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Research Paper

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Kartini Zailanie.

J. Life Sci. Biomed., 6 (1): 01-05, 2016;
pii:S225199391600001-6

Abstract
This study aimed to explore *Padina australis* using UV-VIS, HPLC and Antibacterial. Brown algae (*Padina australis*) obtained from Padike, Talango, Madura. Sampling was done using GPS. Extraction method was done using CaCO3 as neutralizing plus premises of methanol and acetone (7/3 v / v in 300 ml). Analysis was done using UV-VIS and Shimadzu spectrophotometer with a wavelength of 300-800 nm. The disc test showed that the concentration of *Padina australis* extract inhibits the growth of salmonella at a concentration of 10.000 ppm or equal to 4.6 pm ethanol, and at a concentration of 10,000 ppm is equal to 6.23 nm *salmonella typhi* with methanol. Reversed-phase HPLC method with the brand Shimadzu LC-20A (ODS.C-18) used for identification of chlorophyll a fucozanthin 412.5 nm and 439 nm. The identification of brown alga *Padina australis* obtained wavelength value chlorophyll b 444.583 nm, fucoxanthin 450,455 nm, chlorophyll a 618,664 nm, feofitin 665 nm and β caroten 426,451 nm, while the results of the identification of crude brown alga *Padina australis* has a retention time chlorophyll c 6.432 sec, fucoxanthin 10.22 sec, chlorophyll a 38.5 sec, feofitin 56.82 sec and β caroten 62.144 sec.
Key words: Antibakterial, HPLC, *Padina australis*, UV-VIS.

[Full text-PDF]

Research Paper

Phytochemicals and Antibacterial Activities of Soursop Leaf (*Annona muricata*) against *Edwadsiella tarda* (In Vitro).

Anggana Rarassari M and Nursyam Maftuch H.

J. Life Sci. Biomed., 6 (1): 06-09, 2016;
pii:S225199391600002-6

Abstract
Edwardsiella tarda is bacteria in fish caused of Edwardsiellosis disease and it was include to plague bacteria that can harm. *Edwardsiella tarda* infection controlled by using a natural antibacterial that is *Annona muricata*. The aim of this research is to know the content of secondary metabolites in leaves of the soursop (*Annona muricata*) as well as an effective solvent to extract *Annona muricata* to be an antibacterial against on *Edwardsiella tarda* by disc diffusion method. Experimental method used in this research is descriptive and it is based on 2 types of leaves (fresh and dried), 3 different treatment solvent (ethanol, ethyl acetate and chloroform) and three replications. Data obtained from the study was statistically analyzed used statistical applications, SPSS version 21.0. The results of the crude extract of dried *Annona muricata* is in accordance with the results of phytochemical contained phenols, saponins, tannins and flavonoids as well as the best use of ethyl acetate solvent to the extent of inhibition with 15:41 mm.
Key words: Antibacterial, *Edwardsiella tarda*, *Annona muricata*, Phytochemicals, Solvent

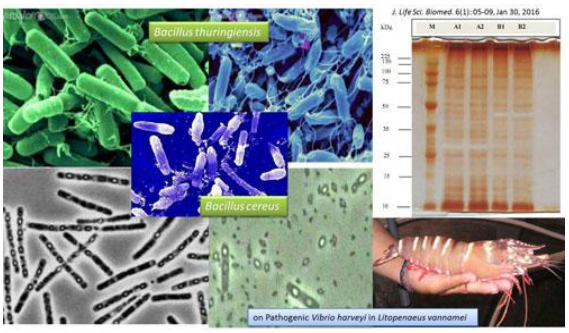
[Full text-PDF]

Research Paper

Antimicrobial Activity of *Bacillus cereus* and *Bacillus thuringiensis* on Pathogenic *Vibrio harveyi* in *Litopenaeus vannamei*.

Masitoh M. M., Hariati A. M. and Fadjar M.

J. Life Sci. Biomed., 6 (1): 10-14, 2016;
pii:S225199391600003-6



Abstract
The bacterial strain of *Bacillus cereus* and *Bacillus thuringiensis* have been known to produce antimicrobial activity against pathogenic *Vibrio harveyi*. The effect of *B. cereus* and *B. thuringiensis* were tested by in vitro and in vivo. In vitro test was used to analyze antagonism characteristic of bacteria

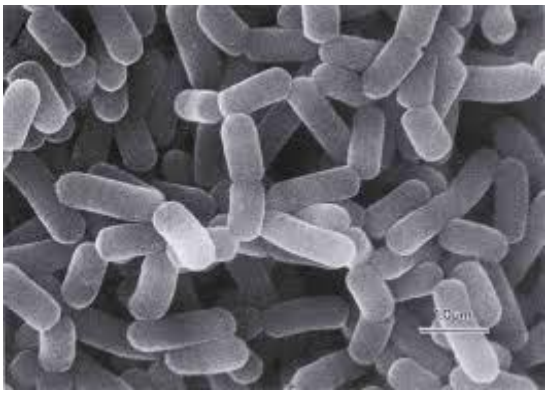
using the paper disc diffusion method. In vivo test was applied to evaluate antimicrobial activity of *B. cereus* and *B. thuringiensis* (10^5 CFU ml^{-1}) on survival rate and histopathology of *Litopenaeus vannamei* challenged with *V. harveyi*. The results showed that *B. thuringiensis* had a greater inhibitory activity of 18.60–35.97 mm. Both Bacillus bacteria treatment resulted in survival rate of 100%, compared with 75% in the treatment without Bacillus. We can concluded that *B. cereus* and *B. thuringiensis* have potential applications for controlling pathogenic *V. harveyi* in shrimp aquaculture.
Key words: *Bacillus cereus*, *Bacillus thuringiensis*, *inhibitory activity*, *V. harveyi*.

[Full text-PDF]

Research Paper

Characterization of Bacteriocin *Lactobacillus casei* on Histamine-Forming Bacteria.

Nugrahani A, Hardoko, Martinah Hariati A.
J. Life Sci. Biomed., 6 (1): 15-21, 2016;
pii:S225199391600004-6



Abstract
Tuna which has undergone a process of decay will be poisoned if being consumed. It caused by the contamination of pathogenic bacteria such as *Escherichia coli, Salmonella, Vibrio cholerae, Enterobacteriacea* etc. Some types of fish contain histidine family scmbroidae high free, such as yellow tail tuna 740 mg/100g of meat, bigeye tuna 491 mg/100 g, mahi-mahi 344 mg/100 g, mackarel 600 mg/100 g, skipjack 1192 mg/100 g and albakor highest to 2 g/100 g. The formation process of histamine in fish is influenced by the activity of the enzyme *L-Histidine Decarboxylase* (HDC). Bacteriocin *Lactobacillus casei* extract is able to inhibit the activity of *Pseudomonas sp, Proteus morgani* and *Micrococcus sp.* The extract of bacteriocin *Lactobacillus casei* has a high temperature stability which has inhibitory activity against bacteria test at a temperature of 90°C. Bacteriocin *Lactobacillus casei* from bacteria *Pseudomonas sp* has the optimum activity at pH 5 with inhibition diameter of 8.25 mm, while the bacteria *Micrococcus sp* has the optimum activity at pH 4 with a inhibition diameter of 9.25 mm. Bacteriocin *Lactobacillus casei* has a molecular weight of 14.34 kDa which included in the group of class III bacteriocins, generally has a large size (> 10 kDa), and can not survive against the heat. Bacteriocins *Lactobacillus casei* extract can inhibit the activity of histamine-forming bacteria growth and have stable properties to high temperature and pH. Future research is recommended to do the production of bacteriocins *Lactobacillus casei* optimization and its application in fishery product.
Key words: Characterization, *Lactobacillus casei,* Bacteriocin, Histamine Forming Bacteria

[Full text-PDF]

Archive

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Study of *Padina australis* using UV-VIS, HPLC and Antibacterial

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ABSTRACT: This study aimed to explore *Padina australis* using UV-VIS, HPLC and Antibacterial. Brown algae (*Padina australis*) obtained from Padike, Talango, Madura. Sampling was done using GPS. Extraction method was done using CaCO3 as neutralizing plus premises of methanol and acetone (7/3 v / v in 300 ml). Analysis was done using UV-VIS and Shimadzu spectrophotometer with a wavelength of 300-800 nm. The disc test showed that the concentration of *Padina australis* extract inhibits the growth of salmonella at a concentration of 10.000 ppm or equal to 4.6 pm ethanol, and at a concentration of 10,000 ppm is equal to 6.23 nm *salmonella typhi* with methanol. Reversed-phase HPLC method with the brand Shimadzu LC-20A (ODS.C-18) used for identification of chlorophyll a fucozanthin 412.5 nm and 439 nm. The identification of brown alga *Padina australis* obtained wavelength value chlorophyll b 444.583 nm, fucoxanthin 450,455 nm, chlorophyll a 618,664 nm, feofitin 665 nm and β caroten 426,451 nm, while the results of the identification of crude brown alga *Padina australis* has a retention time chlorophyll c 6.432 sec, fucoxanthin 10.22 sec, chlorophyll a 38.5 sec, feofitin 56.82 sec and β caroten 62.144 sec.
Key words: Antibakterial, HPLC, *Padina australis*, UV-VIS.

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INTRODUCTION

The Brown algae is very popular in Japan, China and Korea as one of the components of the main intake diet daily [1]. Besides brown algae contain the pigment caroteneoid as a source antioxidants and anticancer [2].

Substances antimikroba is a compound biological or chemical that can inhibit the growth and activity of microbes. Substances antimikroba special for bacteria can be bactericidal (kill bacteria) and bacteriostatic (inhibits the growth of bacteria) [3]. Some algae from the Indonesian coastal areas found to have compound active a character as antimikroba against bacteria patogen [4]. Brown algae type of *Padina australis* have a secondary metabolit like Steroids, Carotene substance bioaktif anti bacterial, fungi, virus or cancer [5].

Padina australis is compounds steroids, terpenoid, polifenol, and saponin [6]. All this chemical allows *Padina australis* to be developed as antibakterial natural because the compounds bioaktif were conceived, is able to inhibit the growth of bacterial. Brown algae can also contain pigments other than as an antibacterial.

The aim of this study was to explore *Padina australis* using UV-VIS, HPLC and Antibacterial.

MATERIAL AND METHODS

Sampling Methods With GPS

Sampling methods with the GPS (Global Positioning System) with the purpose to determine the layout coordinates of the point were observed (113.94444°BT – 7.08795°LS, 113.94231°BT – 7.08913°LS, 113.94548°BT – 7.08911°LS and 113.94347°BT – 7.08999)

Extraction

Samples seaweed *Padina australis* washed and drained, cutted at ± 1.5 cm size with scissors, then the 100 grams sample was weighed and mashed with a mortar pestle. During the process of refining ± 0.5 gram sample was added as a neutralizing CaCO3 because the extraction process will run optimally at neutral pH. *Padina autralis* inserted into the glass beaker covered with aluminum foil. Added methanol (CH 3 OH) and acetone (CH3COCH3) ratio of 7/3 (v / v) in a 300 ml glass beaker is then covered using cling wrap to minimize evaporation of the solvent and covered with aluminum foil to minimize exposure to cahaa. After maceration is then performed filtering (filtration) using Whatman paper no. 42 to separate the results of the filtrate with the filtrate residue thus produced is mixed with clean without residue for 12 hours in order to maximize the results of maceration.

Analysis *Padina australis*

Spectrophotometer UV-Vis and HPLC analysis: UV-Vis spectrophotometry is used to identify pigments, subsequently identified based on wavelength and absorbance values. First dried using a gas powered 104, included in the Cuvet ± 3 ml. Further included in the instrument Shimadzu UV-Vis 1601 tested in the wavelength range 300-800 nm. *Padina australis* analysed using HPLC with a brand Shimadzu LC-20A (ODS C-18). The first step taken in the workplace HPLC system which extracts coarse pigments were dissolved in 5 ml of mobile phase (acetone: methanol: ammonium acetate, 80: 10: 10 v/v). In high performance liquid chromatography, the mobile phase in addition to functioning as a carrier of the components of a mixture to the detector, the mobile phase is one of the critical success factors analysis process [7]. The next phase of 20 mL solution was injected on HPLC pigment with the silence that was the ODS phase (C-18) 5 µm with gradient elution system of methanol, acetone and ammonium acetate (1 M) and a flow rate of 1.0 ml / min.

