Isolation of Lactic Acid Bacteria That Produce Protease and Bacteriocin-Like Substance From Mud Crab (*Scylla* sp.) Digestive Tract

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Abstrak

Isolasi Bakteri Asam Laktat yang Menghasilkan Protease dan Senyawa Bacteriocin-Like dari Saluran Pencernaan Kepiting (Scylla sp.)

Saluran pencernaan merupakan lingkungan kompleks yang terdiri atas berbagai spesies bakteri. Saluran pencernaan biota perairan terdiri atas bakteri aerob dan anaerob yang mampu memproduksi senyawa antibakteri dan enzim. Tujuan penelitian ini adalah untuk mengisolasi bakteri asam laktat yang menghasilkan protease dan senyawa bakteriosin-like dari saluran pencernaan kepiting bakau. Isolasi dan karakterisasi isolat dilakukan menggunakan media MRS. Supernatan netral bebas sel isolat telah diuji dengan menggunakan disc difusi agar terhadap bakteri patogen dan pembusuk. Uji produksi enzim protease telah diuji dengan metode disc diffusion agar menggunakan media kasein agar. Di antara seratus isolat, terdapat 96 isolat menunjukkan zona bening di MRS + CaCO3, katalase negatif, dan bakteri Gram positif. Tiga puluh empat isolat bakteri asam laktat menghasilkkan protease dan hanya empat isolat (yaitu IKP29, IKP30, IKP52, dan IKP94) menunjukkan penghambatan yang kuat terhadap bakteri patogen dan pembusuk. Terdapat tiga pola inhibisi dari keempat isolate terhadap Bacillus subtilis, Staphylococcus aureus, Eschericia coli, dan Salmonella sp. Empat isolat tersebut berpotensi untuk dimanfaatkan sebagai starter pada produksi fermentasi produk hasil perikanan. Penelitian ini adalah penelitian pertama terkait isolasi bakteri asam laktat yang menghasilkan protease dan bakteriosin dari saluran pencernaan dari kepiting bakau.

Kata kunci: *bakteri asam laktat, senyawa bakteriosin-like, protease, Scylla sp.*

Abstract

Digestive tract is complex environment consist of large amount of bacteria's species. Fish intestine bacteria consist of aerobic or facultative anaerob bacteria which can produce antibacterial and enzym. The objectives of this research were to isolated lactic acid bacteria that produce bacteriocin-like and protease from mud crab digestive tract. Isolation and characterization of isolates were conducted employing media MRS. Neutralized cell free supernatant of isolates were tested using disc diffusion agar of against pathogenic and spoilage bacteria to indicate bacteriocin-like-producing lactic acid bacteria. Protease-producing isolate was tested using disc diffusion method in casein agar. Among a hundred isolates, 96 isolates were showed clear zone in MRS+$CaCO_3$,, catalase negative, and Gram positive bacteria. Thirty four isolates produced protease and only four isolates (i.e. IKP29, IKP30, IKP52, and IKP94) showed strong inhibition against pathogenic and spoilage bacteria. There were three patterns of inhibition among three isolates against Bacillus subtilis, Staphylococcus aureus, Eschericia coli, and Salmonella sp. All three isolates showed potential uses for produce starter culture for fishery product fermentation purpose. This is the first report of isolation lactic acid bacteria that produced protease and bacteriocin-like from digestive tract of mud crab.

Keywords: *lactic acid bacteria,bacteriocin-like substance, protease, Scylla sp.*

Introduction

Digestive tract is complex environment consist of large amount of bacteria species. Fish intestine bacteria consist of aerobic or facultative anaerob bacteria (Rurangwa *et al.*, 2009; Talpur *et al.*, 2012). Bacteria isolated from fish intestine showed activity against pathogenic bacteria such as *Vibrio alginolyticus* (Nursyirwani *et al.*, 2011), *V. harveyi., V. parahaemolyticus, Pfiesteria piscida* (Talpur *et al.*, 2012).

Lactic acid bacteria (LAB) are important bacteria for probiotic and biopreservative purposes

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since it can produce organic acid, H_2O_2, reuterin and bacteriocin. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, and often presenting a bactericidal effect against closely related species (Cotter *et al.*, 2005). LAB has been isolated from environment such as mangrove sediment, leaf, and water (Hwanhlem *et al.*, 2014), diary product (Iranmanesh *et al.*, 2014), kefir grain (Zanirati *et al.*, 2015), fermented fish (Hwanhlem *et al.*, 2014; Adnan and Tan, 2007), fish intestine (Kuda *et al.*, 2014; Gurovic *et al.*, 2014; Huang *et al.*, 2014), and blue swimming crab (Talpur *et al.*, 2012) but there are limited study regarding to isolation of bacteriocin-like LAB from digestive tract of mud crab (*Scylla* sp.).

