgL^{-1} initial cell density was higher than the others, whereas the culture with 4 mgL^{-1} cell density result the lowest multiplication of somatic embryo. Fresh and dry weights of somatic embryo in culture with initial cell density 10 mgL^{-1} were 2.93 g and 0.15 g.

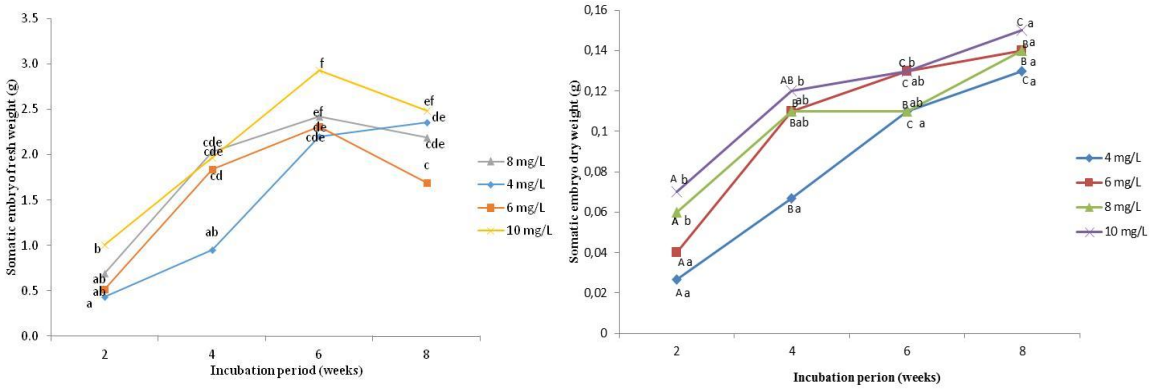

Figure 2. Fresh and dry weight of somatic embryo for 8 weeks in liquid culture; A. Fresh weight, B. Dry weight; The same letter for cell density and incubation period showed no significantly different by Duncan test α <0.05.

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Conversely, fresh and dry weight of somatic embryo in culture with cell density 4 mgL^{-1} was only 2.20 g and 0.11 g (Fig. 2). The optimum multiplication of somatic embryos was obtained 6 weeks incubation period and initial cell density 10 mgL^{-1}.

Beside cell density, BAP also increased the growth of somatic embryo in liquid culture. The growth of somatic embryos in luquid medium with the addition of BAP was better than without BAP (Fig. 3).

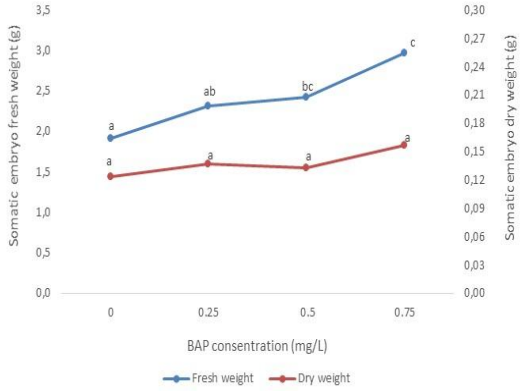

Figure 3. Fresh and dry weight of somatic embryo in liquid culture without BAP in the 6 weeks; The same letter for fresh and dry weight showed no significantly different by Duncan test α <0.05

The growth of somatic embryo on media which added with various concentrations of BAP was observed by measuring the fresh and dry weight at 6 weeks cultures. The growth of somatic embryo on medium with the addition of BAP resulted better than without BAP. The higher concentration of BAP on media, the increasing of somatic embryo the optimum growth was higher. Fresh and dry weight of somatic embryo in liquid medium containing 0.75 mgL^{-1} BAP were 2.97 g and 0.17 g. Otherwise, fresh and dry weight of somatic embryo on media without BAP were only 2.01 g and 0.11 g. However, the optimum growth of somatic embryo in liquid media containing BAP was not obtained yet, because of the growth of somatic embryos on the highest of BAP was given in this research was still increase.

The initial cell density of culture was an important factor of growth needed to obtain maximum growth of cells in liquid medium. Culture with high cell density accelerate the proliferation and vice versa. High cell density absorbed nutrients faster in liquid medium than lower cell density. However, when the number of cells of somatic embryo in liquid medium had already maximum, the cell proliferation should automatically inhibited, because an imbalance between media and nutrients for cell in the medium [7]. The decrease of cell proliferation might also caused by the use of prolonged liquid media which accumulated toxic compound [8].

Previous study reported that prolonged incubation period caused the color changes of media and became finally decreased cell proliferation. Incubation period was also determined cell growth of somatic embryo in liquid media [2]. Culturing somatic embryo for too long period in liquid cultuer caused disturbance of osmotic process within the cell, therefore the water content of somatic embryo became very low. It became one of the factors that decrease proliferation of somatic embryo in liquid culture [10].

The growth regulators BAP in the culture media stimulated cell proliferation in some species. A range different BAP concentration has been used to induce somatic and embryogenesis shoot regeneration of citrus and *Chrysanthemum* [11]. Multiplication of somatic embryos in *Chrysanthemum* showed the best growth at media containing suplement of 1.0 mgL^{-1} BAP [11], whereas in *C. aurantifolia* and *C. sinensis* at media with concentration of 1.5 and 2 mgL^{-1} BAP. The higher concentrations of BAP in liquid culture had a negative effect on embryogenic cell growth. The addition of 3 mgL^{-1} BAP into liquid media descreased the growth of somatic embryo in *C. suhuinensis* [2]. Liquid culture system significantly accelerated multiplication of somatic embryo, thus it has a promising potential as micropropagation technique in citrus trought somatic embryogenesis.

CONCLUSION

The optimum growth of somatic embryo in citrus mandarin were obtained at initial cell density of 10 mgL^{-1} and 6^{th} incubation period culture. Addition of plant growth regulator BAP on media increased multiplication of *Citrus* somatic embryo in liquid culture.

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The Immunomodulatory Effect of *Elephantopus scaber* and *Sauropus androgynus* Extract to Cellular Immune Response in Pregnant *Mus muscullus* Infected by *Salmonella typhimurium*

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Abstract

Pregnancy has a high risk and is more susceptible to infectious diseases. *E. scaber* and *S. androgynus* contains saponins and flavonoids as an immunomodulatory that can increase the body's immunity. The purpose of this study is to determine the immunomodulatory effects of *E. scaber* and *S. androgynus* leaf extract against cellular immune response in pregnant mice infected *S. typhimurium*. This research used seven treatment groups including negative control (K-) mice without injected *S. typhimurium*, positive control (K+) mice were injected *S. typhimurium* and extract treatment with 5 variant doses and dissected on the 12^{th} and 18^{th} days. Lymphocytes was isolated from the blood, then relative number of $CD4^+$ and $CD8^+$ were analyzed with flow cytometry and data continued with ANOVA. Statistical analysis showed that different extract dose and duration of treatment had a significant effect on the number of $CD4^+$ and $CD8^+$ T cells. *E. scaber* and *S. androgynus* act as immunomudulatory effect and treatment with combination of extracts *E. scaber* and *S. androgynus* 50 $mg.kg^{-1}$ BW + 112.5 $mg.kg^{-1}$ BW respectively and treatment with extract of *S. androgynus* 150 $mg.kg^{-1}$ BW are the optimum treatment which can restore immune system conditions such as normal pregnancy without infection.

Keywords: Cellular Immune, *E. scaber*, Immunomodulatory, *S. androgynus, S. typhimurium*.

INTRODUCTION

Typhoid fever is a disease with serious threat in developing countries because it can cause death; which is caused by *S. typhimurium* bacteria [1,2]. In humans, the infection is caused by a decline activity of immune system thus the immune system is incapable to kill the bacteria causing the bacteria to survive, thrive, invade and damage the body's cells [3].

Pregnancy is more vulnerable and have a high risk of infection because in pregnant condition, immunological conditions is unique [4]. Previous research indicated that typhoid fever because of *Salmonella* in pregnancy can cause abortion [5].

Typhoid fever is usually treated with antibiotics and synthetic antibacterial, but either of these medications provides teratogenic effects to fetus. Thus the fetus may be at risk of mental or physical disability [6]. One alternative to solve the problem is using herbs, which is harmless compare to synthetic drugs [7].

E. scaber and *S. androgynus* contains saponins and flavonoids known as a natural immunomodulatory that can enhance the immune system [8]. Flavonoid compounds can improve the activity of IL-2 and lymphocyte proliferation. Lymphocyte proliferation active Th1 cells macrophage activation through cytokines IFN-γ produced by $CD4^+$ T cells and lysis of infected cells by $CD8^+$ T cells [9,10]. Based on this background, this study aims to determine the immunomodulatory effects of *E. scaber* and *S. androgynus* leaf extract against cellular immune response in pregnant mice infected by *S. Typhimurium,* based on $CD4^+$ T cells and $CD8^+$ T cells in the blood.

MATERIALS AND METHODS

Treatment Group

Pregnant mice were obtained from PT. Galaxy Science Jember divided into seven groups (Table 1). Mice were infected with *S. typhimurium* on day 5 after the extract and the extract was continued until dissected on the 12^{th} and 18^{th} day.

Isolation of Lymphocytes Cells

Obtained blood put into propylene tubes that contained 10 ml of RBC lysis and then centrifuged 300 rpm, for 5 minutes 10^0C to obtain pellets. Then added RBC lysis again about 5 mL and then recentrifuged again. The pellet was added 1 ml

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PBS and then centrifuged 300 rpm for 2 minutes 10^0C. Pellets can be added as much as 1 ml PBS then resuspended and divided into 3 microtubes, each 300 mL then centrifuged at 2500 rpm, at temperature of 4^0C for 5 minutes.

Table 1. Treatment Group

Group	Extract (mg.kg^{-1} BW)		Infection (10^7 CFU.mL^{-1})
	E.scaber	*S.androgynus*	
K-	-	-	-
K+	-	-	√
P_1	200	-	√
P_2	150	37.5	√
P_3	100	75	√
P_4	50	112.5	√
P_5	-	150	√

Flowcytometry Analysis

Pellets are added to the monoclonal antibody anti-mouse CD4 monoclonal antibody (BioLegend No. Cat. 100 531). The concentration is 0.01 mg.mL^{-1} and 50 µL phycoerythrin (PE)-conjugated anti-mouse CD8 (BioLegend, No Cat. 100 708) with a concentration of 0.01 mg.mL^{-1}. Then it was incubated for 20 minutes in the ice box, then added 300 µL PBS and resuspended. Later it transferred to the cuvet for flowcytometry analysis.

Data Analysis

This study used a completely randomized design factorial pattern. Data from the flow cytometry analyzed statistically with one-way ANOVA with a significance level of $p < 0.05$ using SPSS, then followed by Tukey's test.

RESULT AND DISCUSSION

Result showed that the relative number of $CD4^+$ and $CD8^+$ T cells at day 12 in pregnant mice without infection *S. typhimurium* lower than infected pregnant mice by the *S. typhimurium* (Figure 1a). On the 18th day of pregnant also showed the same result that the relative number of $CD4^+$ T cells and $CD8^+$ normal pregnant mice lower than infected pregnant mice. It was significantly different (Figure 1b). The increase is due to antigen enters to the body. It can enhance the immune response for the production of immunocompetent cells and increasing the proliferation and differentiation of T cells to antigen elimination which infect the body [11].

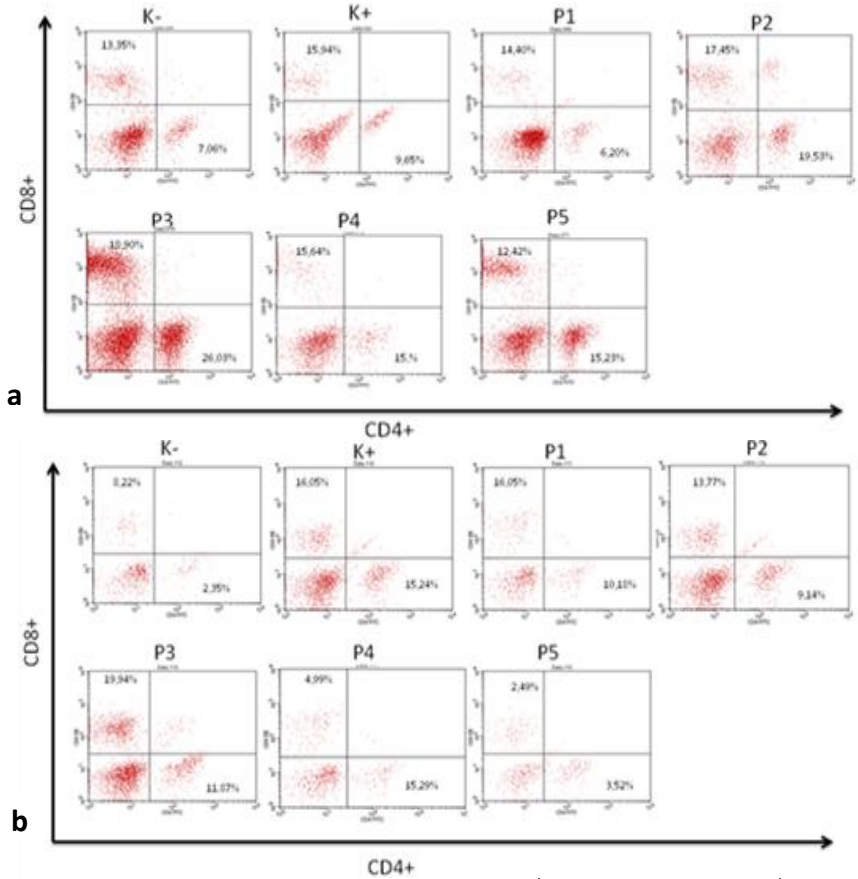

Figure 1. Profile of relative number $CD4^+$ and $CD8^+$ T cells. (a) 12th day of pregnancy; (b) 18th day pregnancy

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The presence of *Salmonella* infection would induce Th1 cell responses and after infection, then $CD4^+$ T cells are activated to increase the number of $CD4^+$ T cells and cytokines [12]. $CD4^+$ T cells contribute to activate macrophages, whereas the $CD8^+$ T cells called cytotoxic T cells kill cells containing microbes in the cytoplasm resulting in a reservoir of infection [13].

The treatment of *E. scaber* and *S. androgynus* extract on day 12 showed a lack of regulation of immune system. It was observed through the number of $CD4^+$ T cells were significantly different compared with the positive control (infected pregnant mice without treatment). $CD4^+$ cell number at treatment *E. scaber* extract 200 $mg.kg^{-1}$ BW has 19.53% and *E. scaber* extract 100 $mg.kg^{-1}$ BW and *S. androgynus* 75 $mg.kg^{-1}$ BW has 26.03% (Figure 2). The relative number of $CD8^+$ T cells on the 12^{th} and 18^{th} day of dissection show that treatment with *E. scaber* extract 100 $mg.kg^{-1}$ BW and *S. androgynus* 75 $mg.kg^{-1}$ BW has 18.90% and 19.94% (Figure 3). This increase is predicted because of the content of the two plants in the form of saponins and flavonoids. Both of these compounds contribute in cell proliferation that is able to induce synthesis the proto-oncogene c-fos and c-myc. The role of proto-oncogene on cell proliferation is increasing the mitogen signal transduction through the increased expression of cytokines IL-2 [14]. Flavonoids can increase IL-2 activity and lymphocytes proliferation [9]. IL-2 can trigger the $CD8^+$ activation to $CD8^+$ produce perforin and granzyme that will destroy infected cells [15].

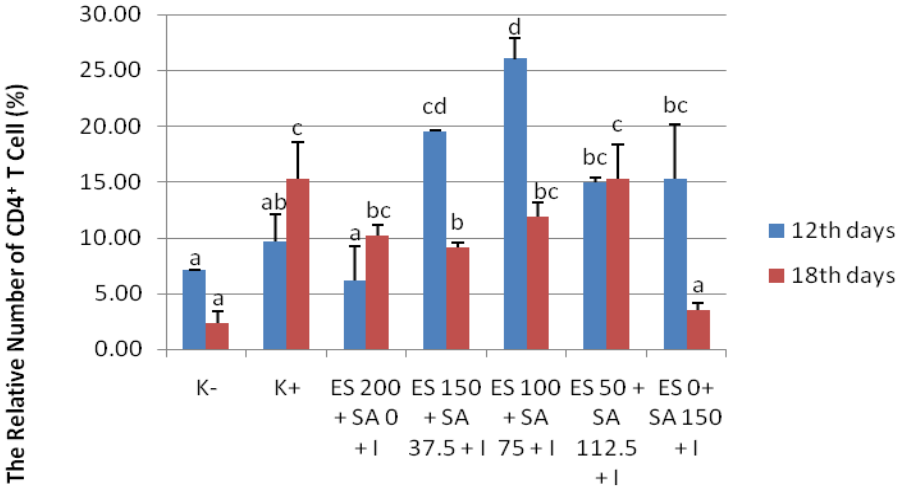

Figure 2. The relative number of $CD4^+$ T cells. The dose and duration of administration of herbs affect the number of $CD4^+$ T cells. Different notation indicates a significant difference ($P < 0.05$).

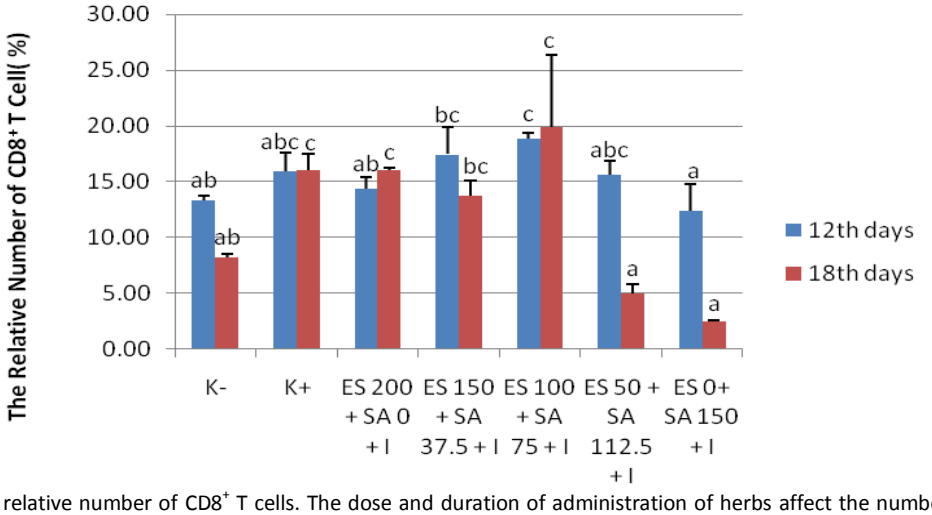

Figure 3. The relative number of $CD8^+$ T cells. The dose and duration of administration of herbs affect the number of $CD8^+$ T cells. Different notation indicates a significant difference ($P < 0.05$).

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The relative number of $CD4^+$ T cells at the 18^{th} day of dissection in treatment *E. scaber* extract 150 $mg.kg^{-1}$ BW has 9.14% and treatment *S. androgynus* extract 150 $mg.kg^{-1}$ BW has 3.52% significantly decreased compared with a positive control (infected pregnant mice without treatment). The relative number of $CD8^+$ T cells also showed the same result the treatment with extract *E. scaber* 50 $mg.kg^{-1}$ BW and *S. androgynus* 112.5 $mg.kg^{-1}$ BW has 4.99% and extract of *S. androgynus* has 2.49%. It significantly decreased compared with a positive control. Saponins and flavonoids in addition act as immunostimulatory also serves as imuno-supresor that suppresses the immune response. Both of these compounds are amphiphilic that can increase the level of Cyclin-Depedent-Kinase (CDK) inhibitor in the form of protein $P27^{KIP}$ that play a role in the regulation of cell proliferation in phase G0/G1 by inhibiting compound G1 Cyclin-CDK resulting in cell cycle does not continue and the cessation of cell proliferation [16].

In pregnancy, the function of humoral and cellular immune suppression that occurred supression of Th1 and Tc cells which will reduce the secretion of IL-2, IFN-γ and TNF-β. Suppression of Th1 response is needed to sustain a pregnancy [17]. In addition, pregnancy hormones such as progesterone, estrogen and prolactin are also affects the immune system that is able to minimize the effects of peripheral NK cells [18]. Treatment with combination of extracts *E. scaber* 50 $mg.kg^{-1}$ BW and *S. androgynus* 112.5 $mg.kg^{-1}$ BW and treatment with extract of *S. androgynus* 150 $mg.kg^{-1}$ BW are the optimum treatment which can restore immune system conditions such as normal pregnancy without infection.

CONCLUSION

E. scaber and *S. androgynus* extract showed a significant difference to the number of $CD4^+$ and $CD8^+$ T cell in infected pregnant mice by *S. typhimurium*. Optimal treatment to help balance the immune system in pregnancy treated with a combination of extracts *E. scaber* 50 $mg.kg^{-1}$ BW and *S. androgynus* 112.5 $mg.kg^{-1}$ BW and treatment P5 with *S. androgynus* extract 150 $mg.kg^{-1}$ BW.