Activity Anti Bacterial

The content of secondary metabolites of seaweed potentially as diverse bioactive metabolites with very broad activity as an antibacterial and antiviral. Sea grass green, red or brown is a potential source of bioactive compounds that are beneficial for the development of the pharmaceutical industry such as anti-bacterial, anti-bacterial, anti-tumor, anti-cancer and agrochemical industries, especially for fungicides and herbicides [8].

The mechanisms that lead to inhibiting the growth of bacteria after being fed extracts of *P. Australis* is the content of bioactive compounds, one of which is the compound phenol and its derivatives. Phenol and derivatives compound binds to a protein on the bacteria through non-specific binding of proteins to form complexes of phenol. At low concentrations, the protein complex formed phenol with weak bonds and immediately undergo decomposition. Phenol then damage the cytoplasmic membrane and cause leakage of the contents of the cell, thereby inhibiting the growth of bacteria. Whereas at high concentrations the substance teesebut coagulated with cellular proteins and the cytoplasmic membrane through lysis. *Padina australis* which contains phenolic compounds, inhibit bacterial growth by interfering with the function of the cytoplasmic membrane. The presence of phenolic compounds is causing the destruction of the cytoplasmic membrane. Ion H of phenol and its derivatives (flavonoids) will attack the polar groups (a phosphate group) so that the phospholipid molecules in the cell walls of bacteria will break down into glycerol, carboxylic acid and phosphoric acid. In such circumstances, phospholipids are not able to maintain the form of the cytoplasmic membrane cytoplasmic membrane consequently will leak and the bacteria will experience growth retardation and even death [6].

RESULTS

The results of the sample pigment suspected as β-carotene, chlorophyll a, chlorophyll b and fukosantin do identification, which is used Spektrofotometer UV-Vis brand of Shimadzu 1601 with long waves are used which 300-800 nm. Analysis with spektrofotometri UV-Vis using the solvent acetone as blanko. The next step is the analysis by using HPLC a Shimadzu LC-20 with A phase of silence (stationer) used that ODS (C-18) and phase motion (mobile) acetone: metanol: ammonium asetat (80:10:10 v/v).

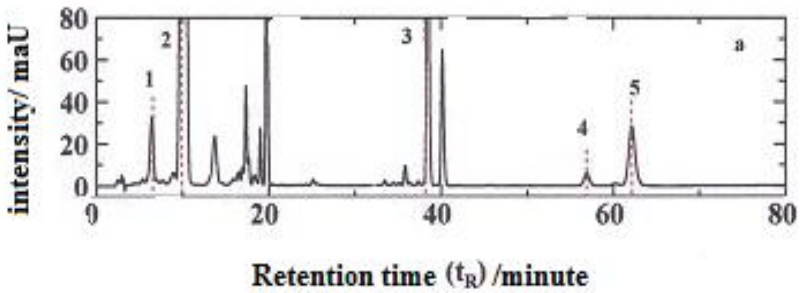

Figure 1. Crude extract chromatograms of *Padina australis*

At each peak or peak indicates a pigment contained in brown seaweed *Padina australis* species. The sequence of each peak or peak in the order of degree of polarity. Of pigment which has the highest degree of polarity to the polarity of the pigment which has the lowest level, because in this study applied a reversed-phase system or a "reversed phase". Reversed phase chromatography using a stationary phase that is less polar than the mobile phase her, because in general the HPLC method using hydrophobic stationary phase.

The next step in the work system that extracts HPLC coarse pigment dissolved in 5 ml of mobile phase (acetone: methanol: ammonium acetate, 80: 10: 10 v / v). Then about 20 mL solution was injected on HPLC pigment with the stationary phase ODS (C-18) 5 µm with gradient elution system of methanol, acetone and ammonium acetate (1 M) and a flow rate of 1.0 ml / min. According to the Son (2004), elution gradient which increase the strength of the mobile phase for chromatographic analysis takes place. The effect of the gradient elution was shortened the retention time of the compounds strong retained on the column. Phytochemical test was conducted on the Uju flavonoids, alkaloids, steroids, terpenoids, saponins and tannins. The test resultsof phytochemical content contained in extracts of *Padina australis* can be seen in Table 1.

Table 1. Testing phytochemical content in the extract *Padina australis*

Type of test	Result	Description
Alkaloid	-	Brown positive. white/yellow and red/orange
Tanin	-	Greenish black positive
Saponin	+	Positively characterized by the presence of foam
Flavonoid	+	Positive green
Terpenoid	+	Positive red-green
Steroid	+	Positive Blue or green

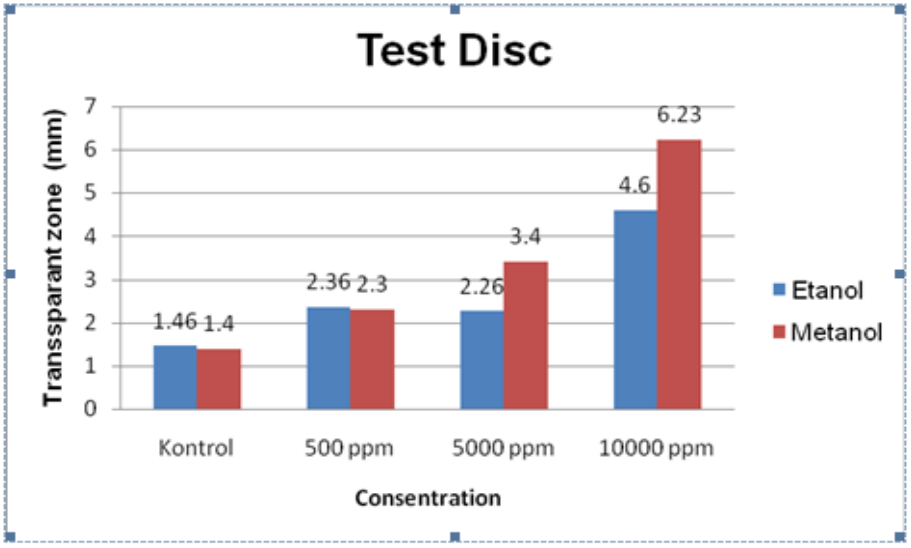

Figure 2. Tes Disc shows the relationship consentration and transparantzone

Test antibacterial activity against *Salmonella typhi* use *Padina australis* extract was used to determine the best solvent between methanol and ethanol in different concentrations in inhibiting the growth of bacteria. The graph above is antibacterial test results with the agar diffusion method or test discs graph using the average yield of 3 replications for each solvent and concentration.

DISCUSSION

To strengthen the results of the TLC, further identification is needed using UV-Vis spectrophotometry. Identification have been a simple process and does not need to make special preparation. Samples included in the cuvet spektro then placed in a special place on the tool then didetektor and spectral pattern will be read and wavelength produced. In addition the sample used for the identification process is very little ± 20 lm. Advantage of identification using UV-Vis spectrophotometer is a simple and very small concentration [9].

The results of the sample pigment suspected as isolate the from the β-carotene, chlorophyll a, chlorophyll b and fukosantin done to identify the next, which is identification with Spektrofotometer UV-Vis with long waves are used which 300-800 nm. Results identification of brown alga *Padina australis* with Spectrophotometer obtained wavelength value chlorophyll b 444,583 nm, fucoxanthin 450,455 nm, chlorophyll a 618,664 nm, feofitin 665 nm and β caroten 426,451 nm.

Using the quiet phase and the phases of the motion are virtually the same [10]. On every peak or peak suggests that the presence of pigment contained in kelp brown type of *Padina australis*. The sequence of each

peak or peak in the order of degree of polarity. Of pigment which has the highest degree of polarity to the polarity of the pigment which has the lowest level, because in this study applied a reversed-phase system or a "reversed phase". Reversed phase chromatography using a stationary phase that is less polar than the mobile phase her, because in general the HPLC method using hydrophobic stationary phase [11].

In Table 1 shows that the extract of *Padina australis* mengandung saponins, flavonoids, terpenoids. Its mechanism of flavonoid compounds thought to denature bacterial cell protein and cell membrane damage irreparably. Flavonoids also are lipophilic which would damage the membranes of microbes. In the flavonoid-containing phenol. Phenol is an acidic alcohol so-called karbonat. Fenol acid also has the ability to denature the protein and cell membrane damage. Acidic conditions by the presence of phenol can affect the growth of bacteria [12].

Terpenoids as an anti-bacterial mechanism that reacts with a transmembrane protein on the outer membrane of the bacterial cell wall, forming a strong bond polymers resulting in the destruction of transmembrane proteins [13]. Damage to the trans membrane protein is a compound entry and exit doors, will reduce the permeability of the bacterial cell wall resulting in bacterial cells will lack nutrients. So that inhibited bacterial growth or death. Saponins as antibacterial mechanism that lowers the surface tension, resulting in the increase in permeability or leakage of cell and intracellular compounds will come out [14].

On the pictures chart the test disc of showing that the concentration of extract *Padina australis* is effective inhibit the growth of *Salmonella typhi* using the solvent ethanol is at a concentration was 10,000 ppm as big as 4,6 ppm. As for the concentration of extract *Padina australis* is effective inhibit the growth of *salmonella typhi* use solvent metanol is to concentrate the 10,000 ppm as big as 6,23 mm. *Salmonella typhi* has the compound a handicap zone at extremely high concentrations. The higher the concentration of a material anti-bacterial activity then an anti-bacteria getting stronger anyway. And the results show that *Padina australis* is able to inhibit the growth of anti-bacteria gram positive and negative [15].

CONCLUSION

The identification of brown alga *Padina australis* with Spectrophotometer obtained wavelength value chlorophyll b 444,583 nm, fucoxanthin 450,455 nm, chlorophyll a 618,664 nm, feofitin 665 nm and β caroten 426,451 nm, while the results of the identification of crude brown alga *Padina australis* has a retention time chlorophyll c 6,432 sec, fucoxanthin 10,22 sec, chlorophyll a 38,5 sec, feofitin 56,82 sec and β caroten 62,144 sec.

Competing interests

The authors declare that they have no competing interests.

REFERENCES

1. Sachindra NM, Airanthi MKWA, Hosokawa M and Miyashita K 2010. Radical Scavenging and Singlet Oxygen Quenching Activity of Extract from Indian Seaweed. J. Food Sci. Technol. 47(1): 94.
2. Mori K, Ooi T, Hiraoka M, Oka N, Hamada H, Tamura M and Kusumi T, 2004. Fucoxanthin and its Metabolites in Edible Brown Algae Cultivated in Deep Seawater. Marine Drugs, 2: 63-72.
3. Fardiaz S. 1992. Food microbiology 1. PT. Gramedia Jakarta Main Library.
4. Meilgaard M, Civille GV and Carr BT. 2007. Sensory Evaluation Techniques Third Edition. CRC Press. New York.
5. Sulistijo 2002. Opportunities and Challenges of Business Development of Seaweed in Indonesia. Proceedings of the National Seminar on Business Aquaculture dated 30 October 2002. Surabaya. Pp.22.
6. Salosso Y, Prajitno A, Abadi AL and Aulanni AM. 2011. Study Potential *Padina australis* as an Antibacterial Natural in controlling bacteria Vibrioalginolitycus in Cultivation of Fish Grouper Rat (*Cromeleptus altivelis*). The faculty fisheries and of marine science. Brawijaya University. Journal Indonesian Natural Material, 7: 7.
7. Auliya P, Wonorahardjo S and Zakia N. 2013. Inluence of Composition Phases of the Motion to the Levels of Benzoic Acid and Caffeine in Coffee Packaging Uses the Method HPLC (High Performance Liquid Chromatography). Of chemical. Faculty of Science. Malang University.
8. Siregar AF, Sabdono A, Pringgenies D. 2012. Potential Antibacterial in Seaweed Extract Against Bacteria Skin diseases Pseudomonas aeruginosa, Staph epidermidis, and Micrococcus luteus. Study of marine science. Faculty of fisheries and oceanography. Diponegoro University. Semarang. Journal of Marine Research. 1(22): 152-160.