Protease is the most important enzyme for industrial and fermentation purpose. Microbial protease are group of enzymes that can have apply in numerous industries, pharmaceutical process, food industry, oil extraction, *etc*. (Borla *et al.*, 2010). Proteolytic bacteria play important role as culture starter for fermentation. Bacterial peptidase function in meat fermentation is break down oligopeptide into amino acid (Sanz *et al.*, 1999). Based on these reason, the objectives of this research were to isolated and characterized LAB that produce bacteriocin-like substance and protease from digestive tract as candidate of culture starter for fishery product fermentation.

Materials and Methods

Twelve mud crabs were purchased from fisherman of Gunung Sari mangrove area, Surabaya in December 2014. Mud crabs were taken with cool box with ice (±5^{0}C) to the laboratory.

Segregation of digestive tract

Digestive tract was taken from the mud crab specimen according to Talpur *et al.* (2012). Briefly mud crabs specimen was sprayed with alcohol 70% prior segregation. Segregation was conducted by scalpel from abdominal part to bucal cavity. Digestive tract of the mud crab was taken directly using sterile tweezer.

Enrichment procedures

Each of digestive tract of total 12 mud crab was taken separately into erlenmeyer flask containing sterile MRS broth and incubated overnight at 37^{0}C prior isolation process.

Isolation of lactic acid bacteria

One mL of diluted samples of enrichment digestive tracts (10^{-1}, 10^{-2}, 10^{-3}) were spread plated onto deMan, Rogosa, and Sharpe (MRS, Merck) medium prepared in sea water (30 ppt) and supplemented with 0.5% $CaCO_3$ then incubated in 37^{0}C for 24 h. Bacteria that showed clear zone around colony was aseptically taken then streaked into MRS medium and incubated 37^{0}C. Pure cultures from plates were tested for catalase and Gram staining procedure. Isolate that showed catalase negative and Gram positive were taken added with 50% glycerol and stored in freezer (-10^{0}C) before used for further test.

Screening of protease-producing bacteria

LAB isolates were thawed and 100 µL of master culture growth in one mL of MRS broth for 18 h. Ten µL culture was dropped in sterile paper disc and put on survace of casein agar (1% skim milk, 1.5% agar, pH 6.5) then incubated 2-3 days. Isolate that produce protease was exhibited clear zone around paper disc.

Antimicrobial activity

Isolate that exhibited proteolytic activity tested for antimicrobial activity test employing disc diffusion agar method against six indicator bacteria (Table 1). Ten µL of 24 h culture was drop on sterile paper disc and put on surface of tryptone soya agar that overlayed by indicator bacteria. Plate was incubated in 37^{0}C for 24 h. Positive antimicrobial activity showed by clear zone surrounded paper disc containing lactic acid bacteria isolates.

Bacteriocin-like substance activity

Isolates that showed antimicrobial activities were cultured in 1.5 mL microtube for 18 h and centrifuge 5000 rpm for 15 minute. Supernatant was transferred into sterile microtube and pH was adjusted to 6.5 before heating 100^{0}C for 3 min to inactivated enzyme and bacteria that next to be called as neutralized cell free supernatant (NCFS). Ten mL of NCFS was transferred into sterile paper disc and put on surface of MRS that was overlay with *L. plantarum*. Positive bacteriocin-like substance activity showed by clear zone surrounded paper disc containing NCFS of LAB isolates.

Result and Discussion

Isolation of lactic acid bacteria

A hundred of isolates that showed clear zone in MRS+$CaCO_3$ medium was isolated. Among 100 isolates, there were 96 isolates that have the characteristics of catalase-negative and Gram-positive. All isolates have a spherical shape with a milky white color or white. These results were similar to those obtained by Nursyirwani *et al.* (2011) who

Table 1. Indicator bacteria that used for antimicrobial activity test

Isolates	Gram
Lactobacillus plantarum	Positive
Bacillus subtilis	Positive
Staphylococcus aureus	Positive
Eschericia coli	Negative
Pseudomonas sp.	Negative
Salmonella sp.	Negative

obtained 21 isolates of lactic acid bacteria in the digestive tract of tiger grouper with a round shape with a diameter of 0.25-2mm with white or beige collour. Jokovic *et al*. (2008) have tried to direct and enrichment method for isolation of lactic acid bacteria in traditional Serbian products made from milk (kajmak). Jokovic *et al.* (2008) was showed that the isolation of lactic acid bacteria by using the enrichment method more effective to obtain better results due to the lactic acid bacteria contained in the product in small amounts.