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Estrous Cycle Response in Mice (*Mus musculus*) with CSE (Crude Sperm Extract) Injected Intraperitoneally

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Abstract

Sperm protein has an important role in fertilization process. It becomes antigenic when it is injected to body and can increase TNF- α secretion. TNF-α in blood vesssel can inhibit estradiol synthesis. Estradiol has a significant role in reproduction cycle, especially in estrous cycle. The study aims to understand the influence of *Crude Sperm Extract* (CSE) to mice (*Mus musculus*) estrous cycle. 16 fertile mices strain Balb-C aged 3-4 months, weighed 20-30 g was divided into 4 groups. P_0 is a control group injected by PBS, group P_1, P_2, and P_3 injected by CSE with 1.5 mg.ml^{-1}, 2 mg.ml^{-1}, and 2.5 mg.ml^{-1}. CSE is injected intraperitoneally during mice's diestrus phase. CSE is injected in day 0, day 12, day 24 and observed daily. The data are descriptively analyzed. The results show that CSE with molecule weight between 26.8-176.8 kDa influences estrous cycle.

Keywords: Crude Sperm Extract, estrous cycle, folliculogenesis, *Mus musculus*

INTRODUCTION

Sperm protein plays an important role in the fertilization process. It becomes antigenic when injected to body and causes immune responses [1,2,3]. Sperm protein will increase the secretion of TNF-a by macrophages-activated CD4 T cells [4,5].

TNF-α will bind to TNF-α receptor in the ovary [6], thus inhibit the synthesis of estradiol through cAMP (Adenosine-3´,5´- Cyclic Monophosphate) and PKA (protein kinase A) pathway. cAMP and PKA are involved in cytochrome P450scc excretion that convert cholesterol to pregnenolon, and eventually estradiol biosynthesis and metabolism [7,8]. Estradiol has a significant role in estrous cycle [9]. Based on the mentioned facts and reasons, the present study aims to understand the influence of Crude Sperm Extract (CSE) in mice estrous cycle.

MATERIALS AND METHODS

Isolation and Charaterization of Bull's Sperm Protein

We obtained 2 ml (900-2000x10^6 cells.ml^{-1}) of bull's sperm from BBIB (Balai Besar Inseminasi Buatan – Center for Artificial Insemination). The sperm was washed with 6 ml PBS (phosphate buffer saline), vortexed and centrifuged (2500rpm, 10 minutes). The pellet was resuspended with 3 ml TCM (Tissue Culture Medium) then vortexed and centrifuged once more (2500rpm, 10 minutes). The pellet was resuspended with 0.5 ml extract buffer and cold-sonicated (50% amplitude, 20 minutes). Total 1 ml suspension was centrifuged (9000rpm, 30 minutes, 4°C) and result's 0.5 ml supernatant was further centrifuged (13.000 rpm, 45 minutes, 4°C) and then resuspended with KCL-HEPES buffer (1:1) and stored in-80°C [10]. The isolated sperm protein was characterized with 12.5% separating gel SDS-PAGE protocol [11].

Animal Treatment

Total of 16 fertile female mice (*Mus musculus*) strain Balb-C, aged 3-4 months and weighed 20-30 g were used as animal model in this study. They were divided into 4 groups: control group (P_0) without CSE injection; P_1 which injected with 1.5 mg.ml^{-1} CSE; P_2 2 mg.ml^{-1} CSE injection; and P_3 2.5 mg.ml^{-1} CSE injection. All injections were all intraperitonal and administered at 0th day, 12th day and 24th day of experiment. CSE is injected intraperitoneally during mice's diestrus phase.

Estrous cycle observation

The observation of estrous cycle was conducted since 0th until 24th days that covers proestrus, estrous, metestrus dan diestrus periods. Vaginal smears were made by using pipette

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with NaCl 0.9% solution. The lenght of estrous cycle was descriptively analyzed.

RESULT AND DISCUSSION

SDS-PAGE characterization reveals that Bull's sperm CSE have 11 protein bands with molecule size 176.8, 63.2, 58.4, 55.3, 52.4, 49.7, 44.6, 38.02, 36.03, 34.1 and 26.8 kDa [12]. These protein bands were all used for treatment in mice.

We identified the phases of mice estrous cycle by its general criteria (Fig. 1). In proestrus phase, vaginal cytology dominated by parabasal cells and estrous phase vaginal cytology dominated by superficial cells. Intermediate and parabasal cells predominate in smears taken during metestrus. The onset of diestrus is marked by a precipitous decline in the number of superficial cells and reappearance of intermediate and parabasal cells [13].

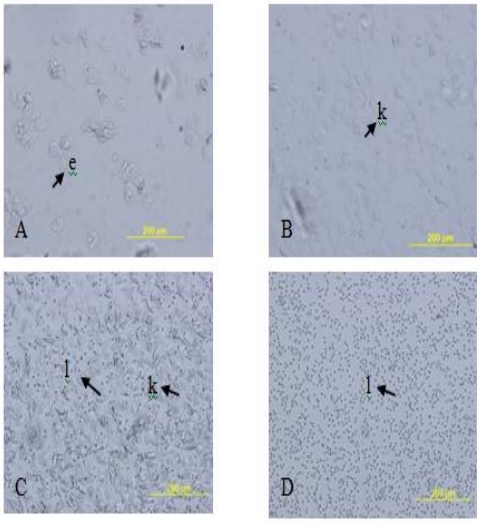

Figure 1. Vaginal cytology representing each stage of estrous cycle

Description:
A. Proestrus, nucleated epithelial cells (e)
B. Estrous, cornification cell (k)
C. Metestrus, cornification cell (k) and leukocyte cell (l)
D. Diestrus, dominated by leukocytes (l).

Estrous cycle signifies sexual activity and organ function, like the function of the ovarium, along with follicle development. We found irregularity in CSE injected group. It characterized by elongation of time in one phase of the estrous cycle, which is diestrus (Table 1, Fig. 2).

Table 1. Average Length of Estrous Cycle Stages on Three Cycles

Phase (day)	Treatment			
	P_0	P_1	P_2	P_3
Proestrus	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Estrous	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Metestrus	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.5± 0.58
Diestrus	2.8 ± 0.7	3.1 ± 0.6	4.1 ± 0.8	4.3 ± 1.6

The data signify that CSE could influence the estrous cycle length, which have elongation in diestrus phase compared to control (P_0) (Fig. 2). The elongation tendencies of diestrus phase in each treatment are 3 days longer (P_1), 4 days (P_2), and 4-5 days (P_3) (Fig. 3). The average duration of normal estrous cycle in mice and rats is 4-5 days [14]. CSE injection in the body may cause immune responses and eventually increase TNF-α secretion in blood by CD4 T cells which are activated by macrophages [2-5]. TNF-α through the blood vessels will bind to TNF-α receptor in the ovary [6].

The increase of cytokine TNF-α in the ovarian inhibit synthesis of estradiol through inhibition of cAMP and PKA pathway. cAMP and PKA involved in regulating the expression of the enzyme cytochrome P450scc. P450scc cytochrome enzymes will convert cholesterol to pregnenolone. Pregnenolone be converted to estradiol [7].

Inhibition of cAMP and PKA will inhibit the enzyme P450scc, so it will inhibit the biosynthesis and metabolism of Estradiol [8]. Inhibition of estradiol can delay the replacement of diestrus phase to the proestrus phase, because the proestrus phase has estradiol in highest levels and the diestrus phase has estradiol is low levels [15]. It will affect on hormone regulation.

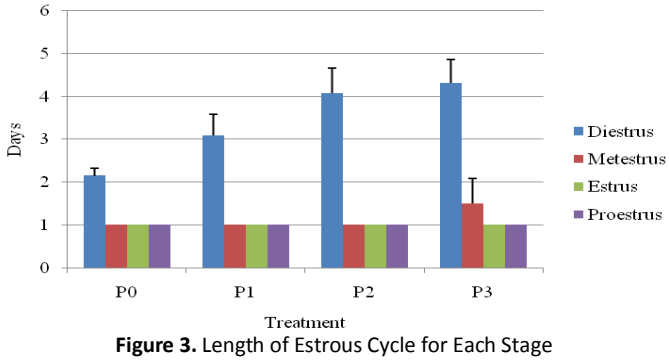

Figure 3. Length of Estrous Cycle for Each Stage

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CONCLUSION

The protein in the bull's sperm CSE have molecule size ranging from 26.8 to 176.8 kDa. Our findings suggested that bull sperm CSE influences the mice estrous cycle, especially in diestrus period elongation. This provides vital prelimenary information for the usage of CSE as the candidate of immunocontraception for human. We infer that it is necessary to measure the estrogen blood level, the antibody formation in the blood and flowcytometry analysis to elucidate if there a TNF-a present in the blood.

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Oocyte In Vitro Maturation with Crude Sperm Extract Protein of Bull's Spermatozoa

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Abstract

Oocyte In vitro maturation (IVM) is one of the important parts for in vitro fertilization (IVF). The success of oocyte maturation is influenced by the composit ion and the quality of IVM medium. Culture medium which used to IVM not only influences the oocyte process to reach metaphase II and proceed the fertilization, but also influences to developmental of an embryo. Crude sperm extract has high-level protein kinase and contains some sperm proteins. Crude sperm extracts expected as natural maturation medium that can increase the success of In Vitro Maturation (IVM). The characterization of crude sperm extracts profile with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Oocyte maturation is observed in the 26th hour from the first culture. The result of crude sperm extract characterization obtained from the protein with the molecular weight is 176.8, 63.2, 58.4, 55.3, 52.4, 49.7, 44.6, 38.02, 36.03, 34.15 and 26.8 kDa. Oocyte maturation with concentration of crude sperm extract 1.5 µg.mL^{-1} with 71.6% matures oocyte and oocyte maturation with concentration of crude sperm extract 2.5 µg.mL^{-1} with 75% matures oocyte.

Keywords: Crude Sperm Extract, In Vitro Maturation, Oocyte

INTRODUCTION

In vitro maturation (IVM) in the oocyte is one of the important phases on in vitro fertilization [1]. One of the obtained benefits from In vitro maturation (IVM) is the availability of adult oocyte as the source of recipient cytoplasm to nucleus transfer program, so that it is possible to increase embryo in vitro production [2].

Efficiency embryo production in vitro is highly affected by the number and the quality of the oocyte which is successful to be mature oocyte [3]. The success of oocyte maturation is affected by the composition and the quality of the used medium [4]. The main compositions which are commonly used to increase the quality of culture medium are protein and hormone [1]. The sperm extract which has high-level protein kinase and it contain some protein in the sperm [5]. The protein kinase can trigger the increase of Maturation promoting factor (MPF) which is the enzyme kinase which has a significant role to continue transition oocyte phase from mitosis to meiosis [6]. Activated MPF (Maturation Promoting Factor) is needed as the beginning of maturation [7].

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In the present research, the crude sperm extract given by oocyte maturation medium aims to determine developmental of Goat oocyte. Thus it can inform the success of IVM which contains some Bull sperm protein as oocyte maturation medium from the different species.

MATERIALS AND METHODS

Isolation and Characterization of Bull's Sperm Protein

The crude spermatozoa extract is isolated and prepared from the masculine bull ejaculator. The semen is obtained from BBIB (Balai Besar Inseminasi Buatan - Artificial Insemination Institution) in Singosari. The fresh bull's semen is taken 2 ml added with PBS (Phosphat Buffer Saline) until 6 ml, centrifuge at 2500 rpm 10 minutes. The pellet is added with TCM 199 Ph 7.4 as 3 mL, then centrifuge at 2500 rpm 10 minutes. The pellet is added with buffer extract as 0.5 mL and mix until homogeny. The obtained suspense is done by cold sonication with amplitude 50% along 20 minutes. Total of 1 mL suspense in centrifuge 9000 rpm along 30 minutes temperature 4°C, the obtained supernatant is taken from 1 mL and centrifuged 13000 rpm along 45 minutes temperature 4°C. The obtained supernatant is added with buffer KCL-HEPES as 0.5 mL or with 1:1 comparison, and then it keeps at -80°C. The iso-

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lated sperm protein extract was characterized with 12.5% separating gel SDS-PAGE protocol.

Oocyte Treatment

Goat Oocyte used is oocyte grade A that have three layers of cumulus. They were divided into 3 groups: control group (P_0); IVM medium without crude sperm extract (medium TCM 199 + 10% Fetal Bovine Serum SA Origin (FBS, GIBCO) and added with the gonadotrophin hormone supplementation as 15 µL FSH and 35 µL LH [9]). P_1: IVM medium with supplementation crude sperm extract concentration 1.5 µg.mL^{-1}. P_2: IVM medium with supplementation crude sperm extract concentration 2.5 µg.mL^{-1}. All groups was insert in incubator at 5% CO_2, 38.5°C of humidity, in drop medium 100 µL per 10 oocytes which is layered by paraffin oil.

Data Analysis

The data of sperm protein character is analyzed descriptively to measure the molecular weight of protein in the bull sperm. The data of oocyte maturation were used to analyze the number of mature oocytes descriptively and then analyzed using One Way Anova.

RESULT AND DISCUSSION

The result of SDS-PAGE characterization shows that the protein in the crude sperm extract has some molecular weight which is 176.8, 63.2, 58.4, 55.3, 52.4, 49.7, 44.6, 38.02, 36.03, 34.15 and 26.8 kDa. Maturation oocyte is the physiologist process which aims to provide oocyte to fertilization. The maturation process of the oocyte in vitro in this research using TCM 199 medium which is added with the LH, FSH, and FBS serum, maturation medium has an important role to the success of oocyte maturation in vitro. Thus the effort of maturation medium with some supplementations maximizes the success of oocyte maturation in vitro. There are three treatments used in this research, which is P_0 as maturation control medium, P1 as the treatment of maturation medium with crude sperm extract concentration 1.5 µg.mL^{-1}, and P_2 as the treatment of maturation medium with crude sperm extract concentration 2.5 µg.mL^{-1}. Maturation oocyte in various treatments showed in Figure 1.

The maturation without treatment (maturation control) shows that from the obtained 75 oocytes, there are 45 mature oocytes observed from oocyte cumulus expansion. Thus, it can be assumed that the success of oocyte maturation is about 60%. Oocyte maturation treatment with crude sperm extract concentration 1.5 µg.mL^{-1} from 67 oocytes, 48 oocytes matured. Thus the success of oocyte maturation is about 71.6%. While treatment of maturation medium with crude sperm extracts concentration 2.5 µg.mL^{-1} from 48 oocytes, there are 36 oocytes matured and the success of oocyte maturation is about 75% (Table 1). The result of this research shows that the crude sperm extract protein can trigger oocyte maturation. Higher concentration gives higher chance for more oocyte to be mature.

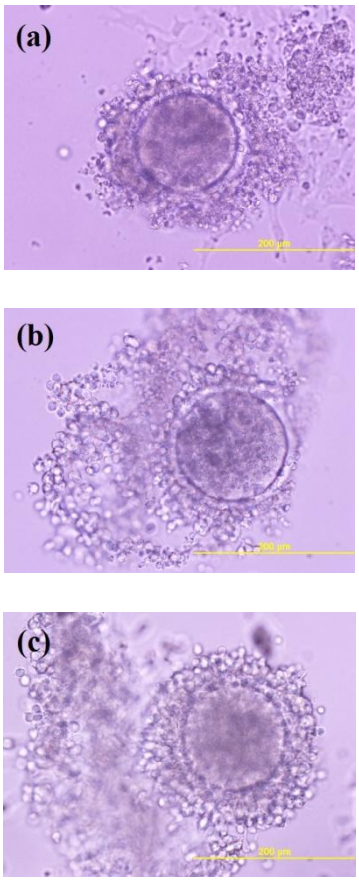

Figure 1. The result of IVM
Description:
(a) Oocyte with the maturation control treatment
(b) Oocyte with treatment CSE concentration 1.5 µg.mL^{-1}
(c) Oocyte with treatment CSE concentration 2.5 µg.mL^{-1}
The observation used the inverted microscope 200x.

Table 1. The result of the goat's oocyte maturation

Treatment	Total Oocyte	Total Mature Oocyte	%
P_0: Control	75	45	60
P_1: SE 1.5 µg.mL^{-1}	67	48	71
P_2: SE 2.5 µg.mL^{-1}	48	36	75

Note: cultured 26 hours in the incubator 5% CO_2 37°C

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The result of analysis of every treatment shows that there is a difference, treatment crude sperm extract concentration 1.5 µg.mL^{-1}l and 2.5 µg.mL^{-1} gives more increases percentage of maturation compared to control treatment. Between treatments of crude sperm extract concentration, the treatment 2.5 µg.mL^{-1} has bigger percentage of success in maturating the oocyte. However, from those treatments, the number of oocyte which is successfully maturated shows few differences, so that the statistical analysis does not show any significant differences from each treatment.

Figure 1 show a morphological change from the three oocyte maturation treatments. The higher concentration of crude sperm extracts treatment, the higher number of mature oocyte. The characteristics of the mature oocyte can be seen from the cumulus expansion, the radiate corona seems shiny, zone pellucid seems clear, ooplasm is hygiene, the granulose cell has good expansion. Good quality of MII oocyte is the clean oocyte, the cytoplasm granular, and the clear zone pellucid [10]. The cumulus cells expansion start to be seen on the incubation of 26-48 hours which is indicated by the presence of cumulus ooforus [11].

The protein kinase can decrease synthesis of Cyclin AMP (cAMP). The decrease of cAMP synthesis is needed to increase the production of *maturation promoting factor* (MPF). The increase of MPF production has significant role to continue the phase from meiosis I to meiosis II in oocyte so that the beginning of maturation [12]. The present research proves that the giving of crude sperm extract can increase the number of a mature oocyte.

CONCLUSION

Crude sperm extract supplementation in a medium can trigger the goat oocyte maturation. The success of maturation with crude sperm extract supplementation concentration 1.5 µg.mL^{-1} is 71.6%. Besides, the success of oocyte maturation with supplementation crude sperm extract concentration 2.5 µg.mL^{-1} is 75%. Crude sperm extract concentration 2.5 µg.mL^{-1} optimizing better for in vitro maturation of oocytes.

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Latent and Eggs Production of Banggai Cardinal (*Pterapogon kauderni*, Koumans 1933) on Various Salinity Levels: Conservation Efforts

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Abstract

The aim of this study is to determine the best salinity level on latent and eggs production of Banggai Cardinal (*Pterapogon kauderni* Koumans 1993) based on first brood and brood production of Banggai Cardinal in the treatment. Water quality measurement was carried out every day to maintain water quality. Water quality measurement showed suitable salinity for Banggai Cardinal breeding. Brood latent showed no significant difference between the treatments. Total of 27 ppt is the level of salinity for Banggai Cardinal reproduction to gain positive respond on eggs production (42.3333 ± 7 eggs).

Keywords: Banggai Cardinal, Eggs Production, Latent, Salinity.

INTRODUCTION

Indonesia has great amount of sea biodiversity which become susceptible due to human activity these days. Trading finfish as fish tank can lead a marine species to be endangered species [1,2]. Cardinal fish is a marine species which spread around temperate and tropical region [3]. Banggai Cardinal is an endemic Indonesian marine finfish species that threatened by human activity (e.g. over fishing). International trading of Banggai Cardinal was 50.000-118.000 each months and the trend tend to increase. Most Banggai Cardinal trades are comes from nature [4]. As result, Cardinal Banggai was on the red list of International Union for Conservation of Nature [5]. Therefore, understanding the breeding method is needed to conserve Banggai Cardinal population in the nature [6].

Banggai Cardinal is a marine fish species [3]. Similar to other marine fish, it is affected by external and internal factor such as physiological aspect of fish brood. Internal factors that affect brood physiology are gonad repoduction, maturity, etc [7]. Whereas, salinity as the main external factor which play important role on fish brood physiology. Salinity affects osmoregulation and metabolism of marine fish [8]. Thus, this study is aimed to determine the best salinity level refer to the production of brood's latent and fecundity of Banggai Cardinal.

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MATERIALS AND METHODS

Brood preparation

On August 2015, Banggai Cardinal brood stock (body length 3±0.2 cm) were collected from Banggai archipelago (South Sulawesi province, Indonesia) and transported to the laboratory in Centre of Marine Aquaclture (Balai Besar Perikanan Budidaya Laut) Lampung within 8 hours. Banggai Cardinal brood stock adapted in 9 aquarium (100x50x50 cm^3) filled with marine water (80%). Total 3 pairs of Banggai Cardinal were adapted in each aquarium for 2 days and feed with artemia 3 times a day (ad satiation).