9. Indharini U. 2010. The Determination Levels of A-Mangostin Infusa Dry on The Rind of the Fruit Mangosteen (*Garcinia mangostana*). A thesis. The faculty of Pharmacy. Muhammadiyah University Surakarta. Surakarta.
10. Leenawaty L and heryanto. 2010. Study composition pigment and of the womb fucoxanthin brown seaweed from the waterways madura with a high-performance liquid chromatography. Marine science march 2010. Vol. 15 (1) 23-32. Engineering industry, Ma Chung University. Malang.
11. Sharif SA. 2009. Application of A Method High-Performance Liquid Chromatography (HPLC) For the Dexamethasone Levels In Tablet Mix with Deksklorfeniramin Maleic. Thesis. University North Sumatra: Medan.
12. Zaraswati D, Eva J. 2012. Effectiveness of Extract Rough Red Algae Eucheuma cottoni As an Antibacterial against Bacteria Pathogenic. Faculty of Biology. University Hassanudin Makasar. Page 1-7.
13. Cowan, M. 1999. Plants Product as Antimicrobial Agent. Clinical Microbiology Reviews, 12 (4): 564-582.
14. Robinson T. 1995. Organic content of higher plants. Publisher. ITB. Bandung. Page 191-213.
15. Michael PJ, Chan ECS, Noel KR, 1998. Microbiology, fifth Edition, Tata McGraw-Hill Publishing Company Limited. 143-146.

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Phytochemicals and Antibacterial Activities of Soursop Leaf (*Annona muricata*) against *Edwardsiella tarda* (In Vitro)

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Fisheries and Marine Science Faculty, University of Brawijaya, Indonesia

ABSTRACT: *Edwardsiella tarda* is bacteria in fish caused of Edwardsiellosis disease and it was include to plague bacteria that can harm. *Edwardsiella tarda* infection controlled using a natural antibacterial that is *Annona muricata*. The aim of this research was to know the content of secondary metabolites in leaves of the soursop (*Annona muricata*) as well as an effective solvent to extract *Annona muricata* to be an antibacterial against on *Edwardsiella tarda* by disc diffusion method. Experimental method used in this research is descriptive and it is based on 2 types of leaves (fresh and dried), 3 different treatment solvent (ethanol, ethyl acetate and chloroform) and three replications. Data obtained from the study was statistically analyzed used statistical applications, SPSS version 21.0. The results of the crude extract of dried *Annona muricata* is in accordance with the results of phytochemical contained phenols, saponins, tannins and flavonoids as well as the best use of ethyl acetate solvent to the extent of inhibition with 15:41 mm.
Key words: Antibacterial, *Edwardsiella tarda*, *Annona muricata*, Phytochemicals, Solvent

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INTRODUCTION

Some obstacles often encountered in aquaculture as disease. *Edwardsiella tarda* is bacteria in fish farming and caused of Edwardsiellosis disease. Edwardsiellosis is a bacterial disease that is a very serious systematic cultivation of eels (Japanese eel) in Japan and Taiwan, Japanase flounder fish and other farmed fish. In the USA, *Edwardsiella tarda* caused of septicemia infectious diseases in fish channel catfish (*Ictalurus punctatus*), host and geographic distribution. These bacteria include in fish pests and diseases of quarantine (HPIK) class II which require high vigilance to prevent of entry and spread of this disease in Indonesia because these diseases relatively can infect and harming quick time [1].

Infection control efforts by fish health management through control measures [2]. *Edwardsiella tarda* bacteria by using a natural antibacterial with environmentally friendly, one of them can use the leaves of soursop *Annona muricata*. In general, according to Octavia [3], soursop leaves is known to contain active compounds that are toxic, this situation allows the soursop can be used as an antibacterial compound. Upendra and Khandenwal [4] stated that extracts of *Annona muricata* has shown antibacterial activity against in eight species of pathogenic bacteria. The investigation clearly revealed the antibacterial properties of this plant can be used in the prevention of diseases caused by pathogenic bacteria.

The purpose of this research is to know the content of secondary metabolites in leaves of the soursop (*Annona muricata*) by phytochemical test and get the kind of effective solvent to extract *Annona muricata* as an antibacterial against *Edwardsiella tarda* by disc diffusion method.

MATERIAL AND METHODS

This study was conducted in November 2014 - January 2015 in the Laboratory of Parasitic Diseases Fish and Fishery Product Technology Laboratory, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang. *Edwardsiella tarda* bacterial identification was done in Fish Quarantine (Station I), Perak, Surabaya.

Extraction of the leaves of *Annona muricata* (fresh and dried) with multilevel maceration method (2x24 hours) using the appropriate solvent polarity in a row, chloroform, ethyl acetate, and ethanol. The method used in this research was descriptive based on the experimental methods. This study consisted of 3 different treatment solvent (ethanol, ethyl acetate and chloroform) and 3 replications, also used as a comparator antibiotic ampicillin as positive control. The parameters observed in this study were the extent of inhibition (mm).

Data obtained from the results of the research statistically analyzed using statistical applications, SPSS version 21.0. *Annona muricata* analyzed descriptively to phytochemical test of secondary metabolites.

RESULTS

Yield of *Annona muricata* extract

The results obtained from the extraction process of soursop leaf *Annona muricata* extract for fresh and dried leaves are different. The extraction of soursop leaves *Annona muricata* as % yield of extract can be seen in Table 1. Based on Table 1, yield of the resulting extract solvent chloroform was the smallest (4.98%), while the solvent ethyl acetate was 7.85% and the ethanol was 8:43%.

Table 1. Yield of *Annona muricata* extract

Solvent	Yield of extract (%)	
	Fresh	Dried
Chloroform	3.37	4.98
Ethyl acetate	5.98	7.85
Ethanol	6.20	8.43

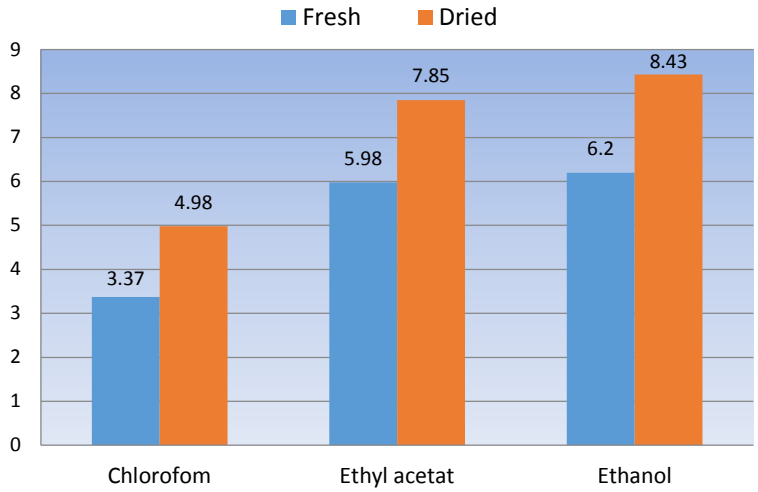

Figure 1. Yield of *Annona muricata* Extract

Yield of extract seen from the chart above that the extract produced from either fresh or dried leaves of the soursop showed that soursop leaf extract with ethanol and ethyl acetate to produce yield of extract was more than chloroform. To fresh extract produce yield of extract relatively smaller because there are many components of water it so difficult to the solvent to enter and extract the compounds contained. This suggests that the bioactive compounds of *Annona muricata* more soluble in ethanol and ethyl acetate as compared with chloroform. Components carried on the extraction process is a component in accordance with the polarity of the solvent, therefore the type of solvent used can affect the amount of yield extract produced [5]. Obi and Onuoha [6] reported that alcohol is the best solvent for extracting the active component of plants.

Inhibitory of *Annona muricata*

Antibacterial activity test used by the test method discs with a diameter of inhibition of bacteria. The amount of inhibition diameter of each extract can be seen in Table 2. Table 2 showed that the average of inhibition produced by extracts of dried leaves of *Annona muricata* on growth *Edwardsiella tarda* greater when compared to fresh leaves extract. This is because the dried leaves of *Annona muricata* more compounds are secondary metabolites that can easily extractable than fresh leaves of *Annona muricata* that there are still have some water [7].

Utilization of different types of solvents (chloroform, ethyl acetate, and ethanol) gives effect to the inhibition of bacterial growth resulting in *Edwardsiella tarda*. Type of solvent ethyl acetate has the greatest inhibitory 15:41 mm compared with solvent ethanol extracts with inhibition of 10.81 mm and chloroform 1:20 mm. This is because the solvent ethyl acetate that is semi-polar can partially dissolve polar and non-polar compounds contained in the sample. Active components that can be dissolved in ethyl acetate and are alkaloids, phenols, hydroquinone, flavonoids, terpenoids, saponins, steroids. Component content more antibacterial compounds contained in the ethyl acetate extract, where in the hydrophobic and hydrophilic components respectively contained in extracts and has a high antibacterial activity. As a result, the highest inhibition was

obtained in the ethyl acetate extract as component hydrophobic and hydrophilic able to enter the cell membranes and inhibit cell metabolism [8].

Table 2. Inhibition Test Results of *Annona muricata* Leaf Extract on *Edwardsiella tarda*

Solvent	Diameter of Inhibitory (mm) ($\bar{x}$ ± sd)	
	Fresh Leaves	Dried Leaves
Chloroform	0.67 ± 0.14	1.20 ± 0.21
Ethyl acetate	3.92 ± 0.13	15.41 ± 0.18
Ethanol	5.74 ± 0.28	10.81 ± 0.30

A compound having the optimum polarity will have a maximum antimicrobial activity, due to the interaction of a compound with the bacteria needed antibacterial hydrophilic-lipophilic balance [9]. Polarity is a compound physical properties important antimicrobial compound. Hydrophilic properties required ensuring the compound soluble in the water phase which is the place of microbial life, but compounds acting on cell membranes require also hydrophobic lipophilic properties, so that the antibacterial compound requires a hydrophilic-lipophilic balance to achieve optimal activity [10].

Phytochemicals of *Annona muricata*

Phytochemical test done to the best extract with ethyl acetate solvent in the leaves of soursop *Annona muricata* method [11] which consist of alkaloid test, steroids test, flavonoids test, tannins test / polyphenols, terpenoids test, and saponin test. Results of phytochemical test ethyl acetate extract of leaves of *Annona muricata* can be seen in Table 3. From the Table 3 it can be seen that the dry extract of *Annona muricata* contained saponins, tannins/polyphenols, flavonoids and steroids with positive results. Phytochemical test results to the ethyl acetate extract of *Annona muricata* according to study of Wisdom et al. [12], where in the phytochemical analysis of the leaves of the soursop their secondary metabolites such as Tanin/polyphenols, steroids, saponins and flavonoids. Flavonoid compounds contained in extracts of leaves of *Annona muricata* potential as an antibacterial for being able to inhibit the growth of bacteria by destroying the cell wall permeability, microsomes, lysosomes and bacterial cells as a result of interaction between flavonoids with DNA [13].

While the tannins/polyphenolic able to act as an antibacterial in the way it reacts with cell membranes, inactivate enzymes - essential enzymes, function and metabolism of the cell's genetic material so hampered and disrupted cell wall synthesis [14]. Saponins are surface active compounds resulting from steroid or triterpene group that binds to sugars, these compounds have a beneficial biological effect that is as hypocholesterolemic and anti-carcinogen and can boost the immune system [15]. Saponins inhibit the growth or kill microbes by interacting with membrane sterols. The main effect is the release of saponin tehadap bacterial proteins and enzymes of the cells [16].