Screening of protease production

Results of screening protease production of LAB 96 isolates showed that 34 isolates (35.41%) able to produce protease. This is shown by clear zones in the medium casein agar. Udomsil *et al*. (2010) have isolated 64 isolates of lactic acid producing protease from fish sauce. Bacterial proteases are generally used to break down oligopeptides into amino acids (Sanz *et al*., 1999). That was one of the important criteria of a LAB isolates could be used as a starter in the production of fermented fish. The same thing, Zheng *et al*. (2014) states that the lactic acid bacteria producing protease is one of the important criteria to produce a scent in the production of fermented fish.

Antimicrobial activity

The The test results showed that microbial activity of 34 isolates that produce protease, there were 24 isolates that showed inhibition zone against indicator bacteria (Table 2). Among 24 isolates there are four isolates that showed high antimicrobial activity against some indicators bacteria, *i.e* IKP29, IKP30, IKP52, and IKP94. There are three patterns of inhibition among the four isolates.

Lactic acid bacteria are able to produce organic acid, H_2O_2, reuterin and bacteriocin that inhibits the growth of other bacteria in the ecosystem (Cotter *et al*., 2005). Organic acids produced by lactic acid bacteria causes the environment becomes acidic so the bacteria are not able to grow at low pH due to it cannot maintain cell homeostasis condition and result in cell death. The ability of lactic acid bacteria obtained from the digestive tract of mud crabs to inhibit bacterial growth of Gram positive and Gram negative is an important character in the application of bacteria as biopreservative agent.

Four isolates of LABs were inhibited the growth of pathogenic bacteria that normally occur in food products, *S. aureus* and *Salmonella* sp. This is in accordance with those obtained by Hor and Liong (2014) which showed that the lactic acid bacteria can inhibit the growth of *S. aureus*. Thelma *et al*. (2014) stated that the lactic acid bacteria inhibited *E. coli, Salmonella paratyphii,* and *Listeria monocytogenes*. Ability of LAB to inhibit pathogenic bacteria, indicating that the four isolates of LAB obtained has potential to be apply as biopreservation. Regarding to the ability of these four isolates to produce proteases, the four isolates can be used as a starter fermented fish products are capable of maintaining fermented food safety.

Production of Bacteriocin-like substance

Neutralized cell free supernatant of four isolates tested its activity against lactic acid bacteria *L. plantarum* were tested. The test was performed employing LAB such as *Lactobacillus sakei* subsp *sakei* JCM 1157 (Hwanhlem *et al*., 2014). The use of *Lactobacillus brevis* AP83, *L. brevis* H56, *L. plantarum* AP76, *L. plantarum* H12 were performed by Ghanbari, *et al*., (2013). Based on this research, *L. plantarum* can be used as an indicator to test activity of bacteria to produce bacteriocin.

Table 2. Antimicrobial activity of five LAB isolate against indicator bacteria

Indicator bacteria	LAB isolates			
	IKP29	IKP30	IKP53	IKP94
Bacillus subtilis	-	-	-	-
Staphylococcus aureus	+	+	-	-
Eschericia coli	+	-	+	+
Pseudomonas sp.	+	+	+	+
Salmonella sp.	+	+	+	+

This was due to the LAB not inhibited by organic acid and H_2O_2 but inhibited by bacteriocin. NCSF of Isolate IKP29, IKP30, IKP53 and IKP94 was inhibits the growth of *L. plantarum* ranging from 3 to 4 mm.

The results indicate that inhibition of bacteria by all four isolates was done by the bacteriocin while also produce organic acids and hydrogen peroxide. Aslim *et al*. (2005) stated that the inhibition of

microbes can be carried out by organic acids, hydrogen peroxide, bacteriocin, or a combination of all three of these substances. All of the four LAB isolates were produced bacteriocin-like substance and protease that can be used as culture starter for fishery fermentation production. This paper is the first report regarding the research of isolation of lactic acid bacteria in the digestive tract of mud crab that able to produce protease and bacteriocin-like substance.

Conclusion

The isolates obtained from digestive tract of mud crab can produce protease and bacteriocin-like substance. These four isolates, namely IKP29, IKP30, IKP53 and IKP94, were obtained and has have the potential to be developed as a starter culture for fermentation of fishery products such as the production of fish sauce and oyster sauce.

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