Treatment

The selected Banggai Cardinal brood stock in solitary aquarium partition (20x30x50cm^3) filled with marine water (80%) was treated with 3 different levels of salinity (27, 30 and 33 ppt) for 3 replications. Marine water diluted with fresh water or added with NaCl (Sigma Aldritch) to gain treated marine water concentration. Salinometer (TDS 10, Dongrun-China) was used as salinity measurement tools. Marine water in solitary aquarium was replaced 75% for every 5 days to maintain water quality.

Water Quality

Temperature and DO measured with Fischer Scientific, Traceable Portable Dissolve Oxygen Meter Pen. The acidity (pH) was measured with Fischer Scientific "accumet" AP110 Portable pH meter. Furthermore, total Ammonia [9] and nitrite [10] were also measured. Water quality measurement conducted every day during laboratory works.

Latent

Latent is the time needed for a fish to breed on treatment conditions. Brood latent is the time it's takes to spawn (days) measured based on Hopkins and Tamaru method [11].

Eggs production

Banggai Cardinal is a mouth breeding marine finfish species [12]. Eggs production are the number of eggs that taken after spawning. Eggs were removed from mouth and counted. Eggs production was measured on mouth breed habit of Banggai Cardinal [13].

Statistical analysis

SPSS 17 for Windows was used for statistical data analysis. Normality analysis was followed by One-way ANNOVA for normal data. Further, we used Tukey post hoc analysis. Statistical analysis was set at $p \leq 0.05$ for differences of treatment.

RESULT AND DISCUSSION

Water quality measurement (Table 1) during laboratory works was considered suitable for the requirement of Banggai Cardinal reproduction conditions. Suitable conditions for organism to live are based on temperature, DO, pH, nitrite and total ammonia [14].

Table 1. Water quality during Banggai Cardinal breed affected different salinity levels

Parameter	Salinity								
	27 ppt			30 ppt			33 ppt		
	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg
Temperature (C)	27	30	28,6	27	30	28.7	27	30	28.6
DO (mg/L)	4.06	5.45	4.79	4.47	5.60	4.99	4.37	5.35	4.85
pH	7.96	8.23	8.09	7.87	8.37	8.05	7.88	8.32	8.04
Nitrite (mg/L)	0.046	0.221	0.133	0.043	0.191	0.141	0.045	0.248	0.138
Total Ammonia (mg/L)	0.039	0.193	0.119	0.041	0.176	0.119	0.034	0.195	0.115

Latent

Fish ability to adapt depends on the existing conditions [15]. Finfish adaptation to the new environment condition could be measured by its spawning [16]. Brood latent showed finfish adapted while brood latent value does not changed much. Brood latent showed no significant difference ($p>0.05$) among treatment during laboratory works (Table 2). It was also means that laboratory condition was suitable for Banggai Cardinal breeding compared to their habitat on the nature. Cardinal Banggai lives on habitat with salinity range 29-35 ppt [17].

Eggs Production

Brood fecundity is the correlation of energy needs for the fish growth and reproduction [18]. Optimum range of energy within could be used to produce eggs in maximal number [19]. Increasing of brood fecundity value on mature finfish expressed their reproduction activity [20]. Laboratory works showed increasing brood fecundity value was affected on various salinity levels (Table 2). Moreover, statistical analysis showed significant difference ($p \leq 0.05$) among treatment. Highest brood fecundity value was on the treatment 27 ppt of salinity. Higher brood fecundity value represented higher reproduction activity [18].

Table 2. Brood parameter of Banggai Cardinal breed affected by different salinity levels

Parameter	Salinity (ppt)		
	27	30	33
Latent (days)	43.1111^{nd}±7	42.1111^{nd}±7	42.6667^{nd}±7
Brood fecundity (eggs)	42.3333^{b}±7	36.1111^{ab}±1	30.6667^{a}±3

*superscript indicated significant differences among treatment, nd = not significant differences

CONCLUSION

Salinity level at 27 ppt was the best condition for reproduction of Banggai Cardinal. It was giving best respond on brood fecundity during laboratory works on this study.

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The Analysis of Hepatopancreas Histologycal Damage in *Neocallichirus karumba* (Poore and Griffin) Shrimp Caused by Heavy Metal Pb Exposure in Madura Strait

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Abstract

Madura strait known as the second busiest shipping lanes in Indonesia. Human activities on the environment can influence the marine ecosystem derived from the household, fishery processing and capture fisheries. It can pollute coastal environments, one of which is heavy metal exposure such as Cd, Hg, Ca, As and Pb. These metals are greatly impacting the life of sea biota. The objective of this research, therefore, is to examine the hepatopancreas damage rate of shrimp *Neocallichirus karumba* due to the exposure of heavy metal Pb in Madura Strait. Method of research is by analyzing Pb content in water, sediment and shrimp by taking sample from 3 different stations. Hepatopancreas damage is then analyzed with SEM-EDX. Result of research indicates that at Station A, Pb pollution in water is the biggest and counted for 0.25 ppm, while those in sediment and shrimp are 5.85 ppm and 1.24 ppm. At Station B, Pb pollution in water is 0.19 ppm, whereas that in sediment is 5.51 ppm, but that in shrimp is 1.04 ppm. At Station C, Pb pollution in water counts for 0.18 ppm, and in sediment, it stands for 5.5 ppm but 0.02 ppm for shrimp. Result of analysis against hepatopancreas damage is explained as follows. At Station A, vacuolization is 20 % and Pb content in organ is 0.520 ppm. At Station B, the parameters are 10% and 0.196ppm. At Station C, it includes 15% and 0.173ppm. Organ damage is straightforwardly related to Pb content in water and sediment. Shrimp age is quite influential to the percentage of organ damage.

Keywords: Heavy metal Pb pollution, hepatopancreas organ damage, Madura Strait, *N. karumba* shrimp.

INTRODUCTION

Since the beginning of the Industrial Revolution, human influence has been a major force affecting marine ecosystems through processes such as global climate change and pollution [1]. Coastal environment is where land ecosystem and sea ecosystem meet, and it is greatly vulnerable to the change of water quality. Sea ecosystem degradation may be due to industrial and domestic pollutions containing chemical compounds of ionic heavy metals such as Cd, Hg, Ca, As, and Pb. The inhabitants of the coast mostly discharge their domestic waste into the water. Fishing activity always involves gasoline. The boats moored along coast harbors in Madura Strait have its paints usually abraded [2].

Lead is rooted from the word plumbum and symbolized as Pb based on Scientific Language. Lead is classified into Class IV-A Metal in the periodic table of chemical substances. It has atom number (NA) of 82 with atom weight (BA) of 207.2 [3]. Lead or Pb is also considered as poisonous, indestructible, and indecomposable compared to others.

The entry of Pb into shrimp is definitely mediated by water. It may enter into respiration channel, such as gill, or through water absorption by body surface, and possibly through foods, particles or waters dissolved by digestion system. Before toxic substance penetrates into a life creature, it must pass through a membrane before entering the cellular room. Cellular membrane's response to heavy metal presence is often in form of membrane damage or membrane permeability, and also the distorted ATP production, which in turn causing the confusion of ionic transfer system [4]. Water sample collected in 2014 from Madura Strait has Pb pollution rate of 0.26 $mg.L^{-1}$, and the causal factor is that Madura Strait is the second busiest sea transportation lane in Indonesia [2].

Neocallichirus karumba is a shrimp species living in the mud along the coast. This species is a digger and also filter feeder. Therefore, it easily accumulates lead through food chain and respiration channel. Further accumulation can be found in the body and hepatopancreas organ [5]. Research is aimed to examine the hepatopan-

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creas damage rate of shrimp *Neocallichirus karumba* due to the exposure of heavy metal Pb in Madura Strait.

MATERIALS AND METHODS

Study Site

This research was conducted in the District of Bangkalan Madura Labang in September 2014. The area of sampling is densely populated areas where people relied on the catch and fish processing, ship maintenance activities. Results or residual waste processing is done on the waterfront without regard to the impact that inflicted. With an area of 35.23 km coastal area can produced 647 tons of fishery products. Data comprise of water samples, sediment and shrimp *N. karumba* collected from 3 (three) different locations (Fig. 1).

Water Sampling

Water samples were taken at the surface and bottom waters. It is meant for accumulated rate of water in the bottom and at the surface would have been different. Water samples were taken from each point using a 500 ml bottle of mineral water which is then preserved with 1 ml HNO3 with Ph range 1.5 and put in a cool box and analyzed using AAS methods. Value Pb content in water is the average value of the sample surface and bottom.

Sediment Sampling

Sediment samples were obtained from 3-point using a small shovel. Sediment samples then inserted into sample container and analyzed at the Chemical Laboratory of Mathematics and Science, University of Brawijaya to be analyzed of heavy metals Pb.

Sampling Shrimps

The shrimp were taken by manually digging at three points location of shrimp that are in the mud of 20-30 cm and then looking for a major hole that shrimp can be pulled out. Phases in sampling of shrimp can be seen in Figure 2 below.

Shrimp sample is sorted based on capture location, and it is stored in box containing sea water and mud as the living media. It keeps shrimp alive which makes them ready for analysis on the heavy metal content and hepatopancreas damage in Biochemical Laboratory, Central Laboratory of Living Sciences (LSIH), University of Brawijaya.

Analysis of the Pollution Source

Analysis of pollution sources includes documentation and interviews of residents in three stations. The interview contains information and activities work of citizens which have a direct impact to the coastal environment.

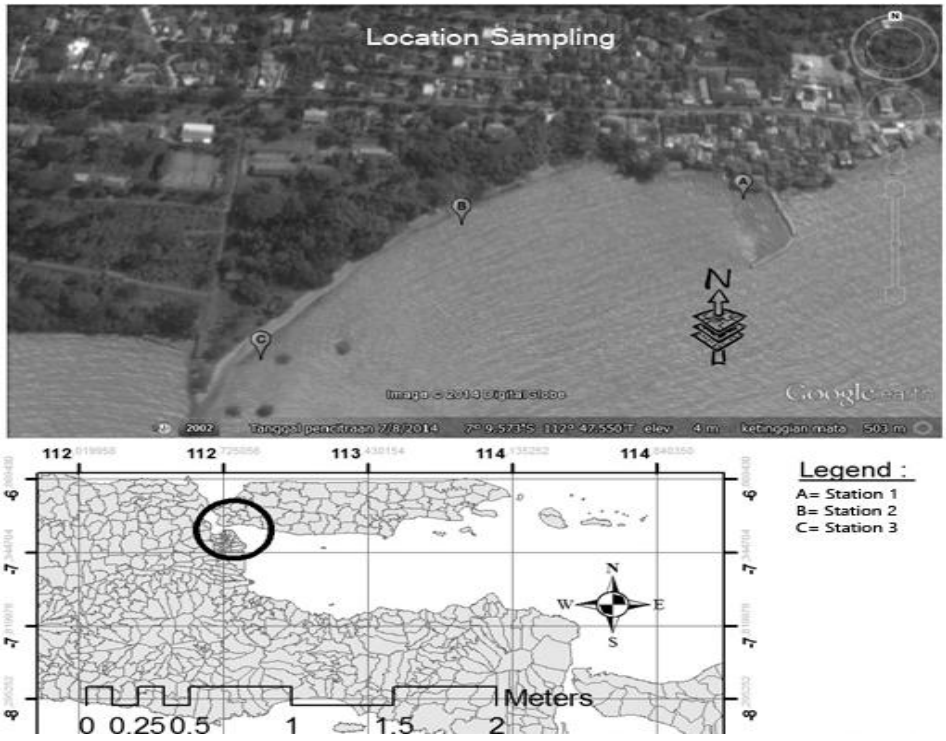

Figure 1. Research Location at the Coast of Madura Strait

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Figure 2. The Process to Collect Shrimp Sample at the Coast of Madura Strait. (a) branched holes of shrimp *N. karumba*, (b) excavating main holes, (c) pulling out the shrimp.

Analysis

The analysis used in this research using descriptive analysis and SEM-EDX. SEM-EDX is a Scanning Electron Spectroscopy Microscopy-Energy Dispersive [6]. It was used to see the shrimp hepatopancreas histological damage of *N. karumba*. Hepatopancreas of *N. Karumba* shrimp then inserted into 96% alcohol and then fixated for 24 hours to get the maximum results [6].

RESULT AND DISCUSSION

The results of measuring the level of Pb pollution in the water, sediment, shrimp and also observation on the hepatopancreas organ can provide information on the impact of heavy metal to damage the environment and organisms that live around the site. Pb contents in Madura Strait can be seen in Table 1.

Table 1. Pb Content in Water, Sediment and Shrimp

Sta	Water (mg.L^{-1})			Sediment (ppm)	Shrimp (ppm)
	Surface	Base	Average		
A	0.17	0.32	0,25	5.85	1.24
B	0.1	0.27	0,19	5.51	1.04
C	0.1	0.254	0,18	5.5	0.02

Pb Content in Water

Household activities, periodical boat maintenance and fish catching holds important role in the location of Pb contents in water. The difference in value of each station can be seen in Figure 1.

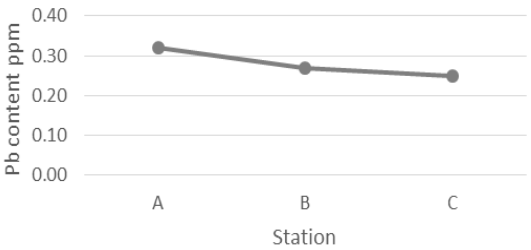

Figure 3. The Pb water content in the Madura Strait

Figure 3 shows difference between each station. Station A has the highest Pb content counted for 0.25 ppm if compared to Station B with 0.19 ppm and C with 0.18 ppm. As reported Pb content in water exceeds 0.005 ppm, and therefore, each station is considered as highly and severely polluted by Pb [7]. Main factors that pollute the location involve domestic activity and fishery capture. The subsistence of the majority is being fisher and processor of the haul, such as fish fumigation and fish marinating. Almost all wastes from domestic, processing and boat maintenance activities are concentrated on the coast. It may trigger the high factor of Pb content at certain location, especially Station A that is highly populated. Abraded boat paints, trash discharge directly by the community, and fishing boat fueled with gasoline and coated with anti-corrosive Pb-loaded paints, are also increas-ing Pb exposure [8].

Pb Content in Sediment

Sediment is a product of deposition derived from land and sea brought by rain and stream. Pb content in sediment is displayed in Figure 4.

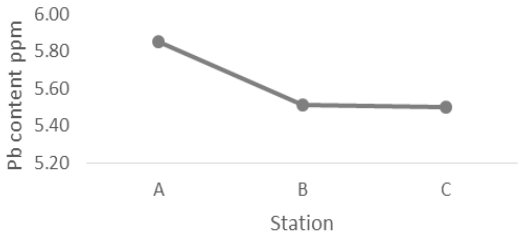

Figure 4. Pb Content in Sediment at Madura Strait

The increased Pb content in water will stimulate the increased Pb content in sediment due to the effect of physical, chemical and biological processes [9]. Stations A, B and C are the location of Total Suspended Solids (TSS) deposition from many locations. Madura Strait is ship lane, but it also accepts factory waste and currently, mud discharge from Lapindo Porong. Kenjeran Coast has been occupied by 60% of

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total population in Surabaya. This occupation contributes significant level of sediment waste that will be finally precipitated and carried over by the stream. The movement of stream into stations has produced small gulf, and it allows small particles to settle. This factor impacts on higher Pb content in sediment than in water and shrimp. The content of heavy metals in sediment increases because heavy metals in water experience dilution due to the effect of tide-ebb pattern which forces them to settle on the base of waters [10,11].

Pb Content in Shrimp

Shrimp *Neocallichirus karumba* is a species that spends almost their live in the mud. This species makes two or more holes in the mud for water circulation [12]. The following is the measured Pb content in shrimp (Fig. 5).

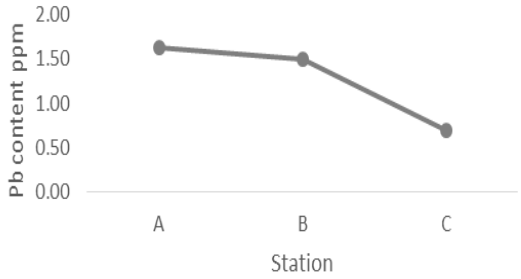

Figure 5. Pb Content in *N. karumba* shrimp at Madura Strait

This shrimp species is *filter feeder* that lives by filtering the water. This organism is *sedentary*, and therefore, hardly avoiding contaminants, and being highly tolerant to certain heavy metals. Therefore, shrimp can accumulate metals higher than other animals [13]. Shrimp *N. karumba* is consumed by immediate community because it is considered to have the ability to recover allergy, itchiness and bladder leakage among children. The impact of consuming organism with accumulated heavy metals is always felt at long-term period [14].

Hepatopancreas Damage

Station A

Result of analysis with SEM-EDX against pictures, spectrums, and table of Pb content in hepatopancreas organ is elaborated in Figure 6. Station A is Jarat Lanjang Village with the highest pollution rate, counted for 1.24 ppm and the category includes in the harmful organisms.

Hepatopancreas histology of shrimp *N. Karumba* is described with 500x magnification. Tubules show the presence of white spots due to 20% vacuolization. This fact is also supported by high Pb content in organ for 0.520 (Fig. 7, Table 2).

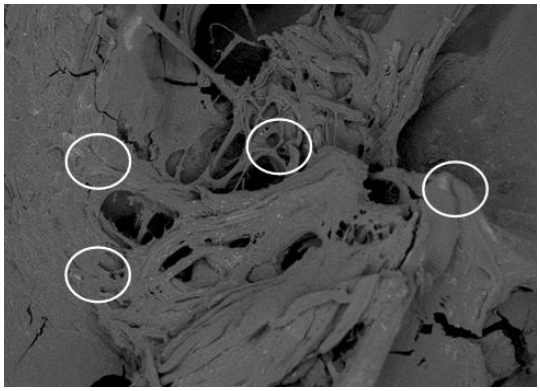

Figure 6. Hepatopancreas Organ of Shrimp *N. karumba* at Station A. **Description**: white circle shows the vacuolization and mineralization

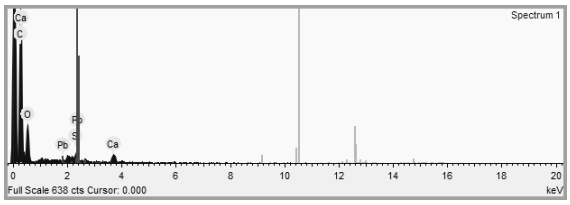

Figure 7. Edx for Chemical Substances in Hepatopancreas

Table 2. Chemical substances in hepatopancreas (%)

Name	C	O	Ca	Pb
Spectrum 1	62.852	33.497	2.093	0.520

Station B

Station B is Jungkar Village. The majority of population works as fishermen. Main pollution source in this station is coming from boat maintenance.

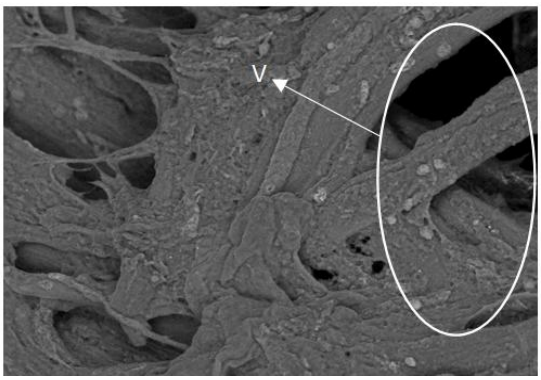

Figure 8. Hepatopancreas Organ of Shrimp *N. karumba* at Station B. **Description: V**= where mineralization is great and causing vacuolization

The description of hepatopancreas histology of shrimp *N. Karumba* has been obtained with 80x magnification. Tubules suffer from 10%

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vacuolization. Pb content is is 0.196 described in the Figure 9 and Table 3.

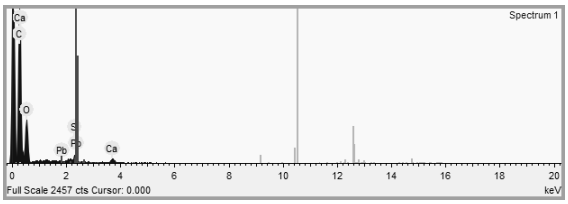

Figure 9. Edx for Chemical Substances in Hepatopancreas

Table 3. Chemical Substances in Hepatopancreas (%)

Name	C	O	Ca	Pb
Spectrum 1	61.171	36.794	1.047	0.196

Station C

Station C is Labinsen (*Laboratorium Induk Senjata*) or Main Weapon Laboratory. The site belongs to Navy base. In this station, fishery or processing activities that pollute environment are not found. Pb content may be still be found but it is a natural factor due to the movement of stream carrying over pollutants and TSS into the station.

The description of hepatopancreas histology of shrimp *N. Karumba* is magnified 80x. Tubules are subjected to 15% vacuolization. Pb content in Station C is 0.173 (Fig. 11, Table 4).