Table 3. Result of Phytochemicals Test to Extract with Ethyl Acetate Solvent in the Leaves of *Annona muricata*

Compound	Result (+/-)
Saponin	+
Tannin/Poliphenols	+
Alkaloid	-
Terpenoids	-
Flavonoid	+
Steroid	+

CONCLUSION

Crude extract of *Annona muricata* in % yield of extract the highest of dry extract with ethanol (8.43%) and ethyl acetate (7.85%). For dry extract according to test results phytochemical classes of compounds containing phenol, saponins, tannins/polyphenols and flavonoids as well as the best solvent to extract *Annona muricata* using ethyl acetate with an area of inhibition that can inhibit the growth of *Edwardsiella tarda* (15.41 mm).

Competing interests

The authors declare that they have no competing interests.

REFERENCES

1. Plumb JA. 1993. Edwardsiella septicaemia. In: Inglis V, Roberts RJ, Bromage NR (eds). Bacterial Diseases of fish. Oxford, UK: Blackwell Scientific Publications. 61-7.
2. Kordi MGH. 2004. Pengendalian Hama dan Penyakit Ikan. Jakarta (ID): Rineka Cipta dan Bina diaksara.
3. Octavia L 2003. Uji Antibakteri, Penentuan Kadar Vitamin C, Dan Gula Total Pada Buah Sirsak (*Annona muricata*). Jurnal Kimia Institut Pertanian Bogor. Vol.2 No.2.
4. Upendra R and Khandelwal P. 2012. Assessment of Nutritive Values, Phytochemical Constituents and Biotherapeutic Potentials of Epiphyllum Oxypetalum. International Journal of Pharmacy and Pharmaceutical Sciences. Vol.4; 287-297.
5. Markham LG. 1988. Fish Hatchery Management. United State Departemen of The Interior Fish and Wildlife Service: Washington DC. 304-306 p.
6. Obi V and Onuoha C. 2000. Extraction and Characterization Methods of Plants and Products. In: Biological and agriscultural techniques. Ogbulie JN, Ojiako OJ (eds). Websmedia publications, Owerri. Pp. 271-286.
7. Marlindaa, M, Sangja dan M, Wuntua D. 2012. Analisis Senyawa Metabolit Sekunder dan Uji Toksisitas Ekstrak Etanol Biji Buah Alpukat (Persea americana Mill.). Jurnal MIPA UNSRAT Online 1 (1) 24-28.
8. Parhusip A. 2006. Kajian Mekanisme Antibakteri Ekstrak Andaliman (Zanthoxylum acanthopodium DC) Terhadap Bakteri Patogen Pangan. Disertasi Pascasarjana IPB. Bogor.
9. Kanazawa A, Ikeda T. and Endo T. 1995. A Novel Approach to Made of Action on Cationic Biocides; Morfological effect on Antibacterial Activity. J Appl Bacteriol 78: 55-60.
10. Branen AL and Davidson PM. 1993. Antimicrobial in Food. Marcel Dekker. New York.
11. Harborne JB 1998. Fitokimia. Penuntun Cara Modern Menganalisis Tumbuhan. Terjemahan Padmawinata, K. Penerbit ITB. Bandung. 354 hlm.
12. Wisdom S, Ugoh GO and Mohammed B. 2014. Phytochemical Screening and Antimicrobial Activities of *Annona muricata* (L) leaf extract. American Journal of Biological, Chemical and Pharmaceutical Sciences, 2: 44-47.
13. Sabir A. 2005. Aktivitas Antibakteri Flavonoid Propolis Trigona sp terhadap Bakteri Streptococcus mutans (in vitro). Maj. Ked. Gigi. (Dent. J.). 38(3): 135-141.
14. Roslizawaty Nita Y. Fakhrurrazi dan Herrialfian R. 2013. Aktivitas Antibakterial Ekstrak Etanol dan Rebusan Sarang Semut (Myrmecodia sp.) Terhadap Bakteri Escherichia coli. Jurnal Medika Veterinaria. 7(2).
15. Meskin MS, Bidlack WR, Davies AJ, Omaye ST. 2002. Phytochemicals in Nutrition and Health. CRC Press, London- New York.
16. Zablotowitcz RM, Hoagland RE, Wagner SC. 1996. Effect of Saponin on The Growth and activity of Rizophere Bacteria. CRC Press, USA.

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Antimicrobial Activity of *Bacillus cereus* and *Bacillus thuringiensis* on Pathogenic *Vibrio harveyi* in *Litopenaeus vannamei*

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ABSTRACT: The bacterial strain of *Bacillus cereus* and *Bacillus thuringiensis* has been known to produce antimicrobial activity against pathogenic *Vibrio harveyi*. The effect of *B. cereus* and *B. thuringiensis* were tested by in vitro and in vivo. In vitro test was used to analyze antagonism characteristic of bacteria using the paper disc diffusion method. In vivo test was applied to evaluate antimicrobial activity of *B. cereus* and *B. thuringiensis* (10^5 CFU ml^{-1}) on survival rate and histopathology of *Litopenaeus vannamei* challenged with *V. harveyi*. The results showed that *B. thuringiensis* had a greater inhibitory activity of 18.60–35.97 mm. Both Bacillus bacteria treatment resulted in survival rate of 100%, compared with 75% in the treatment without Bacillus. It can be concluded that *B. cereus* and *B. thuringiensis* have potential applications for controlling pathogenic *V. harveyi* in shrimp aquaculture.
Key words: *Bacillus cereus*, *Bacillus thuringiensis*, *Inhibitory Activity*, *V. harveyi*.

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INTRODUCTION

Aquaculture is the world's fastest growing food production sector. However, disease outbreaks have caused serious economic losses in several countries. Vibrio species are among the most important bacterial pathogens of cultured shrimp. They are responsible for several diseases and mortalities up to 100% due to vibriosis have been reported [1]. Using antibiotics in potential negative consequences of using antibiotics in aquaculture for the prophylactic treatment of diseases are the development of drug resistant bacteria and reduced efficacy of antibiotic treatment for human and animal diseases [2].

In the search for more effective and environmentally friendly treatments, using bacteria like Bacillus provides a solution to these problems. Bacillus have antimicrobial (bacteriocin) which usually occurs in all growth phases and finishes at the end of logarithmic phase [3]. Luis-Villaseñor et al. [4] investigated that the effect of Bacillus showed trait inhibitory to Vibrio and ability to adhere and grow on intestinal mucus.

The purpose of this study was to investigate antimicrobial activity of *B. cereus* and *B. thuringiensis* against *Vibrio harveyi* under in vitro and in vivo conditions.

MATERIAL AND METHODS

Bacterial strains

A virulent strain of *V. harveyi*, was used as a pathogenic strain. Strain were taken from the stock culture collection of our laboratory. *B. cereus* and *B. thuringiensis*, obtained from isolated from the gastrointestinal of L. vannamei and cultured in duplicate in the general media (nutrient agar with 1.5% w/v NaCl) for 18-24 hours at a temperature of 30°C. Pure isolates were taken after subculture on Tryptic Soya Agar (TSA) was used as an antagonistic strain.

Antagonism assay

The methods of paper disc diffusion assays were used in this study. Both groups of the bacterial strains (the tested strains including the compared strain and the pathogenic strain) were briefly grown in tryptic soya broth (TSB), incubated at 32°C for 24 h. After 24 h, each sterilised paper disc was immersion with B. cereus and B. thuringiensis with different concentration (10^4, 10^5 and 10^6 CFU ml^{-1}). And then placed on the surface of an agar plate (TSA) which was previously inoculated with the indicator pathogen at a concentration of about 10^7 CFU ml^{-1}. The plate was then incubated at 32° C for 24 h and the inhibition zone around paper disc was recorded.

SDS-PAGE electrophoresis test for molecular weight

For detection of bacteriocin activity use polyacrylamide gels and stained with silver nitrate to determine the molecular weight of the separated protein. Low molecular mass standards ranging from 10 to 225 kDa were purchased.

Inhibition of pathogenic *V. Harveyi,* in vivo

L. vannamei were introduced into 5 treatment (each in tripled) filled with filtered seawater at salinity of 20 ppt. The set A was inoculated with *B. cereus* at the concentrations of 10^5 CFU ml^{-1} in rearing medium to facilitate attachment or colonization on the larvae for 24 hours. After one day was then exposed to test pathogen (V. harveyi) at 10^7 CFU ml^{-1} for 2 hour. The set B was inoculated with *B. thuringienis* at the concentrations of 10^5 CFU ml^{-1}. After one day was then exposed to test pathogen (*V. harveyi*) at 10^7 CFU ml^{-1} for 2 h. The set C was inoculated with *B. cereus* and *B. thuringiensis* (combined treatment) at the concentrations of 10^5 CFU ml^{-1}. After one day was then exposed to test pathogen (*V. harveyi*) at 10^7 CFU ml^{-1} for 2 h. The set K(n) received no bacterial inoculums and served as control. The set K(-) was inoculated with pathogen alone at 10^7 CFU ml^{-1} in rearing medium to serve as negative control. The number of CFU ml^{-1} in overnight culture of baceria were standardized from OD measurements at 600 nm. Mortality in each set was recorded for twelve days and no water exchange was done during that period.

Statistical Analysis

Results were presented as mean ± mean standard deviation of three replicates. Statistical analysis was performed using SPSS 20.0. To determine the effect of treatment on the response of each parameter used ANOVA analysis followed by Duncan's multiple range test. Levels of significance are expressed as $P < 0.05$.

RESULTS AND DISCUSSION

Antagonism assay

Antimicrobial activity of *B. cereus* and *B. thuringiensis* against *V. harveyi* were performed by paper disc with different concentrations. The diameters of the inhibitory zones of *B. thuringiensis* was 18.60–35.97 mm (Table 1). We suggested that *B. thuringiensis* showed a greater inhibitory activity than others. The antagonistic effect of these isolates on the growth of indicator pathogen could be determined by the appearance of clear inhibition zones around the paper disc. Bacillus species could produce a large number of antimicrobials [3].

Table 1. Antagonistic activity of *B. cereus* and *B. thuringiensis* strains against *V. harveyi*

Bacteria	Inhibition zones (mm)		
	10^4	10^5	10^6
B. cereus	16.47±0.67	22.80±1.14	31.70±1.96
B. thuringiensis	18.60±1.06	25.13±1.07	35.97±1.58
B. cereus + *B. thuringiensis*	13.79±0.81	20.81±1.47	25.46±0.64

SDS-PAGE electrophoresis test for molecular weight

Direct detection of the bacteriocin was performed by SDS-PAGE. Following electrophoresis, several contaminating proteins were detected in sample. The band had an apparent molecular mass of about 10-225 kDa.

Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by Abriouel et al. [5]. The results (Fig. 1) showed that *B. cereus* had molecular weights of 15.53 kDa and 25.21 kDa while *B. thuringiensis* had molecular weights of 12.19 and 23.25 kDa which there are antimicrobial polypeptides of intermediate size.

Inhibition of pathogenic *V. harveyi* in vivo

Survival rate: The results revealed that *B. cereus* and *B. thuringiensis* lead to reduce shrimp mortality under in vivo conditions. Both Bacillus treatments resulted 100 % survival rate of shrimp, while survival rate of 75% was obtained in not treated shrimp (Figure 2). Bacillus significantly increased survival rate and some digestive enzyme activities of shrimp larvae [6].

