SUBSTRATES BREAK DOWN AND AGARASE ACTIVITY OF *Vibrio* spp AS PROBIOTICS CANDIDATE FOR ABALON

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ABSTRACT

The use of Vibrio as probiotics for fish and shrimp are well known. The purpose of this study was to evaluate the ability of the substrate degradation and agarase activity of some strains of Vibrio as a candidate probiotic for abalone. The ability of agar, starch and casein break down is measured method colorimetricby using UV-VIS spectrophotometer. Agarase activity test is done by calculating the reducing sugars by DNS method. The results showed that isolates Abn1.2 have the highest capability of starch hydrolysis, namely 89.20 and 99.16%, while the lowest degree of starch hydrolysis is Alg3.1 amounted to 76.05 and 90.20% at 48 hours of incubation. All three isolates showed a high capacity to hydrolyze casein. The highest degree of hydrolysis of carbohydrates in *Gracilaria* consistently demonstrated by a combination of isolates Abn1.2 and Alg3.1, ie 52.90% and 59.32% in the amount of inoculum 10^8 and 10^{10} cfu / mL with a 48-hour incubation period. The highest agarase activity by mixed culture Alg3.1-Abn1.2 ie 0593 nkat / mL at hour 32. Thus, the mixed culture strains of *Vibrio natriegens* Alg3.1 and Abn1.2 potential as a candidate probiotic for abalone

Keywords: *Vibrio*, probiotik, agarase, abalon.

INTRODUCTION

Vibrio bacteria group has been known to play an important role in nutrient cycling in aquatic environments through the breakdown of organic matter (Thompson *et al*., 2004). According to Nichols, (2003) and Rieman and Azam (2002) that Vibrio provide unsaturated fatty acids (polyunsaturated fatty acids) are essential for aquatic food chain, which is where many of the aquatic organisms are not able to produce it. Vibrio can also degrade chitin, a homopolymer of N-acetyl glucosamine, which is one of the largest pools of amino sugar in marine. It was also stated that among marine bacteria, Vibrio is an important producer of antibiotics. Inhibitor compound produced by certain Vibrio isolates can reduce other community members such as Alpha-Proteobacteria and Alteromonas (Long and Azam, 2001).

Vibrio has long been exploited for various purposes. Certain species of these bacteria are used for the production of vaccines and probiotics, and can perform bioremediation polyaromatic hydrocarbons (Thompson *et al*., 2004). As probiotics in aquaculture systems, Vibrio used with a variety of reasons, among others, are able to perform the competition with other bacteria (Riquelme *et al*., 1997), add nutrients to provide essential nutrients (Thompson *et al*., 2004), can improve the digestibility with enzymes essential (Tanaka *et al*., 2001), has the ability to colonize the digestive tract and the host body (Sawabe *et al*., 1998, 2003; Thompson *et al*., 2004), and can produce substances that inhibit the growth of pathogenic bacteria opportunistic (Verschuere *et al*., 2000).

One of the criteria that must be met in order for a strain of bacteria worthy of being the candidate probiotics for improvement of physiological function digestive system is its ability to degrade macromolecular components of the feed.

The main macromolecules as a constituent of abalone feed components are agar contained in the cell walls of seaweed. gar-agar is a polysaccharide complex mixture of 1,3-α-1,4-β galactans, composed of agarose and agaropektin polymers are difficult to digest. Introductions probiotic bacteria can produce agarase enzyme will help abalone to break agar into oligosaccharide and D-galactose. Galactose catabolism by enteric bacteria produce large amounts of acetic acid and format, which can be used as a source of energy or a precursor amino acid synthesis by abalone (Thompson *et al*. 2004).

METHODOLOGY

Bacterial strains and culture

Bacterial strain used is a *Vibrio natriegens* strain Abn1.2, Alg3.1 and Alg4.2 strains obtained from the results of previous studies (Faturrahman *et al*., 2012). The bacteria were grown in marine media broth (Difco: Yeast extract 0.1%, Casamino acid 0.5%, 3.0% NaCl, KCl $MgCl_2.6H_2O$ 0.23% and of 0.03%).