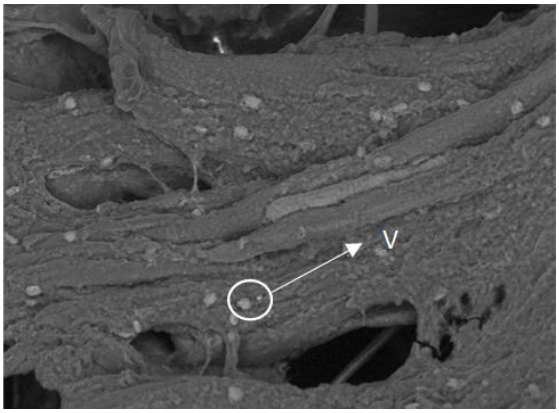

Figure 10. Hepatopancreas of Shrimp *N. karumba* at Station C. **Description: V**= mineralization

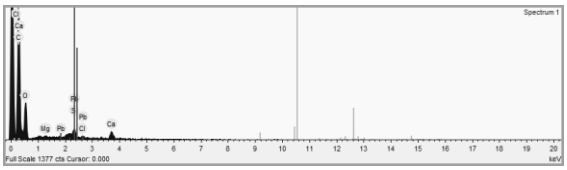

Figure 11. Edx for chemical substances in hepatopancreas

Table 4. Chemical substances in hepatopancreas (%)

Name	C	O	Ca	Pb
Spectrum 1	59.938	35.889	1.921	0.173

The highest damage rate of hepatopancreas organ is found at Station A. The reason is that Pb content in water and sediment at this station is the highest if compared with that in Station B and C. Further evidence is great quantity of white spots (due to mineralization) in different shapes in each station. It triggers what so called vacuolization [15]. Vacuolization is a common disease found in hepatopancreas organ [16]. Moreover, vacuolization, necrosis or cellular death are occurring if lead exposure is left perpetually [17].

Elongated life of shrimp, mollusc, and water animals in the mud may increase the accumulated heavy metals through perpetual food chain [18,19]. Metals in water column can settle into sediment [20]. It then accumulates into the body of the biota. Metals can be heaped into biota through *bioaccumulation* or food chain. The biological metabolism of dangerous metals may influence the growth of water organism. The accumulation of each biota may differ depending its biological characteristic (species, age and physiology) physical and chemical characteristics, and activity in each location.

CONCLUSION

Fishing activities played an important role in the level of contamination at the sites. The polluted waters may increase bioaccumulation of shrimp, which further damages hepatopancreas organ of *N. karumba* shrimp. This damage is straightforwardly related to Pb content in water, sediment and shrimp. It is also proved by the characteristic of shrimp as *filter feeder*. The ecosystem of coast environment shall be maintained because coast community often rely their subsistence on sea commodities. Shrimp *N. karumba* is highly consumed by this community without recognizing the consequence or impact of bioaccumulation.

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The Combination of Entomopathogenic Fungus of *Beauveria bassiana* (Balls) Vuill. with the Insect Growth Regulator (IGR) of Lufenuron Against Reproductive of *Bactrocera carambolae* Fruit Flies (Diptera: Tephritidae)

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Abstract

The study aimed to determine the reproductive ability of fruit flies *B. carambolae* treated with *B. bassiana* and Lufenuron. This study is conducted at the Laboratory of Pest. Department of Plant Pests and Diseases, Faculty of Agriculture, University of Brawijaya, Malang. This study used a completely randomized design with nine treatment and three replications. The study was trying to evaluate the effect of *B. bassiana* and Lufenuron on the reproduction capacity of *B.* carambolae. Results showed that adults of *B. carambolae* to applied combination of *B. bassiana* and Lufenuron immediately after coming out of the pupae until day eighth have the average number of eggs laid is 7.69%, a decrease of fecundity 92.40%, egg fertility by 61.38% and 95.24% decrease of reproduction. Adults of *B. carambolae* applied of *B. bassiana* and Lufenuron on day eighth until day sixteenth (for 8 days), show a decrease in the number of eggs laid by 13.63%, the decrease of fecundity 88.50%, egg fertility by 50.16% and decrease of reproduction by 93.12%.

Keywords: *Bactrocera carambolae, Beauveria bassiana,* Lufenuron.

INTRODUCTION

The fruit fly is a pest so much affecting the horticultural crops. Under condition where the fruit fly populations are high, the intensity of the attack can reach 100% [1]. One type of fruit flies that need attention is *Bactrocera carambolae* (Diptera: Tephritidae). Fruit attacked by B. *carambolae* looks intact from the outside, but the inside of the fruit is actually destroyed as it has been eaten by the larvae of *B. carambolae* [2].

Bactrocera carambolae larvae control using pathogenic microorganisms is more effective because it is environmentally friendly and does not cause resistance on the species. One of pathogenic microorganisms that can be used for larval control of *B. carambolae* is the fungus *Beauveria bassiana* (Bals) Vuill. The pathogenicity of this fungus is not consistent when applied in the field, due to the influence of environmental conditions that do not support especially temperature, humidity, and the intensity of sunlight. The pathogenicity of the fungus *B. bassiana* can be improved by formulation of isolates with the addition of Insect Growth Regulator (IGR).

IGR is a product or material that interferes with or inhibits the life cycle of pests, such that pests cannot reach imago, and unable to reproduce [3]. One of the insecticide active ingredients included in the IGR is Lufenuron. Lufenuron works by inhibiting the synthesis of chitin in the process of ecdysis. In addition to inhibiting the synthesis of chitin, Lufenuron also interferes with the reproductive system of the insects pest target [4]. The combination of *B. bassiana* fungus with the addition of IGR is expected to be a new approach for controlling *B. carambolae* effectively, environmentally friendly. Which in turn does not cause resistance and does not cause the death of natural enemies of both predators and parasitoids as well as to improve the quality of fruits and vegetables [5]. Increased pathogenicity of entomopathogenic fungi by the addition of insecticides can fix isolate and improve the performance of these isolates [6].

According to above previous research, there is a need for research on the combination of entomopathogenic fungus *Beuaveria bassiana* (Balls) Vuill with the Insect Growth Regulator (IGR) of Lufenuron against reproductive of *Bactocera carambolae* fruit flies (Diptera: Teprhitidae). This study aims to determine the

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reproductive ability of fruit flies *B. carambolae* treated with *B. bassiana* and Lufenuron.

MATERIALS AND METHODS

Study Object

Male and female imago were treated in separate cages. Each cage consisted of 10 imago. Imago separation was done because there was a difference in treatment between male and female imago. Male and female imago of *B. carambolae* respectively were treated with *B. bassiana* + Lufenuron with 1.5 $mL.L^{-1}$ concentration. *Beauveria bassiana* was added with Lufenuron for imago of *B. carambolae* for applications. Applications to *B. carambolae* was done by using a saturated sponge and placed on top of the treatment cage. Differences in the treatment of adult males and females were conducted to determine the effect of *B. bassiana* + Lufenuron to the death of *B. carambolae*. Each treatment in the imago is presented in Table 2.

Table 1. Treatment of *B. bassiana* and Lufenuron on the Imago of *B. Carambolae*

Treatment	Application (Day)
♂ BL1 >< ♀ Normal (P_1)	1 - 8
♀ BL1 >< ♂ Normal (P_2)	1 - 8
♂ BL1 >< ♀ BL1 (P_3)	1 - 8
♂ BL8 >< ♀ Normal (P_4)	8 - 16
♀ BL8 >< ♂ Normal (P_5)	8 - 16
♂ BL8 >< ♀ BL8 (P_6)	8 - 16
Control (P_7)	0
Control (P_8)	1 - 8
Control (P_9)	8 - 16

Description :
BL : *Beauveria bassiana* and Lufenuron
BL1 : Application the first day until the eighth day
BL8 : Application eight day until the sixteenth day
♂ : Male Imago
♀ : Female imago

Data Collection and Analysis

Date of the eggs number and formed imago were obtained by direct observation. The calculation was conducted on the eggs number placed by the *B. carambolae* imago and then counts the number of eggs capable of being larva, pupa, up until the imago. Data were analyzed using analysis of variance (ANOVA), if there is a significant difference then continued with Duncan's Multiple Range Test at 5% level.

RESULT AND DISCUSSION

Fecundity of Fruit Flies *B. carambolae*

Fecundity of fruit flies that were applied the combination *B. bassiana* and Lufenuron can be seen in the Table 2. The lowest mean number of eggs was for P_3 (male and female were alike given *B. bassiana* combined with Lufenuron 1.5 $mL.L^{-1}$ in the medium for pupation for effective pupation in suppressing the formation of pupae. The lowest number of eggs was for treatment P_6 (male and female were alike given *B. bassiana* combined with Lufenuron), which was 131.67. All treatments provided real difference to the average number of eggs laid by imago *B. carambolae* on controls. The decline in fecundity of *B. carambolae* imago can be known by subtracting the average number of eggs in the control group with the one in the experiment group divided by the number of eggs in control group and then multiplied by one hundred.

In Table 2, the highest decrease in fecundity of *B. carambolae* imago for those mated on day eighth after the treatment was in P_3 (male and female were alike given *B. bassiana* combined with Lufenuron) which was 92.40%. While the highest fecundity for those mated on day sixteenth after the treatment was in P_6 (male and female were alike given *B. bassiana* combined with Lufenuron) which was 88.50%. This shows that the male and female imago of *B. carambolae* treated with *B. bassiana* and lufenuron was effective in reducing fecundity of fruit fly *B. carambolae*. The decline in fecundity was also due to premature death of imago of B. carambolae given *B. bassiana* and Lufenuron.

Table 2. The Average Fecundity and the Decrease in Fecundity of Fruit Fly *B. carambolae* Treated with *B. bassiana* and Lufenuron on Different Ages

Treatment	Average Number of Eggs (Pcs)	Decrease in Fecundity (%)
♂ BL1 >< ♀ BL1 (P_3)	290.00 abcd	92.40
♂ BL8 >< ♀ Normal (P_4)	213.00 abc	81.40
♀ BL8 >< ♂ Normal (P_5)	154.67 ab	86.50
♂ BL8 >< ♀ BL8 (P_6)	131.67 a	88.50
Control (P_7)	6651.33 i	0.00
Control (P_8)	3813.33 h	0.00
Control (P_9)	1145.33 fg	0.00

Description :
BL : *Beauveria bassiana* and Lufenuron
BL1 : Application the first day until the eighth day
BL8 : Application eight day until the sixteenth day
♂ : Male Imago
♀ : Female imago
Different notation indicates a significant difference ($P<0.05$).

Beauveria bassiana applied to the imago of *Tetranychus urticae* reduces the number of eggs placed by the imago of *T. urticae* up to 98% [10]. Combination of fungus of *B. bassiana* and

Lufenuron 1.5 $mL.L^{-1}$ results in the average number of eggs by 12.42% compared with the untreated imago. This means that the decline in the number of eggs is 87.58% [5]. Application of *B. bassiana* with concentration of spores at 2.0 x 10^7 is able to reduce female fecundity of green leaf hoppers up to 58% [7].

Observation on present studies shows that the infected imago of *B. carambolae* mostly die prematurely. This is because *B. bassiana* enters the insect host's body through the skin, gastrointestinal tract, spiracles, and other openings (Fig. 1). In addition, inoculum of fungi that attach to the body of the insect host can germinate and grow to form a tubular sprouts, then penetrate through the cuticle of the insect body. The penetration is done mechanically or chemically by enzymes or toxins [8].

Figure 1. Imago of *B. caramboale* that Infected with *B. bassiana* and Lufenuron Combination

Fertility of Fruit Flies *B. carambolae*

The results show that there were differences between the mean on fertility of treated and untreated eggs of B*. carambolae* fruit fly. This indicates that *B. bassiana* combined with Lufenuron affected fertility of eggs. Table 3 shows that the lowest percentage of egg hatching on imago of *B. carambolae* mated on day eighth after being treated was in P_3 (female treatment vs male treatment) reaching 55.39% when compared to the imago of *B. carambolae* mated at same age, which was 99.30%. While in the imago of *B. carambolae* mated on day sixteenth after the treatment, the lowest average of egg hatching was in P_6 (male and female treated) which reached 50.16% when compared to the control group of imago of *B. carambolae* which reached 99.27%. Table 3 also shows that the highest decrease in reproduction was in P_3 (male and female equally treated) which reached 95.24%. However, almost all treatments could affect the reproductive decline when compared to the imago of *B. carambolae* in control.

Beauveria bassiana fungus will further produce beauverin toxins making damage to the insect tissue. Within days, the insects will die (Fig. 2). The mycelium of the fungus will come out of the host's body, grow over the host's body, and produce conidium. Insects attacked by *Beauveria bassiana* will die with a hardened body like a mummy and covered by threads of white hyphae. Lufenuron combined with *B. bassiana* will release toxins that cause blood clotting and cessation of blood circulation to the insect that the insect will die [5].

Table 3. The Average Fertility and the Decrease in Reproductive Function of Fruit Fly *B. carambolae* Treated with *B. bassiana* and Lufenuron on Different Ages

Treatment	Average Number of Egg Hatching (Pcs)	Decrease in Reproductive Function (%)
♂ BL1 >< ♀ Normal (P_1)	451.33 ef	85.26
♀ BL1 >< ♂ Normal (P_2)	217.67 cde	92.89
♂ BL1 >< ♀ BL1 (P_3)	145.67 bc	95.24
♂ BL8 >< ♀ Normal (P_4)	98.33 ab	87.07
♀ BL8 >< ♂ Normal (P_5)	67 a	91.19
♂ BL8 >< ♀ BL8 (P_6)	52.33 a	93.12
Control (P_7)	5341.67 i	0
Control (P_8)	3062.33 h	0
Control (P_9)	760.33 g	0

Description :
BL : *Beauveria bassiana* and Lufenuron
BL1 : Application the first day until the eighth day
BL8 : Application eight day until the sixteenth day
♂ : Male Imago
♀ : Female imago
Different notation indicates a significant difference ($P<0.05$).

Figure 2. Imago *B. bassiana* dead Stricken by the Combination of *B. bassiana* and Lufenuron

Beauveria bassiana can produce mycotoxins in the form of beauvericin toxins that cause

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damage to the parts of the egg and the embryo causing disruption in the function of the haemolymph and nucleus of the insects. In addition, *Beauveria bassiana* can also generate secondary bassianolid metabolites like beuverolit, isorolit, and oxalic acid that its mechanism leads to the increase in pH of haemolymph, clumping of haemolymph, and cessation in the circulation of haemocytes as well as tissue or mechanic organ damage such as the gastrointestinal tract, muscles, nervous system, respiratory system and these disorders cause death [9].

CONCLUSION

Combination treatment of *B. bassiana* and Lufenuron influence the fecundity and fertility of *B. carambolae* imago, i.e. 92.40% decline in fecundity and fertility decline 93.12%. The combination of fungus *B. bassiana* and IGR Lufenuron that applied on fruit fly imago *B. carambolae* can inhibit the reproduction of fruit flies experiments in the laboratory. However, it need further research on the field application thus it can be used for pest control in the agriculture practices.

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Water and Chlorophyll Content and Leaf Anatomy of Patchouli Planlet (*Pogostemon cablin* Benth.) Resulted by Shoot-tip Culture Experience Hyperhydricity after Treatment of Modification Ammonium nitrate or Macro salt Concentration on MS medium (Murashige Skoog)

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Abstract

Hyperhydricity is a symptom of abnormal morphological and physiological function which inhibits the regeneration of plantlets. In general, the main symptom of hyperhydricity is a change in the condition of the plantlets which looks clear (Glassy) as a result of low levels of chlorophyll, the high water content in the plantlets, and the abnormal anatomical structure of the leaves. Hyperhydricity can be controlled by reducing cytokinin concentration, increasing gelling agent concentration, and reducing ammonium nitrate and macro salt concentration on medium. Objective of this research was to reduce hyperhydricity in shoot tip culture of patchouli by modification of ammonium nitrate and macro salt concentrtion on MS medium. The various treatment concentrations of ammonium nitrate were 0 mg.L^{-1} (0), 41.25 mg.L^{-1} (¼ concentration), 825 mg.L^{-1} (½ concentration), 1650 mg.L^{-1} (1 concentration) and macro salt MS with 0, ¼ MS, ½ MS, MS with 5 replications. Hyperhydricity on patchouli shoots could be lowered, as indicated by the decrease in water content from 96% to 90-91%, the increase in total chlorophyll content, and the increased number of palisade cells and stomata on the leaf treatment outcome. The concentration treatment of ammonium nitrate showed better results than the concentration of macros salt in increasing the total chlorophyll content, but it did not differ significantly in lowering water levels and increasing the number of palisade cells and stomata. ¼x concentration treatment of ammonium nitrate could increase chlorophyll content of 0.16 to 0.97 mg.g^{-1}, but MS with 1x concentration showed the best result in the increase of number of palisade cells and stomata of the leaves.

Keywords: Ammonium nitrate, Hyperhydricity, Macro Salt, Shoot-tip culture.

INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.) is one of the essential oil producing plants which gives the profit of foreign exchange for more than 50% of the total exports of Indonesian essential oil. The area of planting patchouli in Indonesia declined, in 2009 the total area of 24.536 ha turned to 23.635 ha in 2012. The decrease did not only occur in area of patchouli plant but also on the productivity of patchouli which in 2009, 113.27 kg.ha^{-1} and in 2012, 87.20 kg.ha^{-1} [1]. Low productivity and oil quality were caused by the low quality of plant genetic due to the uncertain quality of seedlings and the development of various diseases [2].

Shoot-tip culture can be used to produce plants having virus-free, genetically homogenous and higher reproduction rate [3]. Plant propagation through shoot-tip culture is able to increase the rate of induction and multiplication of shoots, to improve the quality of produced seeds, however the content of nutrients and liquid medium can improve the hyperhydricity. Hyperhydricity on shoots culture results in leading to the decrease ability of plantlets regeneration [4].

Hyperhydricity is a condition of abnormal morphology and physiology which causes excessive hydration, low lignification, weakening of the stomata function, and lowering the mechanical strength of cultured plant tissue, leading to low regeneration of plantlets. Hyperhydricity symptoms are changes in plantlets conditions which becomes clear as a result of low levels of chlorophyll and high water content in plantlets. Shoots from culture experiencing hyperhydricity have thin cuticle layer, a reduced number of palisade cells, irregular stomata, impaired development of the cell wall, and large intercellular space on the mesophyll cells layer [4]. Several factors can cause hyperhydricity on shoots culture results, such as high salt concentration in the medium,

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high concentration of ammonium in the medium, low concentrations of *gelling agent*, the concentration of microelement and hormonal imbalance, relatively high humidity, low light intensities, the accumulation of gas in the culture bottle, as well as the type of explant used [5].

Salinity on MS medium leads to the top of Salix babylonica experience hyperhydricity and died while transferred to the medium with equal salinity. The decreased levels of NH_4^+ in the media are associated with the increased hyperhydricity and the decreased lignification on *chestnut* and *willow* plants [6]. Hyperhydricity on *chestnuts* that occures in MS medium can be prevented by using ammonium nitrate macro-nutrient in ½ concentration [7]. The decreasing of ammonium nitrate concentration in the WP media of culture *Amelanchier arborea* increased the total number and percentage of shoots [8].

Shoot tip culture of patchouli usually has good multiplication of shoot, in the other hand most of shoot experience hyperhydricity which causes the decrease ability of plantlets regeneration. Since, hyperhydricity can be conducted by the high level of ammonium nitrate and macro salt in medium. Several studies have been done to reduce the hyperhydricity occurrence, by lowering the concentration of ammonium nitrate [7] and lowering the salt concentration in the media to ⅓ concentration of MS [6]. Thus, modification on MS media need to be conducted with expectation patchouli planlet results in has a good regenerations ability.

MATERIALS AND METHODS

Shoot tip culture

Shoot tip used was taken from shoots of cultured patchouli leaves on solid MS medium (Murashige and Skoog) containing 0.1 $mg.L^{-1}$ NAA and 0.3 $mg.L^{-1}$ BAP. *Shoot tip* on cultured patchouli shoots were planted in liquid MS medium and incubated in bright conditions at a temperature of 25 °C for 6 weeks. Hyperhydricity shoots from *shoot-tip* culture results which had the same height and size were cultured on solid MS medium with a concentration of ammonium and macro salt of 0, ¼, ½, and 1x concentrations. The cultures were incubated in bright conditions at a temperature of 25°C. 8 weeks-cultured patchouli shoots were evaluated concerning with its Hyperhydricity by measuring the level of water content, chlorophyll content, and the number of palisade cells and stomata of the leaves.

Water and Chlorophyll Content of Planlet

The water content was calculated based on the difference between wet weight and dry weight of shoots. Chlorophyll content was measured by 0.2 grams leaves which were homogenized and extracted with acetone then its absorbance was measured using a spectrophotometer at a wavelength of 647 and 665 nm. Chlorophyll content was analyzed by Coombs and Hall method [9].