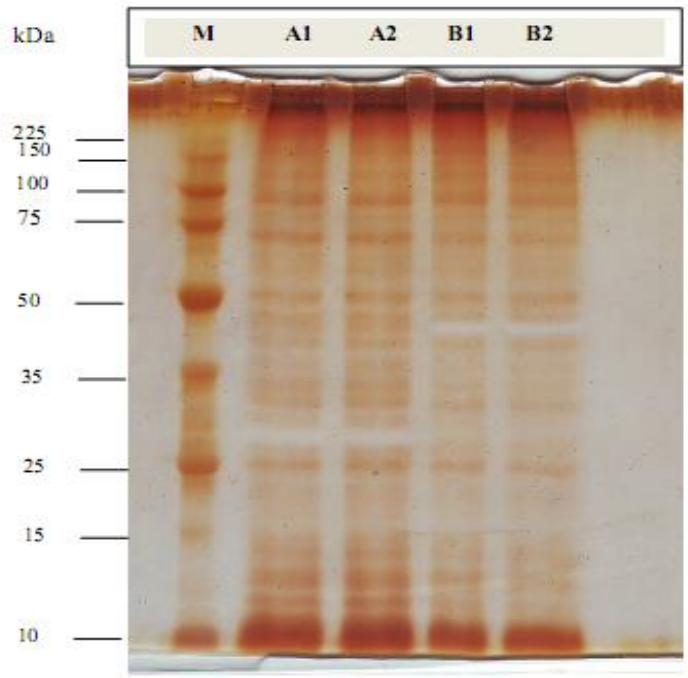

Figure 1. SDS-PAGE analysis protein profile. Line (M): Silver nitrate-stained gel, low range protein standard; line (A1 and A2) protein profile *B. cereus*; line (B1 and B2) protein profile *B. thuringiensis*.

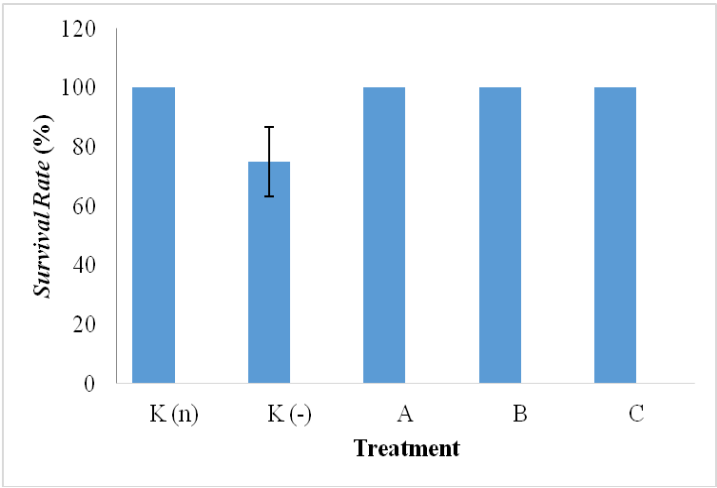

Figure 2. Survival rate recorded during twelve days

Total Haemocyte Counts (THC) : The total haemocyte counts showed significant differences among the treatments. The average of total haemocyte was 5.93 x 10^5 sel mm^{-3} – 89.17 x 10^5 sel mm^{-3} (Table 2). THC of crustaceans plays an important role in regulating the physiological functions including hardening of exoskeleton, wound repair, carbohydrate metabolism, transport and storage of protein and amino acid, haemolymph coagulation and the confinement of invasive organisms by clot formation, phagocytosis, and encapsulation [7].

Table 2. Total haemocyte counts among different treatments

Treatment	THC (x 10^5 sel mm^{-3})
K (n)	12.75 ± 0.75^{d}
K (-)	15.73 ± 0.75^{a}
A	37.3 ± 1.53^{b}
B	89.17 ± 3.75^{a}
C	5.93 ± 0.12^{c}

Fig K(n) : not infected with *V. harveyi* bacteria; Fig K(-): Infected with 10^7 CFU/ml *V. harveyi* bacteria; Fig A: Adding *B. cereus* and infected *V. harveyi*;Fig B: Adding *B. thuringiensis* and infected *V. harveyi*; Fig C: Adding *B. cereus* and *B. thuringiensis* than infected *V. harveyi*.

THC in crustaceans rapidly drops following the injection of foreign material. *B. cereus* and *B. thuringiensis* in *L. vannamei* can be increased significantly after the injection of *V. harveyi*. This study demonstrated promising

results for immune response stimulation in *L. vannamei*. Moreover, Rengpipat et al. [8] explained that *Bacillus* S11 surface antigens, or their metabolites might act as immunogens for shrimp immune defense. *Bacillus* S11 cell wall peptidoglycan might elicit an immune function in shrimps.

Histopathology of Hepatopancreas

The hepatopancreas of control K(n) maintained a good shape and colour. The hepatopancreas of infected larvae were grey and showing vacuolization (Figure 3). The hepatopancreas or digestive gland is a sensitive indicator of diverse physiological states, such as metabolic level, ecdysis phase, nutritional status, disease, etc., so that its use as a general indicator of the physiological condition of the shrimp has been proposed [9].

Figure 3 detected the sites of bacterial presence in the tissue, and the changes caused by the pathogens could be evaluated. Obviously, death will occur when a sufficiently large number of hepatopancreatic cells are damaged by the pathogen, rendering the organ non functional. Robertson et al. [10] explained that the hepatopancreas of chronically infected larvae were grey and aciated, with balls of necrotic tissue. Shrimp with infected by virus or bacteria, pathology of hepatopancreas showing severe necrosis, loss of structure, atrophy of tubule epithelial cells, vacuolation and rounding and sloughing of cells into the lumen [11].

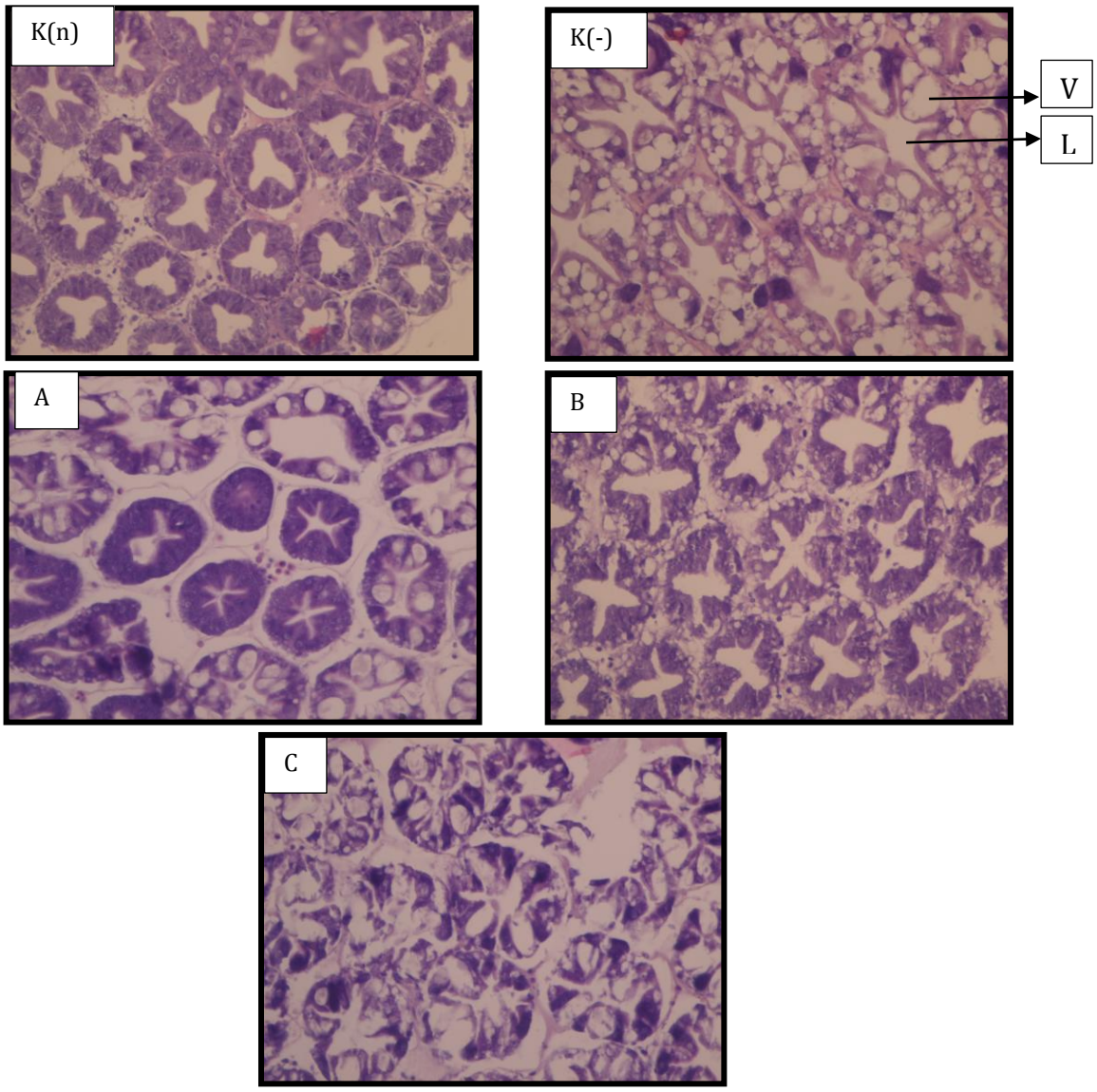

Figure 3. Histopathology of *L. vannamei* hepatopancreas (arrows indicated vacuolation)

Fig K (n): Hepatopancreas of control shrimps group (not exposed to *V. harveyi* bacteria); Fig K (-): Infected hepatopancreas of shrimps exposed to 10^7 CFU/ml *V. harveyi* bacteria. There are many vacuolation of cells (black arrow destruction of hepatopancreas tissue); Fig A: Adding *B. cereus* and infected *V. harveyi*. Some of tissues are healthy (stars/L) also vacuolation is observed; Fig B: Adding *B. thuringiensis* and infected *V. harveyi*. Some of tissues are healthy (stars/L) also vacuolation is observed but low than Fig A; Fig C: Adding *B. cereus* and *B. thuringiensis* and infected *V. harveyi*. There are many vacuolation of cells (black arrow), cells are finally destroyed. L (lumen) and V (vacuolation).

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Competing interests

The authors declare that they have no competing interests.

REFERENCES

1. Karusnagar IPR and Malathi GR. 1994. Mass Mortality of *Penaeus monodon* Larvae Due to Antibiotic Resistant *Vibrio harveyi* Infection. Aquaculture. 128: 203-209.
2. Moriarty DJW. 1997. The role of Microorganisms in Aquaculture ponds. Aquaculture. 151: 333-349.
3. Aly S, Cheik OAT, Imael BHN and Alfred TS. 2006. Bacteriocins and Lactid Acid Bacteria Minireview. African Journal of Biotechnology. 5(9): 678-683.
4. Luis-Villaseñor IE, Campa-Córdova AI and Ascencio-Valle FJ. 2012. Probiotics in Larvae and Juvenile Whiteleg Shrimp *Litopenaeus vannamei*. Intech. 601-602.
5. Abriouel H, Franz CMAP, Omar NB and Galvez A. 2010. Diversity and Applications of Bacillus Bacteriocins. FEMS Microbiology. 35: 201-232.
6. Zhou X, Wang Y and Li W. 2009. Effect of Probiotic on Larvae Shrimp (*Penaeus vannamei*) Based on Water Quality, Survival Rate and Digestive Enzyme Activities. Aquaculture. 287: 349-353.
7. Martin GG, Hose JE, Omori S, Chong C, Hoodbboy T, Mckrell N. 1991. Localization and Roles of Coagulogen and Transglutaminase in Hemolymph Coagulation in Decapod Crustaceans. Comparative Biochemistry and Physiology. 100(3): 517-522.
8. Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S and Menasaveta P. 2000. Immunity Enhancement in Black Tiger Shrimp (*Penaeus monodon*) by a Probiont Bacterium (*Bacillus* S11). Aquaculture. 191: 271-288.
9. Rosas C, Bolongaro-Crevenna A, Sánchez A, Goriola G, Soto and Escobar E. 1995. Role of Digestive Gland in Energetic Metabolism of *Penaeus setiferus*. Biol. Bull. 189: 168–174.
10. Robertson PAW, Calderon J, Carrera L, Stark JR, Zherdmant M and Austin B. 1998. Experimental *Vibrio harveyi* Infections in *Penaeus vannamei* Larvae. Disease of Aquatic Organisms. 32: 151–155.
11. Ambipillai L, Sobhana KS, George KC and Sanil NK, 2003. Histopathological Survey of Cultured Shrimps in Cochin, Kerala. Journal of the Marine Biological Association of India. 45(2): 178-185.