Media Selection for Production Agarase

Selection of the best medium for the production of agarase done by growing the bacteria in media containing agar as a carbon source. Selection of the best media based on the growth of cells (cfu / ml) and a clear zone formed after 24 hours of incubation. The media used is marine broth (MB), sea water medium (SWM), basal salt medium B (BSM-B) and BSM-B supplemented with yeast extract and peptone. Selected bacteria were incubated at 29 ° C for 24 hours. After the cells were separated by centrifugation at 3000 rpm for 30 minutes to obtain a cell-free supernatant. Supernatant removed for examination agarase activity. Agarase best medium for the production will be used for further testing.

Degree of Substrate degradation and Gracilaria Powder

This test is performed to measure the ability of bacteria in degrading macromolecules such as carbohydrates and proteins. The substrate used was starch, agar, casein and powder Gracilaria. At first gracilaria washed and cleaned of dirt, and then dried in an oven at a temperature of 60 ° C overnight. Furthermore, dry Gracilaria blended up into flour and filtered. Testing performed with the following procedures: Bacterial isolates were grown in a liquid medium selected. Before autoclaved, pH adjusted to 7.5 (pH optimum) and added a carbon source such as agar, starch, casein 0.1% (w / v) and powder Gracilaria 1% w / v). They are then incubated at 29 ° C for 48 hours while shaken at 120 revolutions per minute. After the cells were separated by centrifugation at 3000 rpm for 30 minutes to obtain a cell-free supernatant. The cell-free supernatant is then tested on the substrate casein, starch and agar. Ability of substrates degradation measured colorimetric.

Growth Curve and Agarase Production

To produce the maximum β-agarase be making its production curve. The first step is conducted by making inoculum is to take one loop of bacteria, inoculated in 10 ml liquid media selected and incubated at 29°C at a speed of 120 revolutions per minute so as to

achieve logarithmic phase (OD = 0.8). Then as much as 1% starter inoculated production medium Basal Salt Solution plus (called medium B plus, contains g / L: yeast extract, 0.5; peptone 0.2; $NaNO_3$, 2; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0:02; $MnSO_4.H_2O$, 0:02; and $FeSO_4.7H_2O$, 0:02) agarose (1-2 g / L) and incubated at 29 ° C. Sampling was performed every 4 hours as much as 5 mL. Enzyme crude extract is obtained by conducting centrifugation of the sample at a speed of 8000 rpm for 15 minutes. Afer that measurement of pH, concentration of bacterial cells and agarase activity on agarose substrate. Agarase activity test is performed by calculating a reducing sugar with DNS method (Miller 1959) and the concentration of cells was counted by spectroscopic methods (OD at λ=620 nm).

Testing of Enzyme Activity Agarase

The enzyme activity agarase in reducing substrate agarose is determined by the DNS method (Miller 1959) as follows: 5 ml (0.2%, w / v) agarose in 20 mM sodium phosphate (pH 7.5) was heated at 100°C for 2 minutes and then cooled at a temperature of 30°C. The reaction is conducted by mixing 0.5 ml to 0.5 ml specimen enzyme substrate for 30 minutes at a temperature of 29°C. Then added 1.0 ml DNS and heated in boiling water in a water-bath for 10-15 minutes, then cooled in cold water for 10 minutes. Strength reduction agarase monitored with a spectrophotometer at a wavelength of 540 nm. One unit of agarase activity is defined as the amount of enzyme that produces 1 mol galactose per second at 29°C under the conditions described above (Dybkaer *et al*. 2001)..

RESULTS

The ability of all three isolates (*Vibrio natriegens* strain Abn1.2, Alg3.1 and Alg4.2) in hydrolyzing agar are based on the size of the diameter of the clear zone formed. Activity of bacteria in degrading agar can be measured quantitatively by the activity of crude extract or pure enzyme that is produced in liquid culture. Selection of media do to get the optimum enzyme activity. In addition, test the ability to hydrolyze the protein and starch were also conducted on all three isolates. Mixed cultures which showed the highest activity will be selected as a candidate probiotic.