Palisade and Stomata Count

Making the section for counting palisade cells was done by making the crosswise slices of leaves using microtome. Making the section for the observation of the stomata number was done by *clearing*. Leaves were fixed using 70% ethanol for 24 hours, then clearing was performed by soaking the leaves in a solution of 5% NaOH until leaves became clear and visible. Counting the number of palisade cells and stomata of the leaves was done under a microscope with a magnification of 400x.

This research was conducted in the experimental design used a randomized block design. Each treatment was repeated 5 times (bottle) and on each bottle were 4 shoots explants cultured. The obtained data were tested for normality data then performed statistical analysis using SPSS software for Windows 16 and if there was a difference, it would be followed by *Tukey* test at 5% significance level.

RESULTS

Water and Chlorophyll Content of Planlet

Patchouli shoots experiencing hyperhydricity have a structure that looks clear (glassy). Clear green color in leaves that experienced hyperhydricity was caused by the deficiency of chlorophyll and the high water content. The water content in plants can be a marker of a hyperhydricity condition. High level of water is a hyperhydricity trait in plants. The water content of patchouli shoots experiencing hyperhydricity by 96%, while the water content in normal shoots by 85%. Cultured patchouli shoots in media through treatment with ammonium nitrate and macro salt had a tendency of decreased water content. The water content of shoots on media containing with ammonium and macro salts with various concentrations ranged between 90-91%. This suggests that treatment with various concentrations of ammonium and macro salt media (Fig. 1a).

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Shoots experiencing hyperhydricity typically have low levels of chlorophyll. The chlorophyll content of patchouli leaf shoots in hyperhydricity was 0.16 mg.g^{-1} while in normal patchouli leaf was 1.5 mg.g^{-1}. Total of chlorophyll content of patchouli leaves shoots from cultures on MS medium with ammonium nitrate concentration treatment showed an increase compared to those on leaf experiencing hyperhydricity, meanwhile the treatment ¼ and ½ of macro salt concentration showed the results of total chlorophyll content tends to not differ between treatments. The concentration treatment of ammonium nitrate on MS medium produced patchouli leaf shoots with higher chlorophyll content than all of macro salt concentration in the media. The highest chlorophyll content of patchouli shoots leaves was demonstrated by culture results on the concentration treatment of ammonium nitrate into ¼x which was 0.97 mg.g^{-1} (Fig.1b).

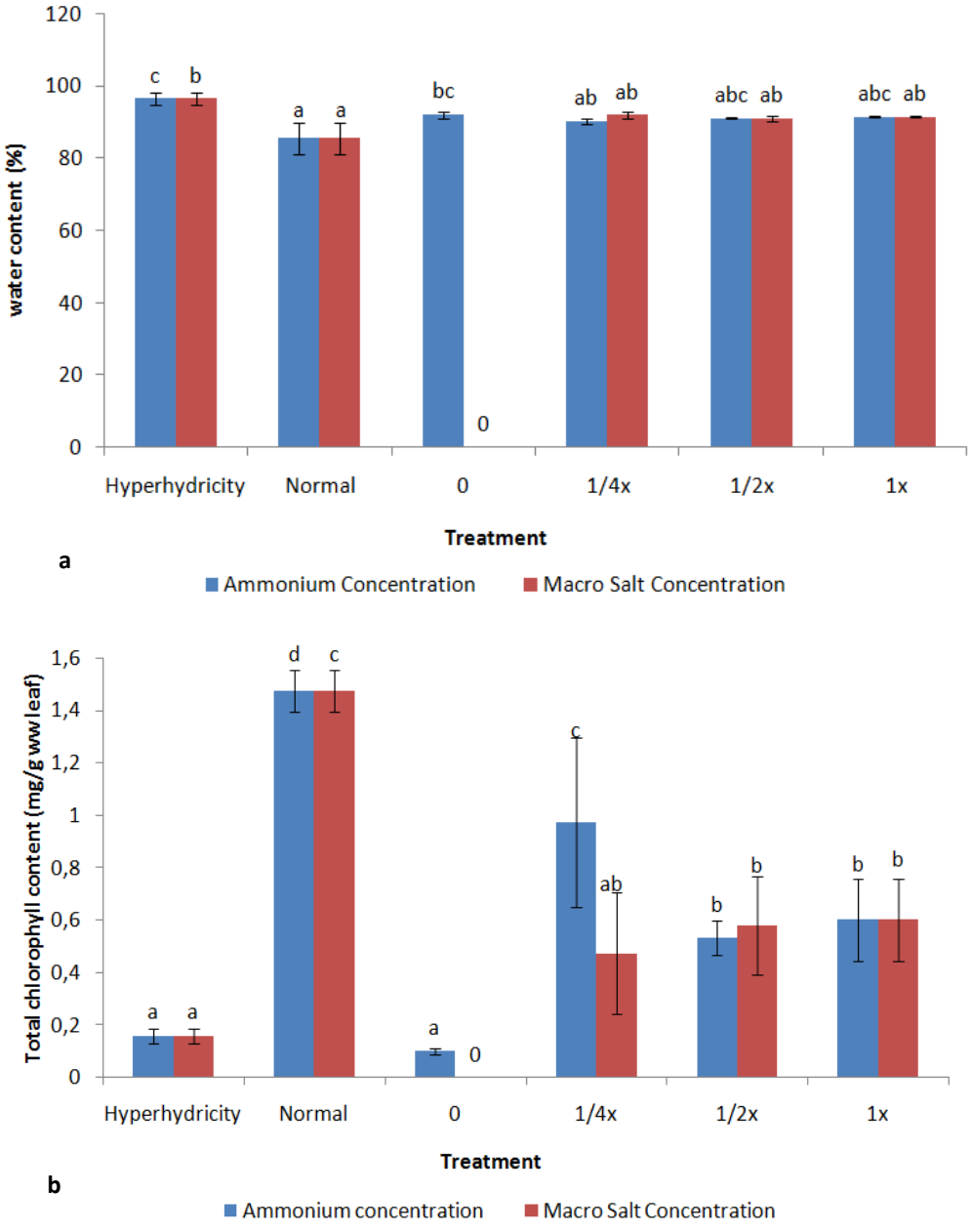

Figure 1. Comparison of water content and chlorophyll content in patchouli shoots experiencing hyperhydricity, normal, and after treatment with the concentration of ammonium nitrate and salt macro on MS medium.
a. water content, **b.** total chlorophyll content.
The same letter in the same treatment showed no difference in treatment results in further test of Tukey.

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Palisade and Stomata of Planlet's Leaf

Patchouli leaves experiencing hyperhydricity had palisade cells which was larger than the palisade cells in normal patchouli leaves. Patchouli leaves experiencing hyperhydricity had a wide space among cells. The composition of palisade cells in the Patchouli leaves experiencing hyperhydricity was more tenuous and not uniform in size. They had less palisade cell than normal leaves (Fig. 2a). Normal plantlets patchouli leaves seemed to have palisade cells which were well-ordered, dense, homogenous, and had narrow space among cells (Fig. 2b). The number of stomata on the leaves experiencing hyperhydricity was less than in normal leaves and after treatment. Stomata on the patchouli leaves experiencing hyperhydricity appeared to have abnormal stomata and guard cells changing shape. Stomata experienced malformations with guard cells which became more elongated than the guard cells in normal patchouli leaves (Fig. 2e). Normal patchouli leaves had rounded stomata and guard cells (Fig. 2f).

Patchouli shoots leaf on the concentration treatment of ¼ ammonium and ½ macro salt concentrations showed characteristics similar to normal patchouli leaves. Both had a palisade cell structure which began to be well-ordered, dense, and homogenous. Both treatments still indicated a space among cells that was wider than normal patchouli leaves (Fig. 2c and 2d). Patchouli leaves coming from the results of the treatment with MS medium containing with ¼ ammonium concentrations and ½ macro salt concentrations appeared to have shape of stomata and guard cells which were similar to normal stomata shape (Fig. 2g and 2h).

Treatment variations in the concentration of ammonium nitrate showed the better results in increasing the number of palisade cells of leaves than treatment variations in the concentration of salt macro, but the treatment 1x concentrations of ammonium nitrate and macro salt demonstrated superior results in increasing the number of cells palisade from 12 to 26 (Fig. 3a) compared to treatment with concentrations of 0, ¼ and ½x. The number of stomata of the cultured patchouli leaves shoot on MS medium with the treatment variations of ammonium nitrate and macro salt indicated that there were an increasing number of stomata compared to the leaves undergoing hyperhydricity, but the treatment 1x concentrations of ammonium nitrate and macro salt showed the highest results of the number of stomata by 64 (Fig. 3b).

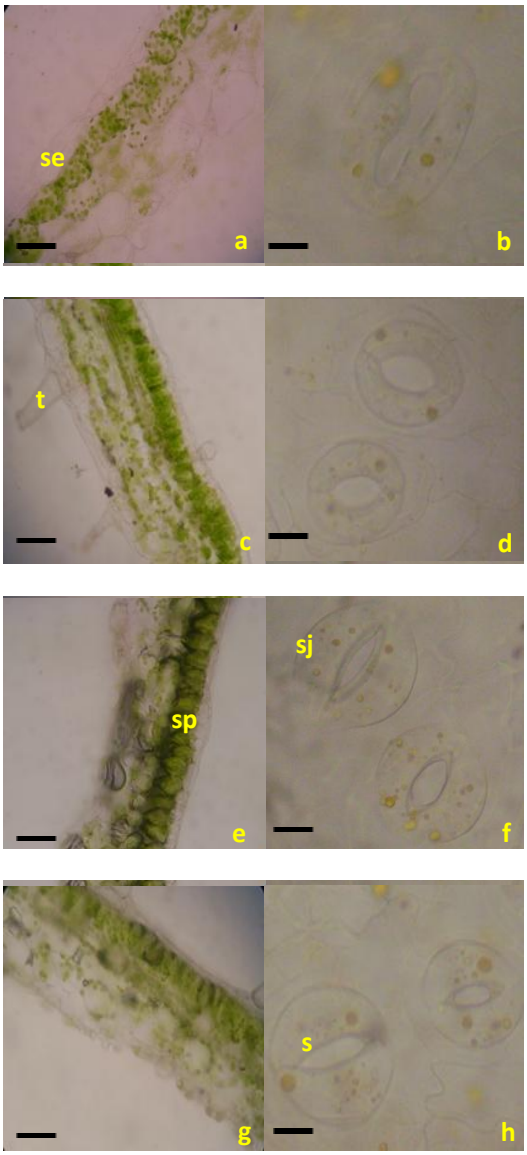

Figure 2. Structure of the leaves anatomy with and without hyperhydricity on patchouli shoots after treatment.
a,b. the cross section and stomata of the leaves in hyperhydricity;
c,d. the cross section and the stomata of the normal leaves;
e,f. cross-section and the stomata of the leaves as the results of the treatment on MS medium with ¼ ammonium concentration;
g,h. cross-section and stoma-ta of leaves as the results of treatment on MS medium with macro salt concentration.
se: epidermis; **t:** trichomes; **sp**: palisade cells; **sj**: guard cells; **s**: stomata. Bar: 15 µm.

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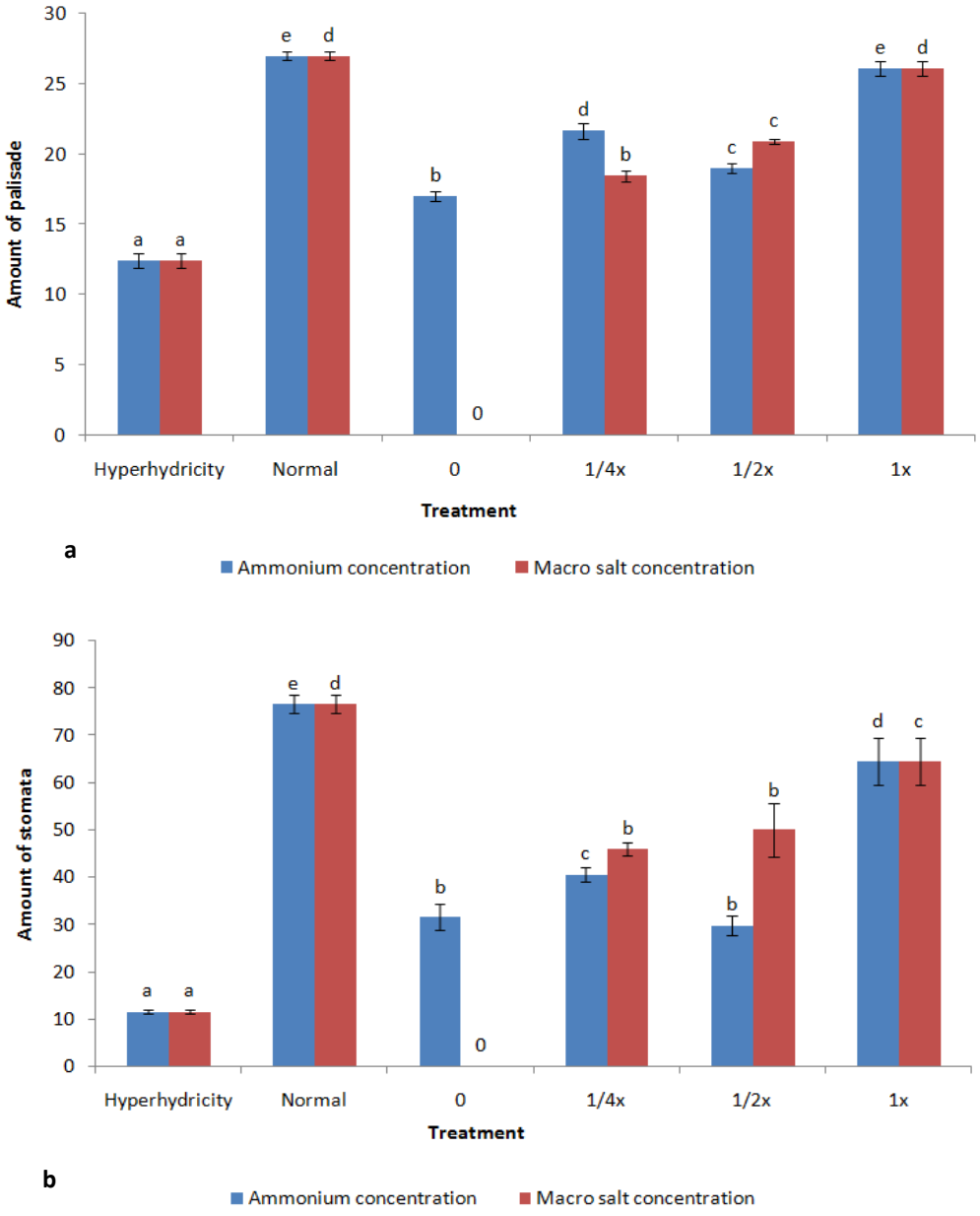

Figure 3. Comparison of the number of palisade cells and stomata on patchouli shoots leaves in the Hyperhydricity, normal, and after treatment with the concentration of ammonium and macro salt on MS medium.
a. the number of palisade cells, **b.** the number of stomata.
The same letter in the same treatment showed no difference in treatment results in further test of Tukey.

DISCUSSION

Water and Chlorophyll Content of Planlet

High water levels in liquid media caused the plant suffering hyperhydricity. The results showed that the plant suffered hyperhydricity had a high water content and it was allegedly also associated with low levels of chlorophyll in the leaves. Accumulation of water content caused the formation of intercellular space that was wider at the mesophyll layer so that the number of palisade cell became less than normal leaves. The small number of palisade cells is assumed to cause the low chlorophyll content, because most of the chloroplasts in the leaves lied in the palisade cells in the mesophyll layer [10].

The chlorophyll content in the patchouli shoots undergoing hyperhydricity was lower than

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in the normal leaves shoots and after treatment. The low levels of chlorophyll was allegedly due to a lack of chloroplasts in leaf shoots experiencing hyperhydricity. The chlorophyll content in the leaves undergoing hyperhydricity was significantly lower than in normal leaves. A previous study reported that hyperhydricity caused a decrease in the number of chloroplasts and the ultrastructural analysis showed the damage to the thylakoid membranes caused by hyperhydricity [5]. Low levels of chlorophyll in the leaves undergoing hyperhydricity were allegedly caused by the lack of appropriate concentrations of nutrients in the media. Levels of chlorophyll in plants were influenced by several factors, such as light, sugar or carbohydrates, water, temperature, genetic factors and nitrogen elements, magnesium, iron, manganese, Cu, Zn, sulfur, and oxygen. It could be used as the basis that the right macro salts comparison can keep levels of chlorophyll in a plant remained normal [11]. The findings of this study indicated that the subcultured patchouli shoots on MS medium with ¼x ammonium had higher levels of chlorophyll than the other treatments. The findings of this study were similar to previous research which reported that the reduction in ammonium concentrations in WPM media of apricot culture produced shoots that had a high chlorophyll content and free hyperhydricity [8].

Palisade and Stomata of Planlet's leaf

The results of this study indicated that the number of palisade cells in the leaves experiencing hyperhydricity was less than in the normal leaves. The small number of palisade cells in the leaves experiencing hyperhydricity was related to changes in the structure of palisade cell which was larger than in normal leaves and the high water content in the leaves experiencing hyperhydricity. The larger size of a cell, the lower number per unit area. The high water content in leaves experiencing hyperhydricity resulted in the accumulation of water on the leaves, causing the wider space among cells.

Changes in the structure of the palisade cells in leaves experiencing hyperhydricity on this study were believed to be related to the mechanism of cell wall formation [12]. The formation of a cell wall in hyperhydricity cell was impaired. Cells undergoing hyperhydricity looked like protoplasts having very thin cell walls. The size became larger and abnormal form of palisade cells in the leaves undergoing hyperhydricity confirmed a relation between hyperhydricity and the disruption of cell wall formation. The disruption of cell wall formation was allegedly due to lack of lignin synthesis [13]. Lignin is synthesized in the cell walls, so the lack of lignin synthesis results in cell walls undergoing hypolignification. Lignin synthesis is affected by a number of enzymatic activity. A decrease of the enzyme performance in the metabolism of phenols, including the reduction of phenylalanine ammonia lyase and increased activity of glutamate dehydrogenase resulted in hypolignification. The increased glutamate dehydrogenase activity was influenced by the concentration of ammonium nitrate in the media [13].

Hyperhydricity was aassumed to be associated with abnormal stomata structure and the declining number of stomata. Abnormal stomata structure which was visible from the modified shape of neighboring cells and the small number of stomata could be assumed to cause regulation of water on the leaves became inefficient, especially in the transpiration process which resulted in the accumulation of water [14]. Accumulation of water on the lacunae leaves caused the hyperhydricity. Accumulation of water in plants that undergo this hyperhydricity was assumed to affect the water content in plants. This was proved by higher levels of water in the plantlets which were experiencing hyperhydricity than in the normal plantlets and after treatment [14].

The amount and structure of palisade cell from treatment results with ammonium and macro salt in MS media seemed to have improved in the structure and the number of palisade cells was similar to the normal leaf, but the treatment with 1x concentration of ammonium and macro salt showed the best results compared to the other treatments. Similar results were also shown in the number of stomata parameters. These results indicated that treatment variations and concentrations of ammonium and macro salt did not have significant effect on the increase of the number of palisade cells and stomata of the leaves. The increasing number of palisade cells and stomata of the leaves was allegedly associated with removing shoots from liquid medium to solid medium. Solid media was assumed to affect the decrease of hyperhydricity in several studies. The seaweed on the media could bind the water to the media so that water absorbed by the explant was not too much. The seaweed was also assumed to modify the availability of dissolved

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nutrients in the media through a chemical reaction so that the nutrients absorbed by explant not excessive [15].

CONCLUSION

Subculture of patchouli shoots undergoing hyperhydricity on MS medium could only lower the hyperhydricity of shoots which was indicated by the decrease of water levels, increase of chlorophyll levels, and the number of palisade cells and stomata. The decrease of hyperhydricity was affected by the concentration of ammonium nitrate and macro salt on MS medium. The best decrease hyperhydricity of patchouli shoots was on MS medium with 1x concentrations of ammonium nitrate and macro salt. The highest increase of total chlorophyll content in the media was at the concentration of ¼x ammonium.

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Zoonotic Potential of Rotavirus from Swine and Bovine in South of Taiwan

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Abstract

Rotavirus was recognized as the virus that responsible for causing acute gastroenteritis, especially young livestock. Taiwan Center for Disease Control (CDC) confirms the majority cases of acute gastroenteritis in Taiwan on February 2015 were caused by rotavirus. In this study, we report the incidence and zoonotic impact of rotavirus strain from Taiwan. This study examined 90 (swine) and 60 (bovine) fecal samples collected from south of Taiwan in March 2015. Detection of rotavirus using VP6 gene by RT-PCR technique with amplicons 379 bp. Zoonotic potential analysis based on nucleotide sequence and phylogenetic analysis. RT-PCR utilizing the primers specific for VP6 gene detected rotavirus with positive reactions 3/30 (10%) in piglets and 1/20 (5%) in the calf. Based on the nucleotide sequences and phylogenetic analysis indicated that 1 of 3 wild strains from swine rotavirus had 85.0% - 91.1% and 1 wild strain from bovine had 78.7% - 85.9% identity relations with human strains. These findings indicated that the wild strains of swine and bovine rotavirus may broadly spread and contribute to zoonotic transmission.