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Characterization of Bacteriocin *Lactobacillus casei* on Histamine-Forming Bacteria

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ABSTRACT: Tuna which has undergone a process of decay will be poisoned if being consumed. It caused by the contamination of pathogenic bacteria such as *Escherichia coli, Salmonella, Vibrio cholerae, Enterobacteriacea* etc. Some types of fish contain histidine family scmbroidae high free, such as yellow tail tuna 740 mg/100g of meat, bigeye tuna 491 mg/100 g, mahi-mahi 344 mg/100 g, mackarel 600 mg/100 g, skipjack 1192 mg/100 g and albakor highest to 2 g/100 g. The formation process of histamine in fish is influenced by the activity of the enzyme *L-Histidine Decarboxylase* (HDC). Bacteriocin *Lactobacillus casei* extract is able to inhibit the activity of *Pseudomonas sp, Proteus morgani* and *Micrococcus sp.* The extract of bacteriocin *Lactobacillus casei* has a high temperature stability which has inhibitory activity against bacteria test at a temperature of 90°C. Bacteriocin *Lactobacillus casei* from bacteria *Pseudomonas sp* has the optimum activity at pH 5 with inhibition diameter of 8.25 mm, while the bacteria *Micrococcus sp* has the optimum activity at pH 4 with a inhibition diameter of 9.25 mm. Bacteriocin *Lactobacillus casei* has a molecular weight of 14.34 kDa which included in the group of class III bacteriocins, generally has a large size (> 10 kDa), and can not survive against the heat. Bacteriocins *Lactobacillus casei* extract can inhibit the activity of histamine-forming bacteria growth and have stable properties to high temperature and pH. Future research is recommended to do the production of bacteriocins *Lactobacillus casei* optimization and its application in fishery product.

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INTRODUCTION

Swordfish which belonging to the family of *scombroidae* will being easily decayed at the room temperature, and also the high water content in fish would be a suitable medium for the metabolism of spoilage bacteria that will causing the process of decay and the fish will no longer fresh anymore. The swordfish that has been decayed will be poisoned if being consumed. It caused by the contamination of pathogenic bacteria such as *Escherichiacoli, Salmonella, Vibrio cholerae, Enterobacteriacea* etc. Poisoning that often occurs by tuna were the histamine *(scombroid fish poisoning)* [1]. The process of formation of histamine in fish is influenced by the activity of the enzyme *L-Histidine Decarboxylase* (HDC). Various types of bacteria are capable to producing the enzyme HDC, including the Enterobacteriaceae, for example: *Enterobacter agglomerans, Enterobacter cloacae, Enterobacter intermedium, Hafnia alvei, Klebsiella pneumoniae,* and *Morganella morganii* [2].

To inhibit the bacterial growth, it is necessary to do the preventive measures in order to slow down the change of histidine so it will not cause the allergie such as by using bacteriocins as an antibacterial agent. Bacteriocins is one of the antimicrobial compounds that produced by lactic acid bacteria. Bacteriocins is defined as the active peptides or peptide complexes that were synthesized at the ribosomes, and also have the activity of bakteriostatic and bactericidal [3]. Bacteriocin that produced by LAB is a secondary metabolite produced by ribosomes, sensitive to proteolytic enzymes and may be inactivated by the digestive tract protease enzyme, generally has a heat resistance (60 ° C or 100 ° C for 30 minutes or more), stable at acid pH and neutral, inactivated at pH above 8.0 [4].

It is known that *Lactobacillus casei* bacteria can produce the bacteriocin that has antibacterial activity against several common pathogens and spoilage microorganisms in food production. It was explained by Chotiah [5] with the result that the crude bacteriocin *Lactobacillus casei* has antagonistic properties against pathogens *(S.typhimurium; E. coli; B. cereus* and *S. enteritidis.* Inhibitory activity against *E.coli* K99 enterotoxigenic and *S.*BCC *aureus* B2062 / ATCC 25923 is not visible. Based on the result above, this research will discuss about the chemical and physical characteristics of bacteriocins *of Lactobacillus casei* and its application on histamine-forming bacteria.

MATHERIAL AND METHODS

The Growth character of *Lactobacillus casei*

The growth curve of bacteria for 24 hours of incubation and sampling every 1 hour to determine the existing phases. A total of 5% (v / v) of the cultivation *Lactobacillus* MRS agar were grown in MRS broth and incubated at 37ºC. Bacterial growth is followed every hour to observe the optical density value or *optical density* (OD) of the starter on MRS media with turbidimetric method with a wavelength of 620 nm [6].

Making the Cultivation

The isolates of lactic acid bacteria that used in the form of agar slant culture MRS broth were rejuvenated with *yeast* addition as much as 0.5% (w / v). Rejuvenation or activation of *Lactobacillus casei* activation were done by growing a loop isolates grown in an agar medium slant into 10 ml MRS broth, then it were incubated at 37 ˚ C for 24-48 hours. After that, 1 ml of MRS Broth cultivation were taken to be grown in 10 ml MRS broth, then it were incubated at 37 ˚ C for 24-48 hours to obtain a cultivation [7]. Rejuvenation is also were done on bacteria test using *Tryptone Soya Agar* (TSA) media.

Extraction of Bacteriocin

The active cultures *of Lactobacillus casei* as much as 10% (v / v) were propagated in the 1000 ml of MRS broth (pH 7.0; glucose 0.25% w / v; peptone 0.5% w / v) for 20 hours at a temperature of 30˚C. Culture centrifuged at a speed of 10,000 rpm for 20 min at 4 ˚ C and neutralized using 1M NaOH to pH 7.0. The solution that were obtained then being filtered using a 0.2 µm membrane filter to obtain the cell-free supernatant [8]. Cell-free supernatant obtained from the extraction process that were precipitated with ammonium sulfate saturated solution of 60% (w / v) and were homogenized with a magnetic stirrer for 24 hours at a temperature of 4 ˚ C. Then, the precipitation solution was centrifuged for 30 minutes at 12000 rpm at 4 º C. The pellets were dissolved in 25 ml of 0.05 M *potassium phosphate buffer* (pH 7.0) [9]. The pellets obtained from the bacteriocins with ammonium sulfate precipitation that were dialyzed using a dialysis membrane (1.0 kDa). The buffer that being used were *potassium phosphate buffer* (pH 7.0) for 18 hours, then buffer have to be replaced at every 6 hours at a temperature of 4˚C to obtained the bacteriocins extract [8]. Bacteriocins solution that obtained by dialysis was centrifuged at ultracentrifugation at a speed of 50,000 rpm for 30 min at 4˚C and the supernatant that obtained were the bacteriocins extract [10].

Biological Characterization (Inhibitory Activity Bacteriocin)

Media MHA which have been sterilized then were put into petri dishes 20 ml each and allowed to solidify at room temperature. The medium were inoculated with 0.1 ml of bacterial suspension test and trimmed with a hockey stick, then allowed to stand to dry for 15 minutes. Bacteriocins extract as much as 50 mL were dripped on paper discs. Then placed on a paper disk media that has been inoculated by bacteria MHA test, then it were incubated at the temperature of 37 ˚ C for 24 hours. The clear zone that being formed indicates the existence of barriers of the growth of test bacteria by supernatant. We then measured the diameter of clear zone (mm) using calipers. The diameter of each inhibition zone was measured three times in different areas and then the results are averaged [11, 12].

Stability against temperature and time

This test were done by heating the bacteriocins as 400µl at a temperatures of 45 ˚ C, 70 ˚ C and 95 ˚ C for 15, 30 and 45 minutes. Then, the bacteriocins extract were tested about its inhibitory activity by disc diffusion method.

Stability against pH

This test were performed by addition of 0.1 M NaOH or 0.1 M HCl to make a difference in the pH level of 2 to 9. The volume of bacteriocins used is 400µl. Then, a solution of NaOH or HCl bacteriocins were homogenized and allowed to stand for a few minutes before being tested to its inhibitory activity against histamine-forming bacteria.

Chemical characterization (Molecular weight Bacteriocin)

Determination of molecular weight bacteriocins were using *Deodecyl Sodium Sulfate Polyacrilamide gel electrophoresis* (SDS-PAGE) [13].

RESULTS AND DISCUSSION

The growth phase of *Lactobacillus casei*

During the growth of lactic acid bacteria, the maximum production occurs at the end of the exponential phase or early stationary phase. The incubation period were used at the 19th hour which this phase occurs production of bacteriocins. The best production of bacteriocins was when it reaches the end of the exponential phase or early stationary phase. The growth curve *of Lactobacillus casei* [14] can be seen in Figure 1.

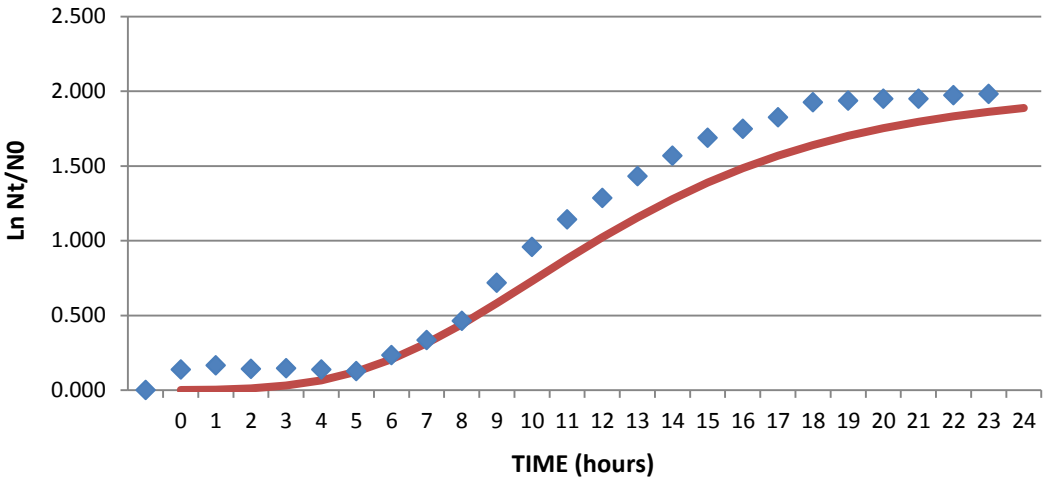


Figure 1. The growth curve *of Lactobacillus casei*

Biological Characterization

The diameter of inhibition zone was looked like diameter of the clear zone around the well which exhibits bactericidal (killing bacteria) or pseudo-diameter zone that showed bacteriostatic properties (inhibit microbial growth). The Clear zone formed by the secondary metabolites or another antimicrobial active compound that were produced. The test results inhibitory activity can be seen in Figure 2.