Keywords: Bovine, Rotavirus, RT-PCR, Swine, Zoonotic.

INTRODUCTION

Rotavirus is enteric pathogen causing acute watery diarrhea in young man and various animal species. About two million hospitalizations and 453.000 deaths in young children below 5 years of age every year [1,2]. Rotavirus is belongs to family reoviridae, icosahedral in structure, 60-80 nm in diameter, non enveloped, possess a one, two or three layered capsid, and containing a genome of 11 segements of double stranded RNA (dsRNA) encoding six structural (VP1, VP2, VP3, VP4, VP6, and VP7) and five or six non-structural (NSP 1-6) proteins [3]. This virus can be transmitted by consuming contaminated food and direct contact with an infected individual or contaminated objects. The symptoms include diarrhea, nausea, vomiting, some stomach cramping, and sometimes people have a low grade fever, headache, muscle aches, and tiredness [4].

There are eight species of rotavirus, referred to as rotavirus A (the majority isolates that infect in mammalian and avian, including human), rotavirus B (identified in human and rat), rotavirus C (human and porcine), rotavirus D, F, and G (identified in chicken), rotavirus E (porcine), rotavirus H (human) [5], rotavirus H was tentatively assigned to a novel rotavirus species [6]. Group A rotavirus is recognized as the most important group that are causes highest prevalence and pathogenesis in human and animals including cattle, swine, horses, dogs, cats, chickens and turkeys [7,8]. Asymptomatic rotavirus infections were also known to occur in pigs in all ages [9]. VP6 is one kind of major structural protein intermediate capsid layer of rotavirus virion, VP6 plays a role as a virulence factor of the virus pathogenesis. Characterization of VP6 gene is encoded 379 amino acid sequences [10].

This study aimed to detect rotavirus from swine and bovine using molecular techniques by reverse transcriptase-polymerase chain reaction (RT-PCR) and then analyze potential zoonotic of the virus. Positive results subsequently cloned using pGM-T vector for ligation, DH5α for transformation, and sent for sequencing, the sequences data were compared with the other strains from different country or species to National Center for Biotechnology Information (NCBI) and followed by phylogenetic analysis using Molecular Evolutionary Genetics Analysis Software (MEGA) version 6.0 and analyzed the homologous identity with multiple sequence alignments using DNASTAR software [11]. The samples were randomly collected from different pigs and cattle farms in South of Taiwan. The data were obtained on March 2015 and it can be used as a reference for control and prevention of the disease that caused by rotavirus in veterinary epidemiology.

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MATERIAL AND METHODS

Specimen Collection

A total of 90 fecal specimens of swine feses and 60 fecal specimens of bovine feses were used in this study. All specimens were collected by random method from different farms in south of Taiwan on March 2015. A number of 90 samples from 3 pig farms and 60 samples from 2 cattle farms were collected. Specimens were collected from 3 groups of swine (sow, fattening, and piglet) and 2 groups of cattle (cow and calf). The stool samples were frozen and stored at -20^{o}C for next processing.

RNA Extraction

Viral RNA was extracted from 1 ml PBS suspensions of stool specimens with Favorgen RNA extraction kit, according to manufacturer's instructions. RNA was eluted with 70 µl of RNase free water and stored at -80^{o}C until use in RT-PCR assays.

Primers and RT-PCR

RT-PCR was performed with My TaqTM One-Step RT-PCR Kit that formulated for first strand cDNA synthesis and subsequent PCR in a single tube. The components of the RT-PCR kit containing 2x My Taq One-Step Mix, forward primer, reverse primer, reverse transcriptase, RiboSaf RNase Inhibitor, and DEPC-H2O.

The primer pairs used in this study are shown in Table 1. 'Primer no. 1' included VP6-F and VP6-R for specific amplification of VP6 genes in swine and bovine rotavirus [12]. 'Primer no. 2' included JRG7 and JRG8 for specific amplification of full length VP6 genes [13].

Table 1. RT-PCR primers for detection of rotavirus and norovirus from swine and bovine fecal samples

Primer	Sequence (5'-3')	Size (bp)
1. VP6-F	5'-GAC GGV GCR ACT ACA TGG T-3'	379
VP6-R	5'-GTC CAA TTC ATN CCT GGT GG-3	379
2. JRG 7	5'-GGC TTT AAA ACG AAG TCT TC-3'	1356
JRG 8	5'-GGT CAC ATC CTC TCA CTA CAT-3'	1356

Condition of RT-PCR reactions for detection VP6 genes were reverse transcription at 42^{o}C or 1 h, after an initial denaturation at 95^{o}C for 5 min, 35 amplification cycles were performed with denaturation at 94^{o}C for 30 s, annealing at 55^{o}Cfor 30 s, and extension reaction at 72^{o}C for 45 s, followed by a final extension at 72^{o}C for 3 min [12]. The amplification products were analyzed by 1.5% agarose gel electrophoresis and visualized by UV light after ethidium bromide staining.

Phylogenetic Analysis

Nucleotide sequences of the VP6 genes were compared with other strains using BLAST search of the National Center for Biotechnology information (NCBI). Phylogenetic analysis based on the nucleotide alignments was constructed using the neighbor-joining method of Molecular Evolutionary Genetics Analysis (MEGA version 6.0) with a pair-wise distance comparison.

RESULT AND DISCUSSION

Distribution of Rotavirus in Swine and Bovine in Taiwan by Age

Distribution of rotavirus positive in group of swine by age was given in Table 2. The largest proportion of swine rotavirus was noted in piglet 3/30 (10%), fattening 0/30 (0%), and sow 0/30 (0%). 1 among 20 samples (5%) were positive in calf and 0% in cow. Based on the positive results, the incidence of positive rotavirus was highest among piglets and calf. Probably because pigs did not receive protective levels of maternal antibody, high levels of passive antibody may temporary protect pigs [17].

Table 2.prevalence of rotavirus from swine and bovine on March 2015

Species	Positive Results	Total
Swine		
- Piglet	3/30	10%
- Fattening	0/30	0%
- Sow	0/30	0%
Bovine		
- Calf	1/20	5%
- Cow	0/60	0%

Sequence and Phylogenetic Analysis

This study has highlighted the significance of incidence and transmission of swine and bovine rotavirus in Taiwan with human strain. Circulation of animal rotavirus strains confirmed potentially zoonotic [18,19].

Transmission to human is possible not only through direct contact with animals, but also indirectly by contact with contaminated surfaces, food, and water. The risk of zoonotic transmission of rotavirus is higher in rural areas with farms under intensive or extensive management. A part of VP6 gene (1,356 nucleotides in length) was able to be amplified in 2 isolated strains. Nucleotides sequences were compared with other strains in GenBank.

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Taiwan wild strain from swine showing 85.0% - 91.1% identity relations with human strains from China (91.1%), Japan (86.0%), Vietnam (85.0%), Japan (85.5%), and Thailand (85.3%), showed on Figure 1. The wild strain of VP6 gene from bovine showing 78.7% - 85.9% identity relations with human strains from China and 78.5% from South Korea, showed on Figure 2.

Phylogenetic tree and homologous identity comparison analysis showed bovine rotavirus had lower transmission risk than swine (Fig. 3 and 4). Some study mentioned that bovine had low incidence and transmission risk for rotavirus [20].

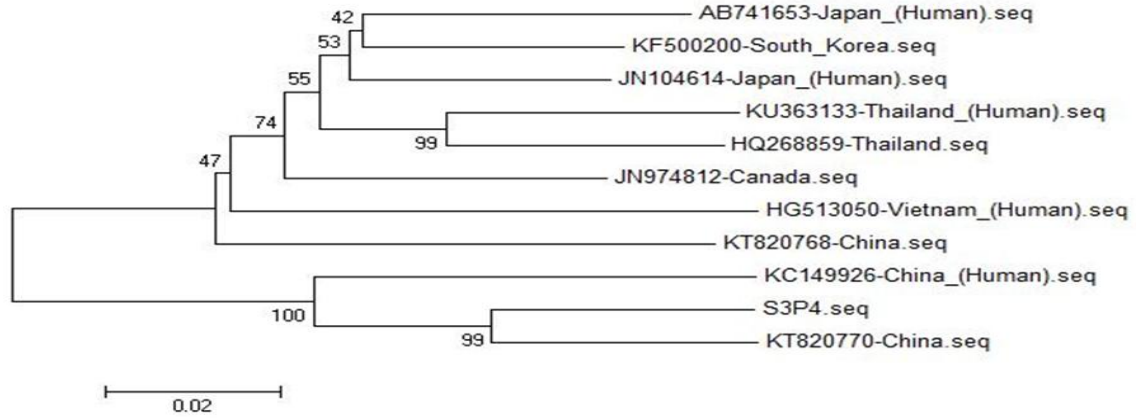

Figure 1. Phylogenetic tree of swine rotavirus based on the VP6 gene (1356 bp) analyzed by MEGA version 6.0 to show the distance of identity relations. Wild strains from Taiwan: south of Taiwan (S3P4). GenBank accession number: KC149926, AB741653, HG513050, JN104614, KU363133, HQ268859, JN974812, KF500200, KT820768, KT820770.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11		
1		91.1	86.0	85.0	85.5	85.3	85.2	86.3	86.3	86.0	94.3	**1**	S3P4.seq
2	9.6		85.3	84.9	85.8	86.6	85.6	86.4	86.5	84.7	90.9	**2**	KC149926-China_(Human).seq
3	15.0	16.1		90.6	93.6	91.5	91.9	92.1	93.5	90.4	85.9	**3**	AB741653-Japan_(Human).seq
4	16.3	16.5	10.3		91.0	89.2	89.2	90.6	89.5	89.5	84.9	**4**	HG513050-Vietnam_(Human).seq
5	15.7	15.4	6.8	9.8		91.9	92.4	93.3	94.1	91.4	85.2	**5**	JN104614-Japan_(Human).seq
6	16.3	14.7	8.9	11.6	8.4		94.0	92.0	92.9	89.3	85.5	**6**	KU363133-Thailand_(Human).seq
7	16.4	16.0	8.4	11.6	7.8	6.4		92.0	92.6	90.2	85.7	**7**	HQ268859-Thailand.seq
8	15.0	15.0	8.2	9.9	6.8	8.6	8.7		93.0	90.5	86.3	**8**	JN974812-Canada.seq
9	15.0	14.9	6.6	11.2	5.9	7.6	7.9	7.5		89.8	86.2	**9**	KF500200-South_Korea.seq
10	15.1	16.9	10.6	11.6	9.4	11.5	10.4	10.1	10.9		85.8	**10**	KT820768-China.seq
11	5.9	9.9	15.2	16.5	16.1	16.1	15.8	15.0	15.2	15.4		**11**	KT820770-China.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**		

Divergence

Figure 2. Homologous identity comparison of swine rotavirus

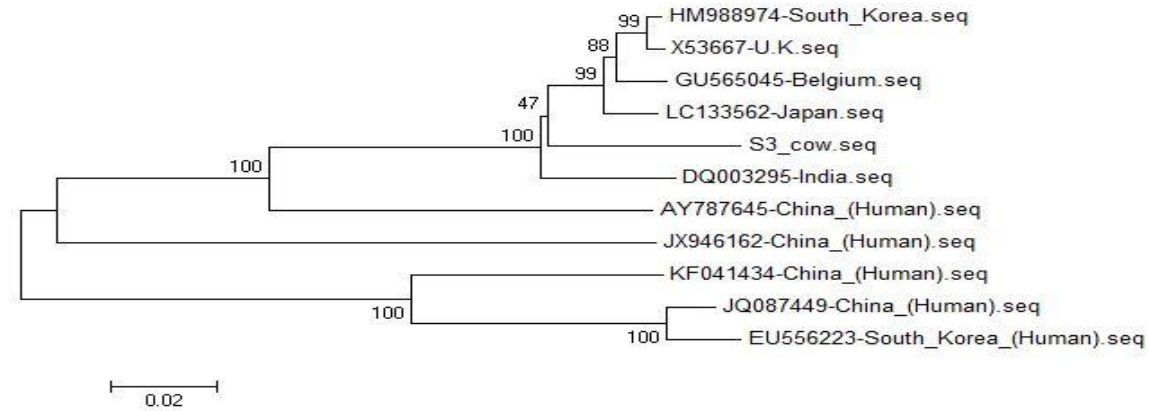

Figure 3. Phylogenetic tree of bovine rotavirus based on the VP6 gene (1356 bp) analyzed by MEGA version 6.0 to show the distance of identity relations. Wild strains from Taiwan: south of Taiwan (S3_cow). GenBank accession number: AY787645, JQ087449, JX946162, KF041434, EU556223, DQ003295, GU565045, HM988974, LC133562, X53667.

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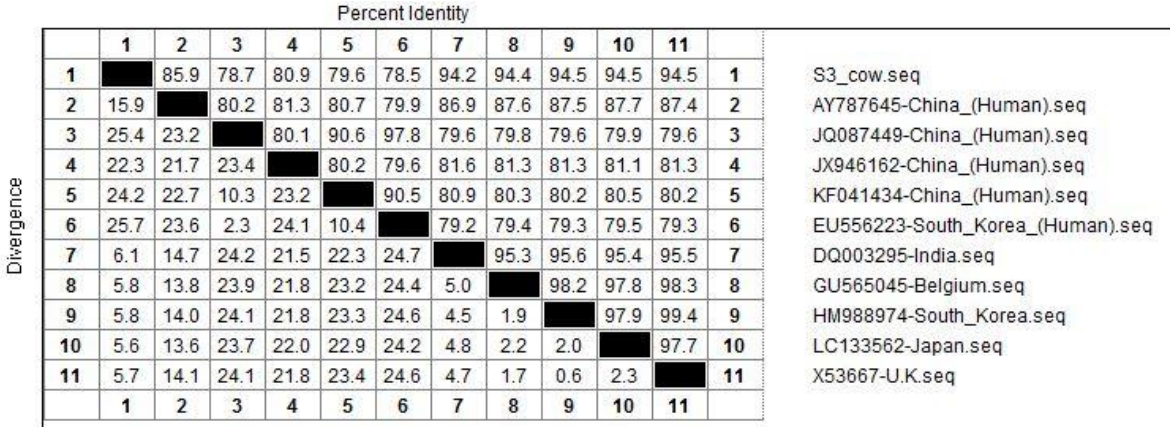

Figure 4. Homologous identity comparison of bovine rotavirus

These results reflect 2 possible issues: the first is that VP6 rotavirus maybe transferred directly from pig to human or from humans to pig, in which provides zoonotic source for swine rotavirus outbreak. Second, pigs may be co-infected with a human and a swine strain of rotavirus simultaneously [21].

CONCLUSIONS

Evidence for zoonotic transmission of wild strain rotavirus from Taiwan both in swine and bovine showed in phylogenetic tree and homologous identity comparison analysis. This information will be useful in the rasionalization of genotypes for vaccines to protect Taiwan pigs and cattle. An effective vaccine may potentially reduce zoonotic transmission.

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Effectiveness of Indigenous Lead (Pb) Reducing Bacteria Consortia of Waste Water Treatment in Agar Flour Industry

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Abstract

Lead (Pb) is known as toxic element in environment. It is produced from processing of Agar Flour industry activities. Enhancement of Pb in the wastewater of agar flour is very dangerous for human health. Effect of lead damage some organ e.g. kidney, liver, and hair. Environment standard of lead concentration on waste water based on Governor of East Java Regulation No. 72 of 2013 is 1 $mg.L^{-1}$, otherwise, initial lead levels of waste water are 3.114 $mg.L^{-1}$. The aim of the research is reducing the level of lead to be harmless for the environment. One method to decrease a high concentration of lead in wastewater is biosorption. Consortia of *Bacillus alvei* and *Bacillus pumilus* as indigenous bacteria are used to decrease Pb level in the wastewater. The experiment was carried out with varies of wastewater concentration and bacteria 3 %, 4%, 5%, 6%, and 7%. Consortium of *B. alvei* dan *B. pumilus* at 7 % concentration have the highest potency 93.58 % to reduce lead reach 0.2 $mg.L^{-1}$.

Keywords: Bacteria, Consortia, Concentration, Pb, Waste of Agar Flour

INTRODUCTION

Industry development in Indonesia has negative impact on ecosystem. The Effects were characterized by pollution. Pollution came from pollutants [1], such as hazardous industrial waste water which badly manage. It is also found at agar flour industrial waste water in Malang city. This pollution cause many problems in environment.

Problems occured bacause heavy metal (Pb) found in the waste of agar flour industry. These lead (Pb) is truly toxic, non biodegradable, potentially pollute the environment [2,3]. It is also carcinogenic and highly toxic [4]. Therefore, to control environmental pollution by Pb, it is necessa-ry to restrict maximum content of Pb in the waste water that discharged into the environment. Based on observation, Pb levels in the waste water of agar flour industrial are 3.114 $mg.L^{-1}$ which too high compared to the standard quality 1 $mg.L^{-1}$ according Governor of East Java Regulation No. 72 of 2013.

The concentration of Pb higher than the standards would be harmful to living organisms, especially indirect impact on the human health, it can damage the brain which reduce the intelligence of children [2]. Lead cause interference on nervous system, reproductive system and urinary tract [5]. Lead enter the waters through efflorescence in the air with the help of rain water [3]. Alternative treatments should be done to avoid such health problems, especially treatment for waste problem.

Biotechnology offers a solution to the polluted environment. There are several treatments that are offered in biotechnology, in this case the solution used for this study is using bacteria for bioremediation of Pb contaminated environment [6,7]. Indigenous bacteria are used as a natural agent to reduce waste water, biosorption principle is used to minimize the impact of Pb [8]. Biosorption is biological process using dead cells and living cells, caused surface of cell to bind metals. Biosorption is known as the other different mechanism of bioremediation [9]. In this case, biosorption is a metal ion absorption process using indigenous bacteria as biological agents. All microbial such as bacteria has ability to absorb heavy metal from water solution. Cell walls play an important role in binding metals ions. The advantages apply biosorption method using bacteria are giving low cost due to use bacteria as biological origin of materials, it can remove heavy metals in large quantities, and metal recovery [10].

Consortia of bacteria are known to reduce the levels of Pb in waste water better than individual bacteria. Using the bacterial consortia was more

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effective in reducing waste [11]. Thus, this study use a consortia of bacteria to assay the rate of reducing Pb.

MATERIALS AND METHODS

Starter of Bacteria Isolates

Indigenous bacteria were used for treatment. Indigenous bacteria obtained from industry of agar flour waste water, such as *B. alvei* and *B. pumilus.* Culturing bacteria used Nutrient Agar and multiply bacteria used nutrient broth. Culture starter for lead reducing experiment consist of tree starter of individual and both species of B*. alvei* and *B. pumilus*. Consortia starter of B*. alvei* and *B. pumilus* was developed using combination of both species at 24 hours age with the same volume and cell density is 3 x 10^8 cell/mL of each species.

Treatment

The concentration of bacteria in the waste water used were 3 %, 4 %, 5 %, 6 % and 7 %. As a comparison this study use two control treatments, positive control and negative control. Positive control is concentration of Pb on sterilized waste added with sterilized aquades, while the negative control is concentration of Pb on unsterilized waste added with sterilized aquades. Each treatment and control take place in shaker with 100 rpm for 7 days. After 7 days, Pb concentration on each treatment was measured using Atomic Absorption Spectrometry (AAS)**.** The last result of the Pb concentration will compare with Governor of East Java Regulation No. 72 of 2013 [13].

Statistical Data Analysis

Statistical analysis in this study using SPSS 22.0 for Windows for analysis percentage of Pb reducing among treatments . If the level of significance <0.05, the research hypothesis is accepted and the null hypothesis is rejected. If the data showed significant results, then conducted a further test of Duncan.

RESULTS AND DISCUSSION

Biotechnology in the environment fields provides an important role to help the existing problems in the neighborhood. The aims of processing waste water is decreasing concentration of hazardous waste, thus it can fulfill as the standards quality. Method used for this problem is using bacteria as a bioremediation agent to reduce hazardous metals concentration such as Pb in the waste water.

Capability of indigenous bacteria to reduce the toxic effect of Pb could be effectively used in environmental biotechnology. Indigenous Bacteria is a mixture of a wide variety of beneficial microbe which originally lives in a particular area. It have potential in the process of biodegradation, bioleaching, composting, and nitrogen fixation [14]. In this study, we used bacteria that are a native bacterium of agar flour waste water. Bacteria that isolated and identified from waste water are *Bacillus alvei* and *Bacillus pumilus*. These bacteria tested in laboratory scale to determine its benefits for reducing Pb which contained in the waste water.