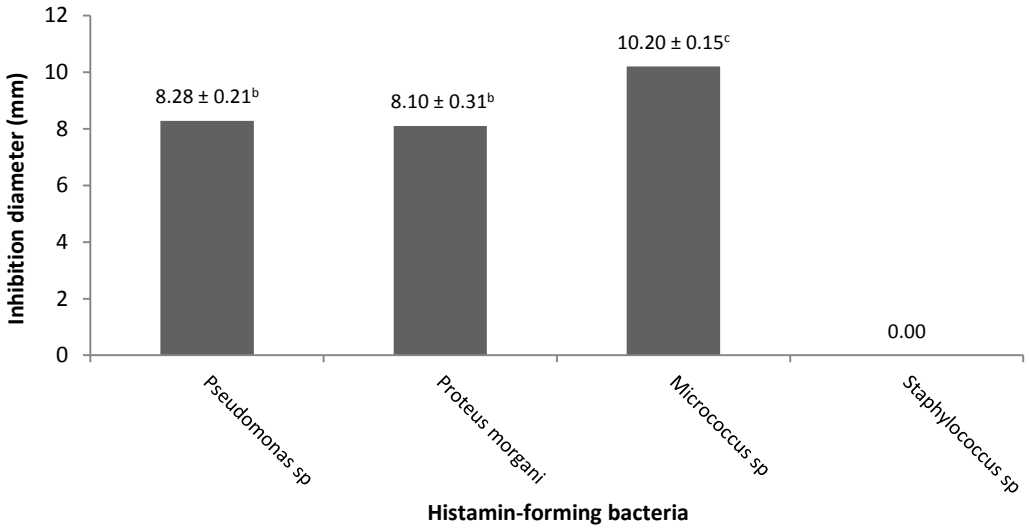
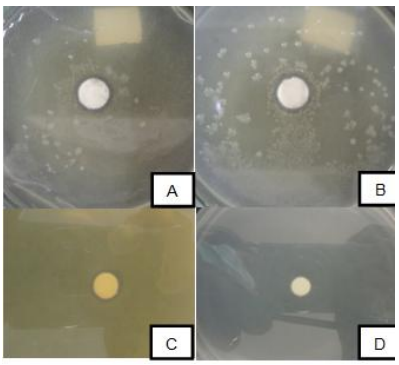


Figure 2. Graph bacteriocins extract inhibitory activity *of Lactobacillus casei*

On inhibition test bacteria *Staphylococcus sp* (Figure 3) there is no clear zone so that it can be stated that the bacteriocins *of Lactobacillus casei* can not inhibit the test bacteria *Staphylococcus sp* due to *Staphylococcus sp* has resistance to acids and has a strong cell wall (covalently bonded) so that this bacteria is more resistant to acids and other substances that were produced by antagonic *Lactobacillus casei* [15]. Bacteriocins extract of the bacterium *Lactobacillus casei* test has inhibitory activity which has inhibition diameter between 6-11 mm.



Description:

(A) *Pseudomonas sp*

(B) *Proteus morgani*

(C) *Micrococcus sp*

(D) *Staphylococcus sp*

Figure 3. Results of bacteriocins inhibition diameter *Lactobacillus casei* against histamine-forming bacteria

Based on the results of bacteriocin inhibitory activity against histamine-forming bacteria, bacteriocins can be concluded that the extract can inhibit the growth of *Lactobacillus casei* histamine-forming bacteria. Inhibition of the enzyme *L-histidine Decarboxylase* will affect in delayed or no product formation so that leads to the reduction or even the histamine can not be produced [16]. Bacteriocins will affect the membranes, DNA synthesis and protein synthesis. In general, bacteriocins showed bactericidal or bacteriostatic activity against other bacteria that are closely related to the producing strain. The main mechanism of bacteriocin was varied, they are the formation of pores in the cytoplasmic membrane or cell wall biosynthesis and inhibition of enzyme activity (RNAse or DNAse) in target cells [5].

Stability against the temperature

From the test results of the temperature characteristics of these bacteriocins, bacteriocins obtained inhibitory activity in bacteria *Pseudomonas sp* results can be seen in Figure 4 and the bacteria *Micrococcus sp* that the results can be seen in Figure 5.

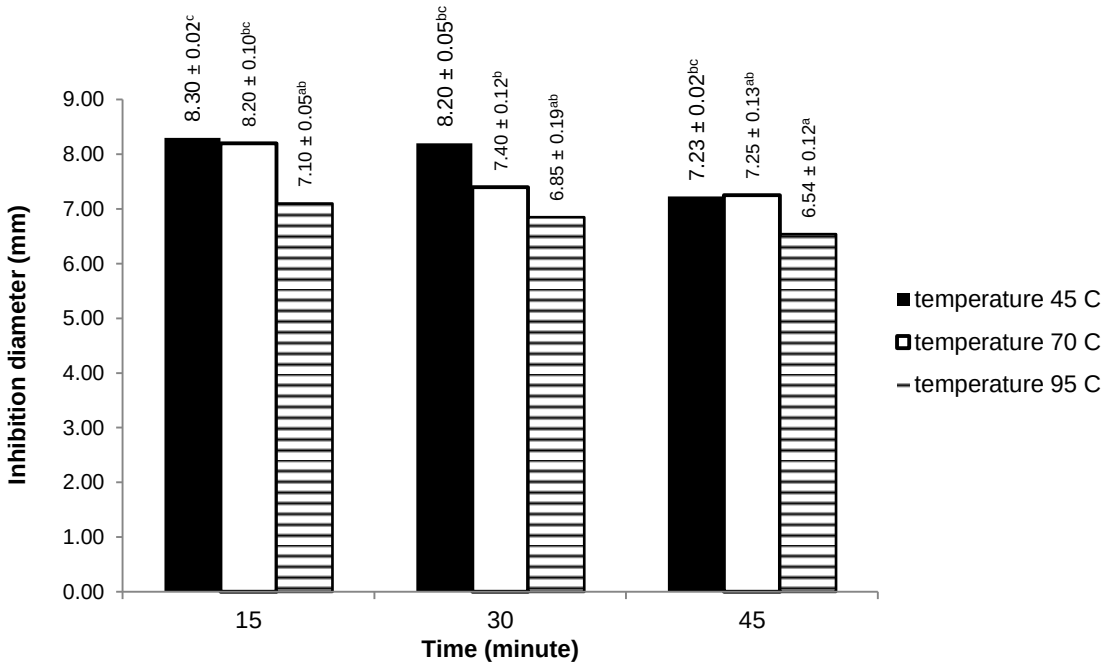


Figure 4. Graph bacteriocin inhibitory activity against the treatment temperature and heating time in bacteria *Pseudomonas sp*

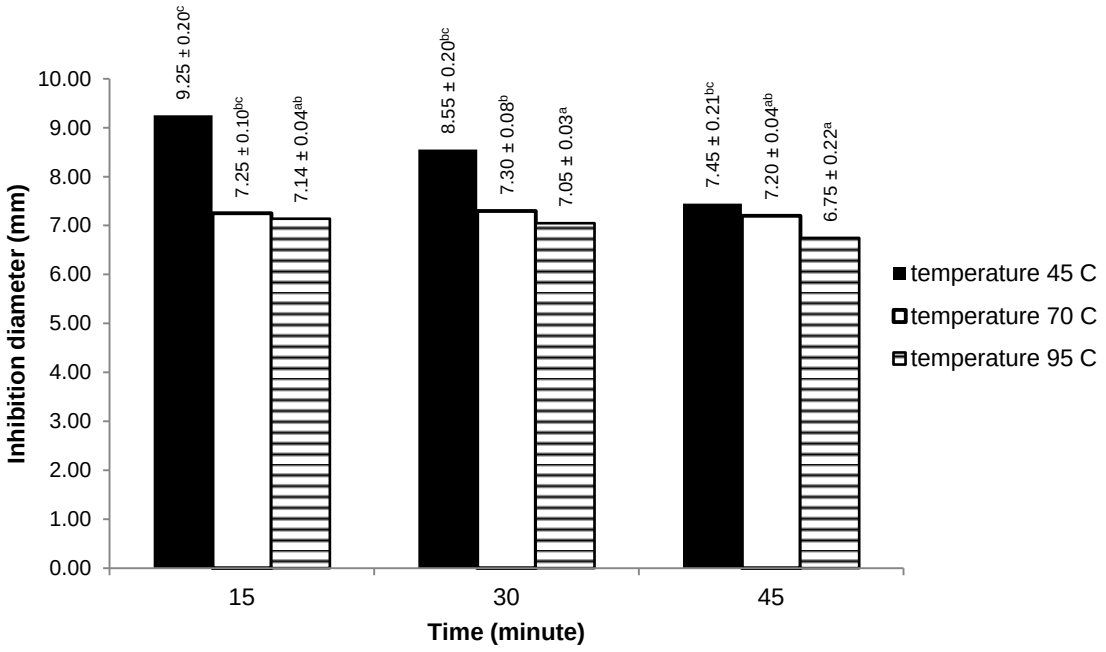


Figure 5. Graph bacteriocin inhibitory activity against the treatment temperature and heating time in bacteria *Micrococcus sp*

Bacteriocin *Lactobacillus casei* did not lose the inhibitory activity in the temperature range of 45 ° C, 70 ° C and 90 ° C, however, these bacteriocins decreased inhibitory activity against both the histamine-forming bacteria, the *Pseudomonas sp* and *Micrococcus sp.* Bacteriocin inhibitory activity decline continued with the increasing of heating temperature and the longer it has been used. It also can be seen in Figures 4 and 5 that the higher temperature was used, the smaller of diameter of the inhibition of bacteriocins against histamine-forming bacteria, as well as time. The longer it is used, the smaller the diameter of the inhibition of bacteriocins against histamine-forming bacteria. Bacteriocins of lactic acid bacteria resistant to the temperature of 100 ° C for 30 minutes even until the temperature of the autoclave. This is because in these bacteriocins may exist compounds - small globular compounds and their strong hydrophobic regions. It can be distinguished that from bakteriophage that is not heat resistant to autoclaving [17].

Stability against pH

The pH factor is often a consideration for preservatives to be used in food, especially food for livestock with low pH conditions such as beef, *ham,* meatballs, milk, butter, cheese etc. [18]. From the results of testing the characteristics of bacteriocins against this pH value, obtained bacteriocin inhibitory activity on histamine-forming bacteria *(Pseudomonas sp* and *Micrococcus sp),* the result can be seen in Figure 6.