Test result (Fig. 1) showed that indigenous bacteria can reduce Pb concentration. Initial Pb concentration of waste water is 3.114 $mg.L^{-1}$, after 7 days each single treatment able to reduce Pb levels. Increasing of culture concentration at 7 days experiment showed increased of percentage of Pb reducing. Starter of *B. alvei*, *B. pumilus,* and consortia of both species are able to reduce of Pb concentration until 0.910 $mg.L^{-}1$ (70.78 %), 1.544 $mg.L^{-1}$ (50.41 %), and 0.200 $mg.L^{-1}$ (93.58 %) respectively at culture concentration 7 %. The figure 1 showed percentage of Pb reduction among treatments. Consortia of *B. alvei* and *B. pumilus* at 7 % culture concentration in 7 days experiment have highest percentage of Pb reduction.

Effectiveness of bacteria consortia is due to the synergistic action of both species in the broth culture or due to different metabolism pathway by individual bacteria [15,16]. *Bacillus alvei* and *Bacillus pumilus* are indigenous bacteria that are exploited from waste water. Indigenous bacteria of both species from Pb contaminated habitat was adapted and it can still grow on their environment. Reducing Pb levels by bacteria consortia produced significantly higher and more efficient results [17].

Indigenous bacteria have the ability to reduce Pb concentration in the agar flour waste water significantly. In this case, *Bacillus* identified from agar flour waste water. *Bacillus* is Gram positive and aerobic. Gram positive bacteria have the ability to bind heavy metal compared to Gram negative bacteria, because its cells wall structure, it contains peptidoglycan, teichoic, teichuronic acid that responsible for Pb binding. Phosphate and carboxyl group in the cells wall also plays an important role to bind Pb [17,18,19]. *Bacillus* used by many researchers to investigate the

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reduction of Pb, because it has high potential of reduction to remove heavy metal and also as biosorbent, for reducing Pb based on biosorption principal [20,21].

Biosorption of Pb by consortia of *B. alvei* and *B. pumilus* was highest at 7% culture concentration. Culture concentration indicates the number or density of bacterial cells that affect the biosorption process. The culture concentration 7 % in 7 days experiment have highest potency to Pb reduction, Pb level reduce to be 0.200 mg.L^{-1} (thus absorbed Pb reached 2.914 (mg.L^{-1}) or 93.58%). The results appropriate with the aim of biosorption to reduce concentration of environmental pollutant [22].

Degradation of wastes by bacterial consortia is highly significant [17]. Based on stastistical data analysis, value of Pb reduction by bacteria decrease significantly and also it fullfill quality standard of Governor of East Java Regulation No. 72 of 2013 [13], that Pb level after treatment harmless than Pb level before treatment.

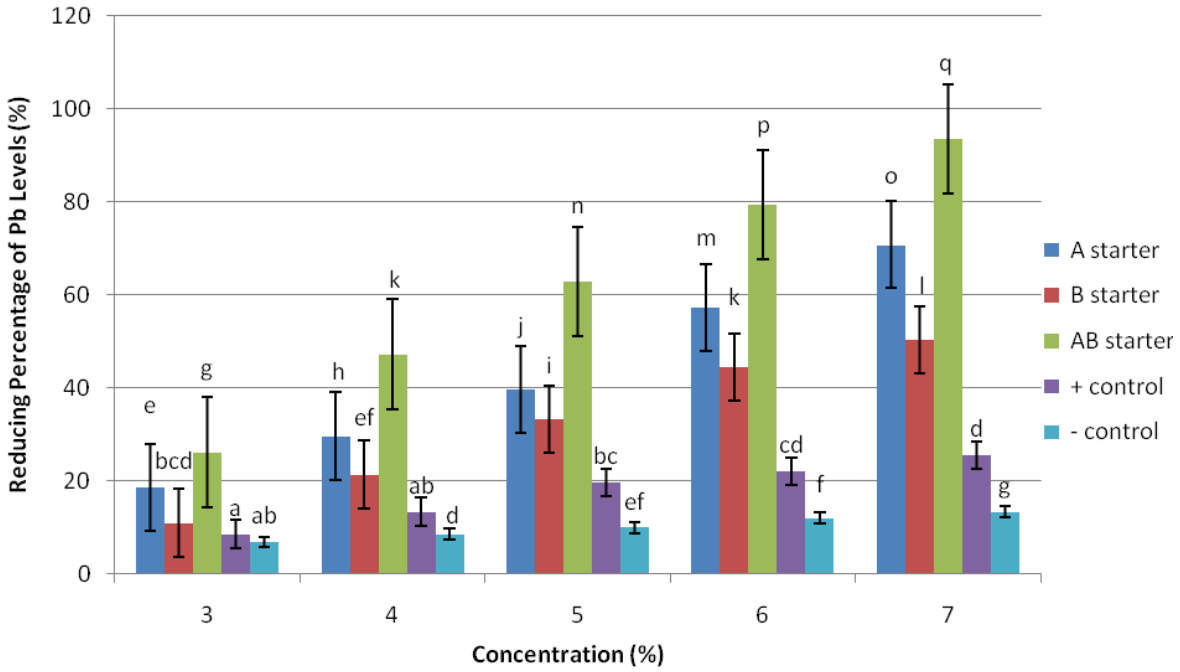

Figure 1. Reducing of Pb levels with each concentration and starter

CONCLUSIONS

Consortia of *B. alvei* and *B. pumilus* reduce Pb concentration higher than individual species. The culture concentration of the bacteria consortia at 7% have highest potency 93.58%., it reduced Pb concentration to be 0.200 mg.L^{-1}; it fulfill standard quality Based on Governor of East Java Regulation No. 72 of 2013.

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Antimicrobial and Antioxidant Activity of Endophyte Bacteria Associated with *Curcuma longa* Rhizome

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Abstract

Most cases of bacterial resistance towards antibiotics, encourage various efforts to gain new sources of antibiotics. Endophyte bacteria is a micoorganism has important role as the producer of bioactive compounds. Endophyte bacteria from *Curcuma longa* with antimicrobial and antioxidant activities have not been studied yet. *Curcuma longa* has been utilized as the main ingridients of traditional herbal medicines (*jamu*). The objective of this research was to investigate the antimicrobial and antioxidant activity of endophyte bacteria associated with *Curcuma longa* rhizome. Based on morphological characteristics of bacterial colonies, eight endophyte bacteria was isolates from *Curcuma longa* rhizome. Screening of endophyte isolate has antimicrobial activity was done using agar well diffusion method. The culture supernatant of each endophyte isolate was dropped on agar well against pathogenic bacteria *Salmonella enterica* ser. Typhi, *Staphylococcus aureus* and yeast *Candida albicans*. Three endophyte isolates K_3, K_2 and M_{1b} showed antimicrobial activity against pathogenic bacteria and yeast. Isolate K_3 showed strong antimicrobial activity againts *C. albicans* and *S. aureus*, however isolate K_2 and isolate M_{1b} showed antimicrobial activity againts *Salmonella enterica* ser. Typhi and *S. aureus*, respectively. Those endophyte bacteria also had antioxidant activity shown by scavenging ability toward DPPH radical with consecutive percentage of isolate K_3 (72.3 %), K_2 (51.3 %) and M_{1b} (64.6 %). Isolate K_3 showed the highest antimicrobial and antioxidant activity. Based on biochemical characteristics using Microbact 24E kit, isolate K_3 was identified as *Paenibacilus alvei* and isolate K_2 as *Enterobacter agglomerans*.

Keywords: antimicrobial, antioxidant, *Curcuma longa*'s rhizome, endophyte bacteria.

INTRODUCTION

Recent main health care issues include the rise of antibiotic resistances and the rise of chronic and degenerative disease in countries throughout the world regardless of income level. The rise of antimicrobial resistance need the discovery and/or production of novel anti-microbial. Antioxidants, that have capability scavenging free radicals, are known to play important roles in preventing the degenerative, ROS-linked diseases. As the human population growth and the increase awareness on healthy life, people prefer natural compounds. Thus, the exploration of novel source of natural bioactive compound is unavoidable. One of the most promising source of natural bioactive compound is endophyte [1].

Endophytes are microorganisms, often bacteria, actinomycetes or fungi that live in healthy plant tissue intercellularly and/or intracellularly without causing any apparent symptoms of disease. Endophyte bacteria are found in roots, tubers, rhizome, nodule, stems, leaves, flowers, ovules, seeds and fruits of various plant species. In general roots have greater numbers of endophytes than above ground tissues [2]. Many evaluations of bacterial endophytes have shown that they are widespread in numerous plant kingdom. A single plant may have several different endophyte bacteria. The structure of bacterial endophyte communitas are varied, dynamic overtime, and attributed to plant source, plant age, tissue type, time of sampling, season and environment [3].

The endophyte bacteria beneficial to its host by promote plant growth and yield, suppress pathogens, help plants to tolerate biotic stress or abiotic stresses, help to remove contaminants, solubilize phosphate, or contribute in fixing nitrogen. Endophytes bacteria are also known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity. It has reported over two thousands natural products have been isolated from endophytes associated with medicinal plants, including alkaloids, flavonoids, glycosides, phenolic acid, xanthones, steroids, terpenes, tetralones, coumarins, quinones, lactone, polysaccharide, peptides. Such bioactive

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metabolites are found to apply as agrochemicals like insecticidal, growth-promoting, and their potential in the pharmaceutical like antibiotics, antioxidants, antitumor, antidiabetics, antiparasitics, antithrombotic, anticancer and immunosuppressants agents [4].

Medicinal plant is well known as source of precious bioactive compound. Endophytes that have long time associate with medicinal plant may participate in metaboloc pathway or gain some genetic information to produce specific bioactive compound similar to the host plant. Plant that have ethnobotanical history should be sourced of endophyte microbe. Therefore, it is needed and important to study and explore medicinal plants and endophyte microbes that live in [4]. *Curcuma longa*'s rhizome, commonly called as turmeric has been widely used as a spice and has a long history of medicinal use in the treatment of a variety of human diseases especially in Asia regions. This study was aimed to analyze the antimicrobial and antioxidant activity of endophyte bacteria associated with turmeric rhizome.

MATERIALS AND METHODS

Study Area

Sample of turmeric rhizomes were collected from Mondo Village, Mojo District, Kediri Regency. The soil type in the research site is aluvial, along the area of Brantas Watershed. Soil acidity in the plant site is 6.34. Optimum pH of soil for most plants range 5.5 – 7.0 and nutrient will adsorbed well in the range pH 5.5 – 6.5 [5]. Refer to the soil pH at the sample site, it has qualified for the plant to grow well. Healthy ten months old plants were selected as source of rhizome for endophyte bacteria isolation.

Surface Sterilization of Turmeric rhizome

Rhizomes of turmeric were washed with running tap water. The procedure includes sequential immersion of rhizomes parts in 70% ethanol for 3 minutes, sodium hypochlorite 2% for 5 minutes and 70% ethanol for 30 seconds, then rhizomes was washed using sterilized distilled water for five times [6]. The last twice washing solutions were plated on Nutrient Agar (NA) to confirm the effectiveness of sterilization treatments. The surface of turmeric rhizomes were pilled out using aseptic technique and the inner tissues of rhizomes were macerated using a sterile mortar and pestle [7].

Isolation of Endophyte Bacteria from *Curcuma longa* Rhizome [8,9]

Total of 10 g turmeric rhizomes were extracted then performed a serial dilution in saline solution (0.85% NaCl) and plated out in Nutrient Agar (NA) to recover endophyte bacteria present in the rhizome. All the plates were incubated at 28-30°C (room temperature) for 48 hours. The isolated bacteria were preliminary characterized according to their morphological characteristics. The distinct colony types were picked up from Nutrient Agar (NA) plates and were purified through three rounds of streaking and single colony was selected an refresh in the same medium.

Test Microorganisms

Pathogenic strain yeast of *Candida albicans*, Gram-positive bacteria *Staphylococcus aureus* and Gram-negative *Salmonella enterica* ser. Typhi clinical isolates were used as test microorganism in this study. All pathogenic strains were obtained from Department of Microbiology, Medical Faculty, University of Brawijaya. After 18-24 hours of incubation at 37^{o}C (for bacterial strains) in NA and 30^{o}C (for yeast strain) in PDA, a loopful of each test strains was suspended in sterile distilled water water until obtained 1 x 10^{6} cfu.mL^{-1} for bacteria and 10^{5} cfu.mL^{-1} for yeast.

Assays of Antimicrobial Activity

Isolated endophyte bacteria from turmeric rhizomes were cultured in 5 mL Nutrient Broth (NB) medium at room temperature (28-30^{o}C) for five days. After five days, culture medium was centrifuged at 4000 rpm for 15 minutes and supernatant was screened for antimicrobial activity by agar-well diffusion technique on NA media that was previously seeded with test pathogens. Supernatant (50µL) was added into wells (7 mm) formed by cork borer on the NA medium [10]. Sterile NB was set as control. As a positive control for antimicrobial activity towards test microorganism, we used amoxicillin antibiotic dose 10 µg.mL^{-1} [11] for *S. enterica* ser. Typhi and dose 25 µg.mL^{-1} [12] for *S. aureus*. While for the antimicrobial activity on yeast, we used anti fungal nystatin 12 µg.mL^{-1} as positive control [13]. The plates were incubated in suitable temperatures for 24-48 hours; the zone of inhibition was measured and recorded.

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Assay of Antioxidant Activity by Scavenging DPPH Free Radical

Endophyte bacteria culture (in NB medium) were centrifuged at 4000 rpm for 15 minutes, 4°C and then the supernatants were assayed their antioxidant activity by scavenging DPPH free radical methode [14] described with any modification. The supernatant (0.5 mL) was added to 3 mL of 0.1 mM DPPH in methanol solution. Methanol 1.5 mL was then added thus the final volume of solution was 5 mL. For control, supernatant of each sample was replaced by steril Nutrient Broth (NB). Methanol was used as blank. Discoloration of DPPH radical solution was measured at 517 nm in triplicate after incubation in the dark for 5 hours. Ascorbic acid was used as the positive control. Percentage of scavenged DPPH radical was calculated using following formula

$$\% \text{ Scavenging} = \left[\frac{A_0 - A_1}{A_0}\right] * 100$$

A_0 is the absorbance of control and A_1 is the absorbance of sample (supernatant of endophyte bacteria culture) or standard. Ascorbic acid was taken at various concentrations as a known antioxidant for comparative analysis. Then the percentage of scavenging were plotted against respective concentrations used, and from the graph, EC_{50} was calculated.

Statistical analysis

The experimental results of biological activity tests were expressed as mean ± standard deviation (SD) of three replicates. The results were processed using Microsoft Excel 2007 and SPSS software. The data of antimicrobial activity assay results was analyzed using Kruskal-Wallis test followed by t-test and Tukey test whereas antioxidant activity using Anova following Tukey test.

RESULTS AND DISCUSSION

Based on the morphology characteristics of colony, we obtained eight isolates K_1, K_2, K_3, K_4, M_{1a}, M_{1b}, M_5 and M_6 of endhopytes bacteria. Each pured isolate was tested further for the antimicrobial and antioxidants activities.

Antimicrobial Activity

Three of eight isolates of turmeric endophyte bacteria has inhibition activity towards pathogenic test microorganism. Isolate K_3 inhibit the pathogenic yeast *C. albicans* (Fig. 1a) and pathogenic bacteria *S. aureus* (Fig. 1b). Otherwise, isolate K_2 inhibit the pathogenic bacteria *Salmonella enterica* ser. Typhi (Fig. 1c) and isolate M_{1b} inhibit *S. aureus* (Fig. 1d).

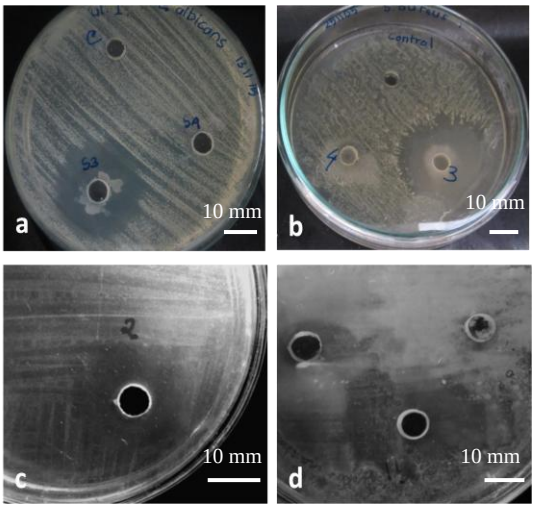

Figure 1. Inhibition zone of endophyte bacteria isolates Isolate K_3 towards *Candida albicans* (a) and *Staphylococcus aureus* (b); Isolate K_2 towards *Salmonella enterica* ser.Typhi (c); Isolate M_{1b} towards *Staphylococcus aureus* (d).

The results showed that the inhibition zone of isolate K_3 towards *C. albicans* was greater than antifungal nystatin (12 µg.mL^{-1}). It also showed similar results for inhibition zone of isolate K_3 to *S. aureus*, which is greater than amoxicilin (25 µg.mL^{-1}) and isolate K2 to *Salmonella enterica* ser. Typhi than amoxicilin (10 µg.mL^{-1}). Otherwise, the inhibition zone of isolate M_{1b} to *S. aureus* was relatively similar to the inhibition zone of amoxicillin (25 µg.mL^{-1}) (Fig 2).

Microbes produce any substance for defense systems or survival mechanism. These include antibiotics, bacteriocins, metabolic by-products, lytic agents, numerous types of protein exotoxins, and short chain fatty acid [15]. This study found that Isolate K_3 and M_{1b} (Gram positive bacteria) showed inhibition of growth towards the pathogenic bacteria *S. aureus* as Gram-positive bacteria and showed no inhibition towards Gram-negative bacteria *S. enterica* ser. Typhi. In contrast, isolate K_2 showed inhibition to Gram- negative bacteria *S. enterica* ser. Typhi and showed no inhibition to the Gram-positive bacteria *S. aureus* and yeast *C. albicans*. These properties are similar or corresponding to the nature of bacteriocins that they have a relatively narrow killing spectrum and are toxic only to bacteria closely related to the producing strain. But further test is needed to confirm that the

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substance is bacteriocin. In addition more than 99% of bacteria can produce at least one bacteriocin and within a species tens or even hundreds of different kinds of bacteriocins are present [15].

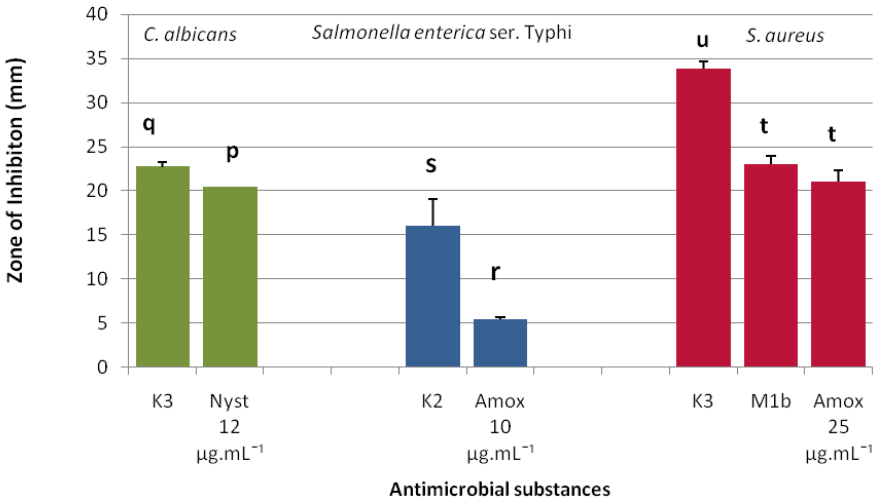

Figure 2. Antimicrobial Activity of Endophyte Bacteria Turmeric Rhizomes towards the Pathogenic Test Microorganism

Antioxidant Activity

The test of antioxidant activity on the eight isolates of endophyte bacteria showed that all isolates has the ability of scavenging to DPPH radical (Fig. 3). Three isolates with the highest antioxidant activity are isolate K_3, K_2, and M_{1b}. Bacterial growth curve were made for the three isolates to obtain optimum time for sampling to test the antioxidant activity.

Antioxidant compound produced by the endophyte bacteria, as reported by scholar, is consisted of various substances. Antioxidant substances produced by endophyte bacteria are EPS [16], surfactin [17], L-asparaginase [18], carotenoid pigment [19], and several enzymes [20]. Most of the compounds were produced maximally at the end of exponential phase. Thus the sample for antioxidant activity was collected at the 14th hour (the end of exponential phase).

Before the test of antioxidant activity, OD of liquid culture of endophyte bacteria was equated. The test of antioxidant activity showed that isolate K_3 has the highest ability of antioxidant activity compared to the other two isolates (Table 1) and then the EC_{50} of K_3 isolate was determined.

Efficient Concentration or EC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour)[21]. Isolate K_3 supernatant had EC_{50} value 70.26 µL.mL^{-1} and vitamin C (as standard) had EC_{50} value 3.71 µg.mL^{-1}. In this study isolate K_3 supernatant was still in original liquid and had not evaporated yet or extracted in to concentrate, so it was intelligible that the EC_{50} value was too lower than vitamin C. For next study may be required further processing of the supernatant.

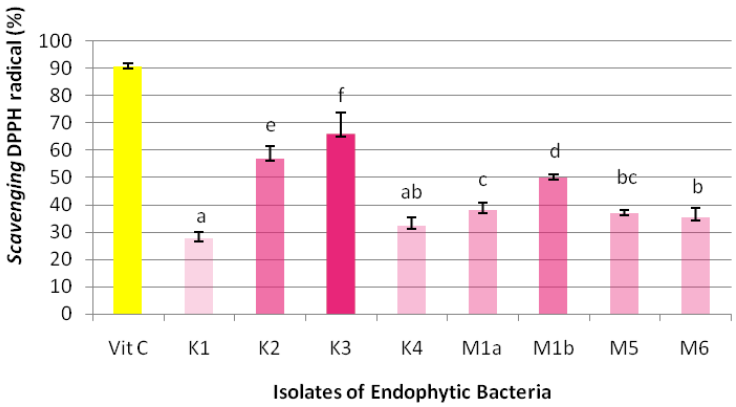

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Figure 3. Antioxidant Activity of *Curcuma longa* Endophyte Bacteria Isolates

Tabel 1. Antioxidant activity of 3 isolates

No	Isolates	Scavenging DPPH radical (%)
1	K_2	51.3 ± 3.1
2	K_3	72.0 ± 1.7
3	M_{1b}	64.7 ± 2.5

Note: Each value is represented as mean ± SD (n=3)

Species Identification

Isolates K_3 and K_2 had both antimicrobial and antioxidant activity significantly , therefore need to characterize these isolates furthermore. Isolate K_3 and K_2 was characterized based on the biochemical characteristic using Kit Microbact System 24E. Biochemical characteristics of isolate K_2 were analyzed by the software Microbact System 24E and isolate K_3 were analyzed refer to Identification flow chart from Microbiology Laboratorium, The University of Ottawa Canada base on Bergey's Manual of Determinative Bacteriology [22]. The results of characterization showed that isolate K_2 was assumed as *Enterobacter agglomerans* with 99.9% similarity*,* whereas isolate K_3 is *Bacillus alvei* or *Paenibacillus alvei* with 91.2% similarity.

Paenibacillus alvei are rod-shaped, Gram-positive, motile, spore-forming , catalase-positive bacteria and grow on simple media (NA/NB). *Paenibacillus alvei* are common found in honeybee colonies , soil , milk, mosquito larvae, the wax mot, humans and very rarely pathogenic for vertebrates. It has reported that *Paenibacillus alvei* produce antimicrobial substance: paenibacillin P and paenibacillin N [23], peptide AN5-1 [24], cyclic lipopeptides [25], depsipeptide [26]. Some of the antimicrobial substance show active againts pathogen *S. aures* and *C. albicans* [23,24,26,27] and consistent with this findings, this study showed endophyte bacteria from *Curcuma longa's* rhizome, isolate K_3 that assumed as *Paenibacillus alvei* show antimicrobial activity to *S. aureus* and *C. albicans*. Isolate K_3 also show antioxidant activity, it promote previous research that *Paenibacillus alvei*'s metabolite have antioxidant activity Exopolysaccarides (EPS) [16,28,29]. *Enterobacter agglomerans* are rod shape, Gram negative, motile, non-sporforming bacteria. These bacteria first were isolated from plants, vegetable, fruits, seeds, and they are also commonly found in the ecological niches such as water, soil, sewage, feculent material, foodstuffs, clinical specimens [30]. It has reported that *Enterobacter agglomerans* produce antimicrobial substance that have inhibition growth to any pathogen bacteria and fungi. Some of them have inhibition growth to *Salmonella sp.* like herbicolin O [31], phenazine [32], and consistent with these finding, isolate K_2 that assumed as *Enterobacter agglomerans* has antimicrobial activity to *Salmonella enterica* ser.Typhi. Isolate K_2 also has antioxidant activity, it promote previous research that *Enterobacter agglomerans* has free radicals-scavenging ability [33].

CONCLUSION

The study obtained eigth isolates of endophyte bacteria from the *Curcuma longa* rhizomes. Three isolates of endophyte bacteria have antimicrobial activity, i.e. isolate K_3 to *C. albicans* yeast and *S. aureus* bacteria; isolate K_2 to *S. enterica* ser. Typhi, and isolate M_{1b} to *S. aureus*. All isolates of endophyte bacteria from *Curcuma longa* rhizomes has the antioxidant activity. The highest antioxidant and strong antimicrobial to Gram positive pathogenic bacteria activity was showed by isolate K_3 which identified as *Paenibacillus alvei*. The strong antimicrobial activity to Gram negative pathogenic bacteria and had high relative antioxidant activity was showed by Isolate K_2 which identified as *Enterobacter aglomerans* by biochemical characterization.

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The Influence of Fermentation Time in the Physical and Chemical Composition of Fermented Soybean Husk by Using *Aspergillus niger* on the Quality of Raw Feed Materials

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Abstract

Soybean husk (*Glycine max L*. Merrill) a soybean processing waste as raw material for *tempe* obtained after the process of boiling and soaking soybeans. The main problem in the use of soybean husk (*Glycine max L*. Merrill) as feed material is its crude fiber content which is fairly high. This study aimed to observe the fermented soybean husk using *Aspergillus niger* to improve the quality of the raw feed materials. This was conducted by using completely randomized design (CRD) analysis and repeated three times; the time optimization of *Aspergillus niger* in 2, 4, and 6 days based on chemical analyses (moisture, protein, fat, ash, crude fiber and feed containing carbohydrates (NFE) and physical assessment fermentation (smell, texture, moisture and hyphae) were analyzed descriptive qualitatively. The results showed that 4 days fermentation of soybean husk using *A. niger* is successful gives the highest score based on physical characteristics texture, aroma, moisture, and the formed hyphae and the most effective treatment for decrease in crude fiber is 13% and increase in NFE contained in the largest on 4 days fermented soybean husk by *Aspergillus niger* with a long time 4 days.

Keywords: *Aspergillus niger*, fermentation, soybean husk.

INTRODUCTION

Soybean husk is a waste that is produced from the process of boiling and soaking soybeans which were used as the materials to make *tempe*. After going through the process, the husk will be separated and will normally be thrown away by the *tempe* producer.

Based on the analysis in the Laboratory of Biochemistry and Nutrition Fish, Faculty of Fisheries and Marine Sciences, Brawijaya University that soybean husk (*Glycine max L*. Merrill) has a water content of 12.45%, 14.32% protein, 38.35% crude fiber, 2.32% fat, 4.14% ash and 2.42 kkal.g^{-1} energy. Therefore, soybean husk still has the potential to be used as a feed for animals considering that it has a high protein and energy [1].

The main problem in the use of the soybean husk (*Glycine max L*. Merrill) as a raw material is fairly high cellulose content of around 33.49% [2]. Further explained that the soybean husk (*Glycine max L*. Merrill) contains 10-20% hemicellulose, 29-51% cellulose, 1-4% lignin and 6 -15% pectin [3].

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Technology to improve the quality of materials the feed is fermented [4]. Generally all fermentation end products usually contain compounds that are simpler and easier to digest than the original material thus increase the nutritional value [5]. The use of agricultural waste products as fermentation substrate is due to the mass-produced, the cost of which used lower and rich in nutrients [6].

Cellulase enzyme complex is composed of cellobiohidrolase, endoglucanase and β - glucosidases which all act synergistically to convert complex carbohydrates lignocellulosic biomass into glucose efficiently [7]. Cellulase can be produced by fungi, bacteria, and ruminants. Production of commercial enzyme normally uses fungi or bacteria. Fungi can produce cellulases include genus *Trichoderma*, *Aspergillus*, and *Penicillium* [8]. *Aspergillus niger* has been widely used because it produces the three fundamental enzymes required for cellulolysis [9].

Previous research the use of *A. niger* can decrease crude fiber is already done. Declared by the proximate analysis note that the content of crude fiber grout tofu before it is fermented in the amount of 24.03% and crude fiber content of the grout tofu out after fermentation between 0.04 to 0.16% [10]. This is supported by other research results [11], that the content of crude

fiber grout tofu 21.29% and after fermented decreased to 17.29%. A study mentioned that the fermentation 2 to 4 days can decrease the crude fiber [12].

The high fiber content and the lack of other nutritional content of the constraints of local feed use this as a source of alternative feed prospective. Thus, this study aims to observe different time duration in the physical and chemical composition fermentation soybean husk to improve the quality of fish feed as the feed materials.

MATERIALS AND METHODS

Soybean Husk Fermentation

Briefly, dried and ground soybean husk 100 g each were placed in a 600ml beaker glass and autoclaved at 121°C for 20 min [13]. After that, the soybean husk was densified by using dilution of 10^6 [14]. The soybean husk *(Glycine max L.* Merrill) was then added molasses in the ratio of 1:1 with the given mold dose [15] and the substrate was then stirred until it became homogeneous and mixed with sterile water until the water level reaches 70% [11]. Then, the tray was covered with *plastic wrap* and laid in the incubator at a temperature of 30°C with pH 5 [16].

Physical Assessment Fermentation

Physical Assessment Fermentation is used in order to determine differences in physical quality which appear, on the soybean husk that fermented and non-fermented. The scoring media of fermentation soybean husk started from 1 to 4 (Bad, Less good, Good and Excellent) where greater score indicated good fermentation [17]. In the scoring media of fermentation soybean husk is shown in table 1 below.

Table 1. Physical Assessment Fermentation

Score	Lumps (%)	Scent (%)	Water Steam (%)	Hyphae (%)
1	<10	>10	<10	<10
2	<10 - <25	>10 - <25	<10 - <25	<10 - <25
3	>25 - <40	>25 - <40	>25 - <40	>25 - <40
4	>40	<40	>40	>40

Notes: **Lumps** (Soft <10, Few <10-<25%, Some>25-<40%, A lot of >40%), **Scents** (No <10%, Slight >10-25<%, Normal >25-<40%, Strong <40%), **Water steam** (Dry <10%, Normal >10-<25%, Less >25-<40%, Moist >40%), **Hyphae** (No <10%, Few >10-<25%, Several >25-<40%, Many >40%).

Chemical analysis

The parameters observed in this research include chemical compounds contained in fermented soybean husk before and after being fermented through proximate test (moisture content, ash content, crude protein, crude fat, and crude fiber. The proximate analysis which was tested with the analysis of water content at a temperature of 105°C by using the oven for 6 hours, while the protein analysis was analyzed by using Kjeldahl method. Simultaneously, the fat was also analyzed by using soxhlet and petroleum ether in order to dissolve the fat. Crude fiber was assessed by using a solution of H_2SO_4 and NaOH as the solvent and the analyzation of the ash was carried out by using a furnace with a temperature of 600°C for 2 hours [18].

Statistical analysis

Statistical analysis used the analysis of variance (ANOVA). ANOVA was used to test the effect of the treatment and then further used the least significant difference (LSD) test at the level of 5%. In the other hand, the data of organoleptic test were analyzed descriptively qualitative.

RESULTS AND DISCUSSION

Successful Rate Fermentation

Based on the results, the best fermentation time was on the 4th day. The physical observation of the fermentation soybean husk includes texture, aroma, moisture, and the formed hyphae. The data scoring of fermentation soybean husk can be seen in Figure 1 below:

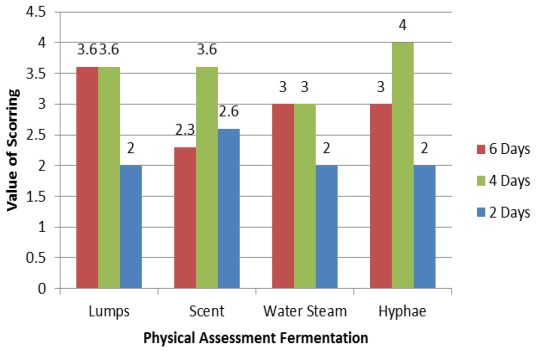

Figure 1. The average scoring value of physical assessment time fermentation containing ■ 6 days ■ 4 days ■ 2 days fermentation soybean husk.

The high scoring value indicated that the fermentation process has been going very well. The best result of the scoring occurred on the 4th day. On the 4th day gives the highest score based on physical characteristics lumps, scent, water steam, and the formed hyphae.

Moreover, the texture or lumps of the fermented soybean husk indicated the best result on the 4th day compared to other days. Fermentation causes the changing nature of the feed

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material including the texture; it is as a result of the content cleavage in the foodstuff caused by microorganisms that are in there [19]. Establishment of a texture is also influenced by water content, fat content, type and amount of carbohydrate food products [20].

Another thing is that in the 2nd and 4th day, the aroma of the fermentation soybean husk showed the best fragrance that is slightly sour and fragrant but it created an acid aroma and an odor of ammonia on the 6th day. Good fermentation has a sour and fragrant aroma [21]. Effect on the hydrolysis time increased levels of NH_3 (ammonia) giving rise to acid aroma [22]. Feed given additional fermentation may be associated with aroma and flavor that can affect the appetite of the animal [23].

In the fermentation with *A. niger,* water steam was formed on the 4 and 6th day because of an exothermic reaction when the process of organic material cleavage occurred. Fermentation process will produce CO_2 and heat as a result of the organic material breakdown [24]. The water content also affected the growth of mold and dynamics that occur during the process ensilase because water is required for the synthesis of protoplasm microorganisms and dissolved organic compounds [25].

Based on the results of the 2nd day, there were some very heavy hyphae throughout the fermentation media, and besides that, several spores grew well in some of the fermentation points. On the 6th day, hyphae began to decrease because the spores grew very much. Previous research also demonstrated that Coconut oil cake and palm kernel oil cake that were fermented with *A. niger* would have hyphae by 90% and spores by 10% on the edge of the spores on the 4th day [26]. Hyphae thrive but few spores that grow on the fourth day, so that the material is more easily digested and utilized because the spores increase fiber content material [5].

Improvement Nutrient Content

The results of the chemical analysis can be seen in Table 2 below. Based on the results of proximate treatment C (fermented for 4 days) are considered better, it can be seen from the decline in crude fiber contained in the largest C treatment is 13%. This observation is not as good as the research of leaves lamtoro fermented with *A. niger* crude fiber decreased by 46.61% for 3 days [5]. The decrease in crude fiber occurred due to *A. niger* has three essential enzymes needed for celluloses [9]. The increased crude fiber during the final phase of fermentation vegetable waste was due to the utilization of the nutrients provided by the mold and then the reduction can be attributed to breaking the non-starch polysaccharide for mold protein [27]. Crude fiber is part of a carbohydrate that difficult to digest by the digestion of fish, the higher the fiber in the diet then lowers the energy [28]. High crude fiber will give a sense of satiety because of the composition of complex carbohydrates that stops the appetite that caused a decline in food consumption [29]. The fiber content is too high will suppress growth [30].

Based on the proximate result of an increase in the value of the protein with the length of fermentation time, testing crude protein fermented soybean husk is the highest by a long period of 6 days at 17.02%. Vegetable waste and fermentation by using *A. niger* S14 for 8 days increased the protein by 38% [27]. Increased protein content after the fermentation process probably derived from *A. niger* which has synthesized the urease enzyme to break urea into ammonia and CO_2. This ammonia was then used for the mold to form amino acids (protein) [31].

In the 6th day fermentation, higher *A. niger* content was given and it resulted in fat degradation. It happened because the mold has achieved an exponential growth [12]. The decreased fat in fermented palm kernel oil cake flour occurred due to the conversion of fat into a single protein biomass [32]. Lipase enzyme produced by fungi greatly affects crude fat content after fermentation substrate because the enzyme lipase will remodel fat to be used by fungi as an energy source [33]. Microbial lipases have been used as a catalyst in producing oleochemicals-based products include fats or oils such as triglycerides modified low-calorie [34], so even though the resulting reduced oils or fats rich in EPA and DHA [35].

Increased NFE in the 4th day fermentation due to increased glucose as a result of fermentation of *A. niger* hydrolyze cellulose. Glucose levels continued to rise from 8 up to 64 hours, but declined to 72 in rice straw fermentation using *A. niger* [36]. *Aspergillus niger* also produce ß-glucosidase enzyme that is strong that this enzyme serves to accelerate the conversion of cellobiose widened glucose [37]. Carbohydrates are used as energy source of non-protein replacing protein as an energy source. If the feed shortage of non-protein energy then the fish will use a portion of the protein to insufficient energy needs [38]. Feed containing carbohydrates (NFE)

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exact to reduce the use of protein as an energy source known as protein sparring effect [39].

In the trials, the dry matter loss was about 7% in the present study. 20% dry matter loss after 96 h of fermentation of mixed oil cakes with *A. niger* 616 [40]. In a similar study with wheat bran, has also reported significant ($p < 0.05$) reduction in dry matter throughout the fermentation period for wheat bran using *A. niger* S14, suggesting utilization of nutrients present in the substrate by fungi for its growth and metabolic activities [41]. Feed given additional fermentation may be associated with aroma and flavor that can affect the appetite of animal and an additional fermentation in feed also provides an additional element of essential amino acids [23].

Table 2. The results of the fermentation soybean husk proximate analysis

Composition	Treatment			
	A	B	C	D
Dry content (%)	87,34±1,56[a]	82,75±0,58[b]	83,21±0,57[b]	82.12±0,39[b]
Ash (%)	4,14±0,30[a]	3,67±0,37[a]	3,91±0,31[a]	3.84±0,70[a]
Protein (%)	14,32±0,64[a]	15.13±0,05[a]	15,55±0,11[a]	17.02±0,18[b]
Fat (%)	2,32±0,43[a]	2,13±0,09[a]	2,03±0,14[a]	2.03±0,1[a]
Crude fiber (%)	38,35±0,65[d]	37,52±0,28[c]	33,45±0,39[a]	35.72±0,30[b]
NFE (%)*	40,87±0,78[a]	41.55±0,73[a]	45,06±0,67[b]	41.39±0,71[a]

Table 2. Result of observation towards chemical composition including dry content, ash, protein, fat, crude fiber and NFE in different time fermentation soybean husk towards A. (0 days), B. (2 days), C. (4 days) and D. (6 days). Values are means±SEM of three replicates (n=3); means in the same row followed by the same superscript letter are not significantly different (DMRT, $p>0.05$). *NFE (Nitrogen Free Extract) = 100-Protein-Fat-Fiber-Ash.

CONCLUSION

The results show that 4 days fermentation of soybean husk using *Aspergillus niger* is successful gives the highest score based on physical characteristics texture, aroma, moisture, and the formed hyphae and chemical analysis result the best time fermentation is 4 days the most effective treatment for decrease in crude fiber and increase in NFE contained in the largest on 4 days fermented soybean husk by *Aspergillus niger* with a long time 4 days. All of these changes enhance the value of the soybean husk as an animal feed, including aquafeed.

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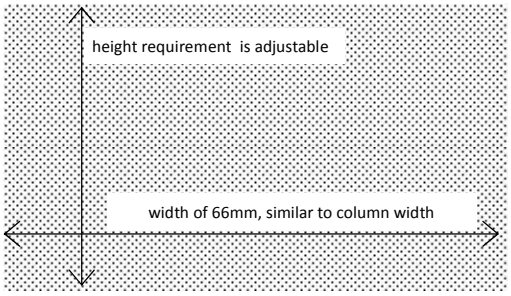

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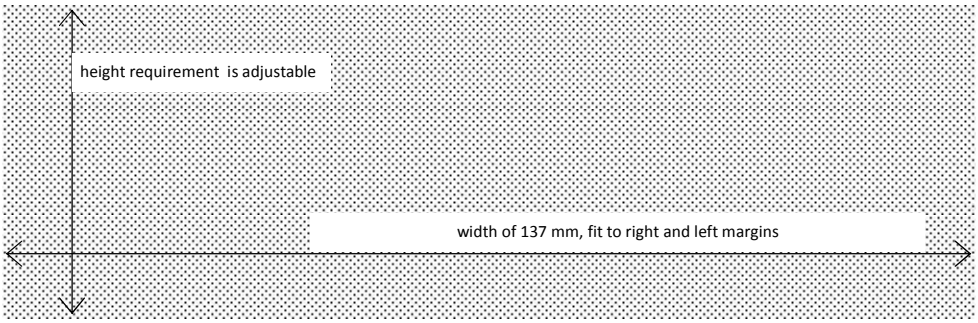

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