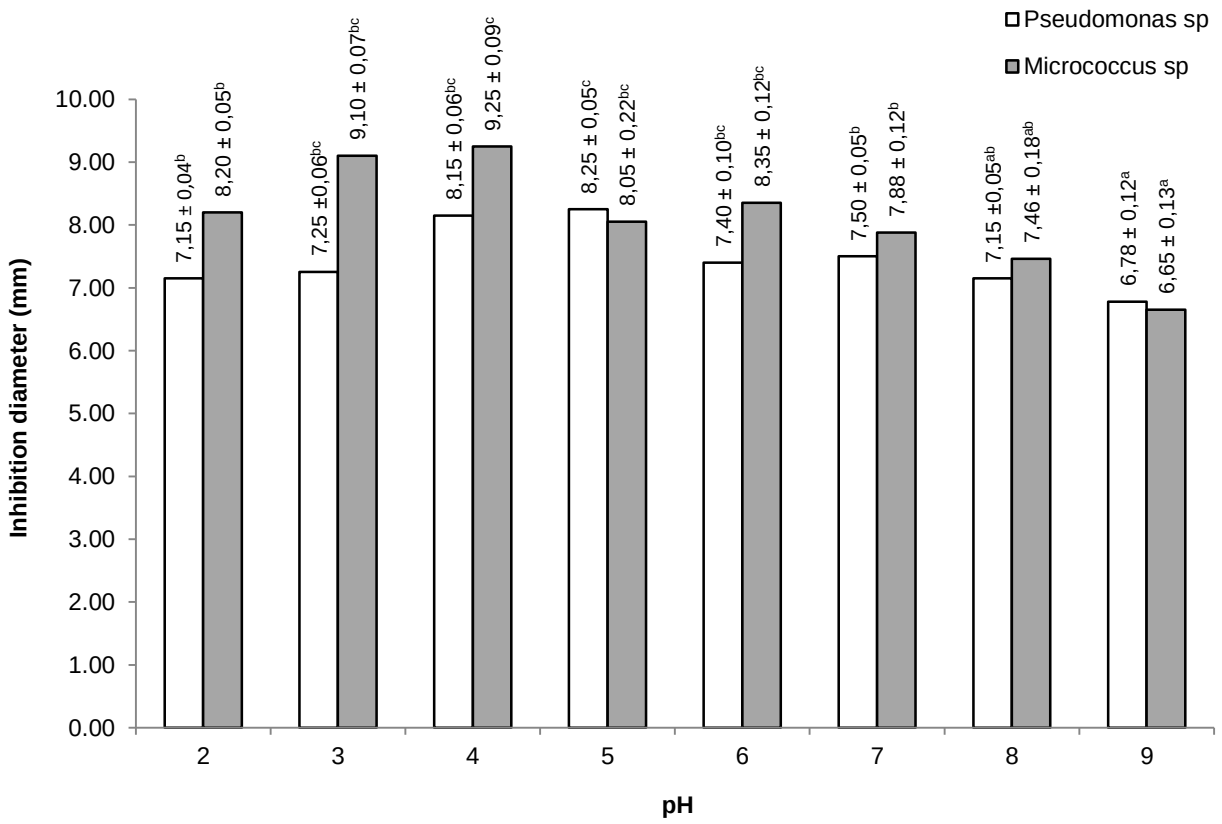


Figure 6. Graph bacteriocins extract inhibitory activity against pH

Bacteriocin *Lactobacillus casei* bacteria *Pseudomonas sp* test has optimum activity at pH 5 with inhibition diameter of 8.25 mm, while the test bacteria *Micrococcus sp* has optimum activity at pH 4 with a diameter of 9.25 mm inhibition. Bacteriocins produced by *Lactobacillus acidophilus* has optimum activity in the range of pH values of 4-5. Bacteriocin inhibitory activity decreased with increasing pH values (pH approaching the base) and active at acidic pH. The higher of the pH so the bacteriocin activity will be reduced, as seen in bacteriocins lost piscicolin activity at high pH near the pH 8 [19].

Chemical characterization (Molecular Weight)

Bacteriocins *of Lactobacillus casei* has a molecular weight of 14.34 kDa which can be seen in Figure 3. The molecular weight of bacteriocins were different by *Lactobacillus* species such as have a broad molecular weight range. Based on the size, morphology and physical, bacteriocins [20] produced by the bacterium *Lactobacillus casei* are grouped in Class III bacteriocins, which were generally large (> 10 kDa), and are not heat resistant consisting of two types. Type IIIa is bakteriolisin which is an enzyme of bakteriolitic. Examples are studied in this type is lisostaphin. Type IIIb is non-lytic type bacteriocins, one of which is helvetisin J (37 kDa) that were produced by *Lactobacillus helveticus.*

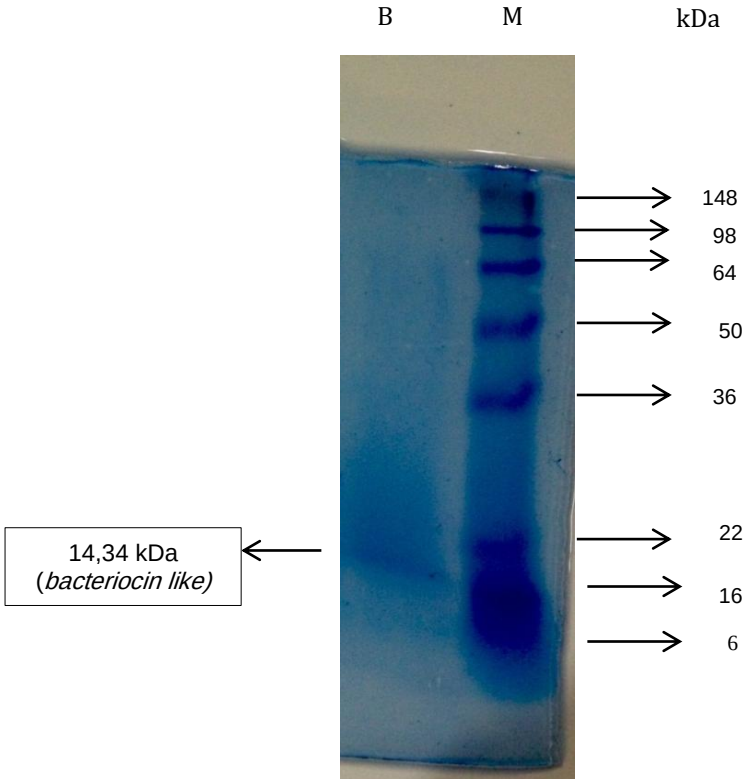


Figure 7. Result of elektroforesis SDS-PAGE

CONCLUSION

Bacteriocin *Lactobacillus casei* extract is able to inhibit the bacteria *Pseudomonas sp, Proteus morgani* and *Micrococcus sp.* Extract of bacteriocin *Lactobacillus casei* has high temperature stability which at a temperature of 95 ° C still has inhibitory activity against bacteria test. Bacteriocin *Lactobacillus casei* bacteria *Pseudomonas sp* test has optimum activity at pH 5 with obstacles measuring 8.25 mm, whereas the test bacteria *Micrococcus sp* has optimum activity at pH 4 with obstacles measuring 9.25 mm. Bacteriocin *Lactobacillus casei* active at acidic pH. Bacteriocin *Lactobacillus casei* has a molecular weight of 14.34 kDa were included in the group of class III bacteriocins, generally large (> 10 kDa), and are not heat resistant.

Competing interests

The authors declare that they have no competing interests

REFERENCES

1. Meryandini, A. 2009. Isolation bacterium and characterization enzyme. Makara Sains. 13 : 33-38.
2. Mangunwardoyo W, Romauli AS, and Endang SH. 2007. Selection and Testing of Enzyme Activity *L-Histidine Decarboxylase* from Histamine-Forming Bacteria. Makara Sains. Vol.11. No.2, pp. 104-109.
3. Jeevaratnam K, Jamuna M., and Bawa AS. 2005. Biological preservation bacteriocin of lactic acid bacteria. J. Indian Journal of Biotechnology. 4 : 446- 454.
4. De Vuyst L and Vandamme EJ. 1993. Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications. London : Blackie Academic and Professional,
5. Chotiah S. 2013. Exploration and conservation of genetic resources bacteriocin producing microbial pathogens bacterial growth inhibitors in cattle. Veterinary Research Institute. Bogor.
6. Hadioetomo. 1990. Microbiology Basic Vol. I. Jakarta : Erlangga.
7. Usmiati S and Marwati T. 2007. Selection and optimization of production processes bacteriocin of *Lactobacillus sp.* J. post-harvest. 4(1): 27-37.
8. Ogunbanwo ST, Sanni AL, and Onilude AA. 2003. Characterization of Bacteriocin Produced by *Lactobacillus plantarum* and *Lactobacillus brevis* F1*OG1.* African J.Biotechnol. Vol.2. No.8, pp. 219-227.
9. Ohmomo S, Murata S, Katayama N., Nitisinprasart S, Kobayashi M, Nakajima T, Yajima M and Nakanishi K. 2000. Purification and some characteristics of enterocin on-157, a bacteriocin produced by *Enterococcus faecium* 157. Journal of Applied Microbiology. 88: 81-89.

10. Fadda S, Patricia A, Fabienne B, Monique Z, Regine T, Graciela V, Marie C and Champornier-Verges. 2010. Adaptive response of *Lactobacillus sakei* 23K during growth in the presence of meat extracts: a proteomic approach. International Journal of Food Microbiology. 142: 36-43.
11. Nurlia. 1997. Influence Replenishment Bacteriocins and Combined Bacteriocins Production Bacterium Acid Lactate to amount Bacterium in Milk Pasteurization. Thesis. Program Post Scholar. Institute Pertanian Bogor. Bogor.
12. Nurliana. 2009. Prospects Food Traditional Aceh Food Health Exploration Compound Antimicrobials from Oil Pliek u and Pliek u. Graduate Forum. 32(1): 1-10.
13. Fatchiyah, EL Arumingtyas, Widyarti S and Rahayu S. 2006. Analysis Biology Molecular: DNA Isolation, PCR, Immunobloting, and Isoenzyme. Malang : University of Brawijaya.
14. Jimenez-Diaz R, Rios-Sancherz RM, Desmazeaud M, Ruiz-Barba JL and Piard JC. 1993. Plantaricin S and T ; two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. Applied and Environmental Microbiology. 59(5):1416-1424.
15. Suseno IT, Sutarjo S, and Anita K. 2000. Drink Probiotics Nira Siwalan: Older studies Storage to Antimicrobial power *Lactobacillus casei* on some Bacterium Pathogens. Journal Technology Food and Nutrient. Vol.1. No.1.
16. Wendakoon CN and Sakaguchi M. 1995. Inhibition of amino acid decarboxylase of *Enterobacter aerogenes* by active components in Spices. J. Food Prot. 58(3): 280-283.
17. De Vuyst L and Vandamme EJ. 1994. Antimicrobial Potential of Lactic Acid Bacteria In: DeVuyst L and EJ Vandamme. Bacteriocins of Lactic acid Bacteria: Microbiology, Genetic, and Application. London. Blackie Academic Professional.
18. Jay JM. 2000. Modern Food Microbiology 6th Edition. Maryland: Aspen Publishers, Inc. Gaithersburg.
19. Jack RW, Wan J, Gordon, Harmark K, Davidson BE, and Hillier AJ. 1996. Charaterization of chemical and antimicrobial properties of pisicolin 126, a bacteriocin produced by *Carnobacterium piscicola* Jg 126. J. Appl. Environ. Microbiol, 62(8): 2897-2903.
20. Lee H and Kim HY. 2011. Lantibiotics, class I bacteriocins from the genus *Bacillus*. J. Microbiol Biotechnol. 21: 229-235.

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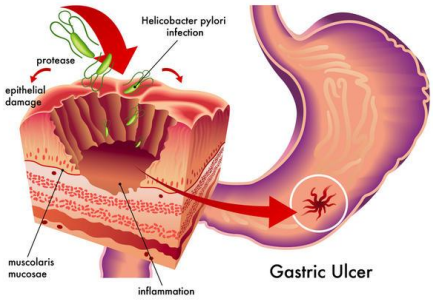

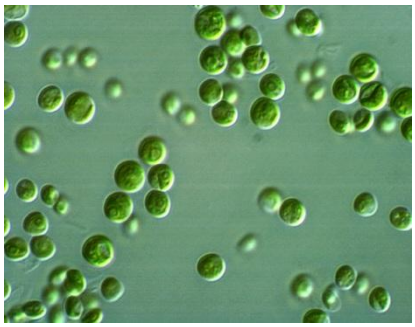

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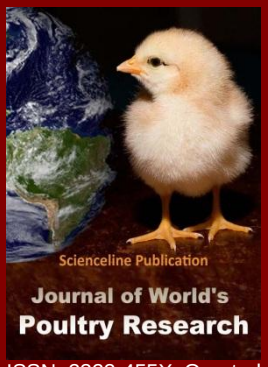

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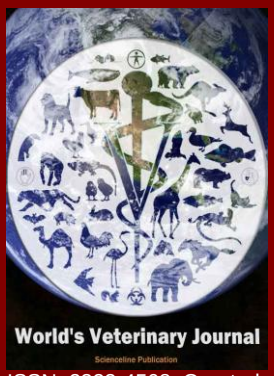

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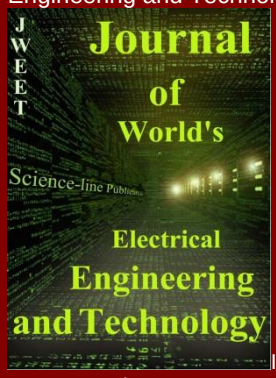

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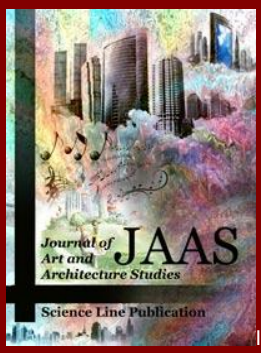

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