The Best Media for Growth and Production

Table 1. Growth and qualitative enzyme activity of Alg3.1 isolate in different medium for 24 hour incubation

Growth Media	Growth (cfu/ml)	Clearing zona (mm)
MB	6.0×10^7	26.5
SWM	7.4×10^7	32.5
BSM-B	4.1×10^7	21
BSM + peptone + yeast exstract	1.1×10^8	38

note : MB, *marine broth;* SWM, *sea water medium;* BSM, *basal salt medium B*

Degree of Substrates Degradation

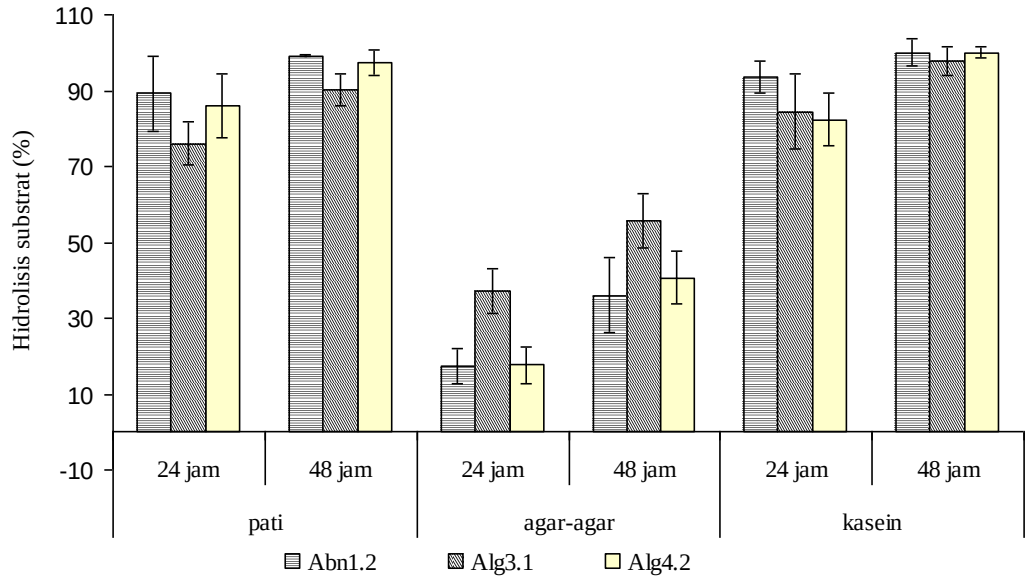

Figure 1. The degree of hydrolysis of starch, agar and casein by agarolitik bacteria were incubated at 29 ° C for periods of 24 and 48 hours of observation. Data are presented as an average of two replications (±S.E)

Degree of Gracilaria Carbohydrate Degradation

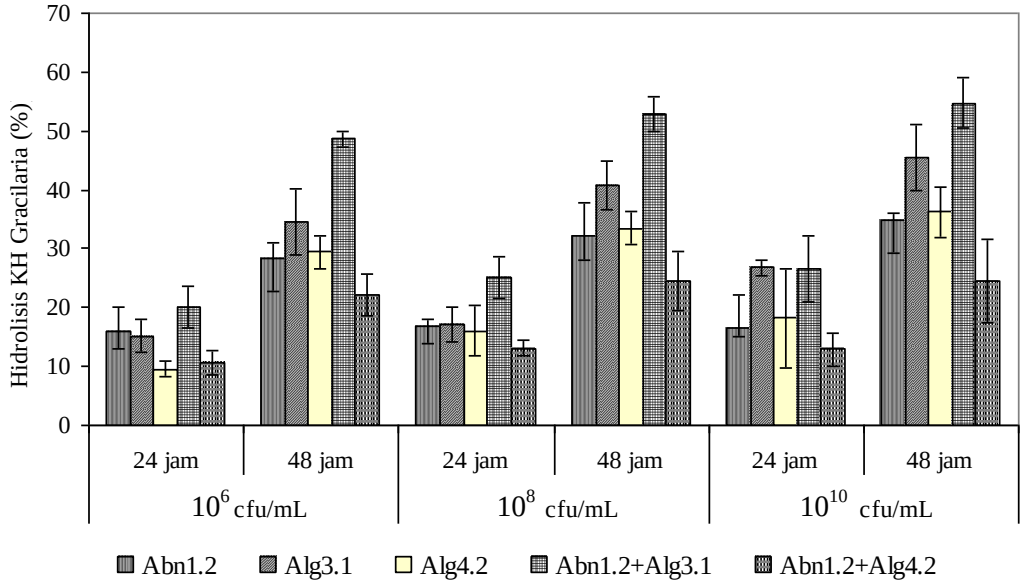

Figure 2. The degree of hydrolysis of carbohydrates *Gracilaria* sp. (%) by bacteria agarolitik on a different amount of inoculum over a period of 24 and 48 hours of observation. Data are presented as an average of two replications (±S.E)

Agarase Enzymes Activity

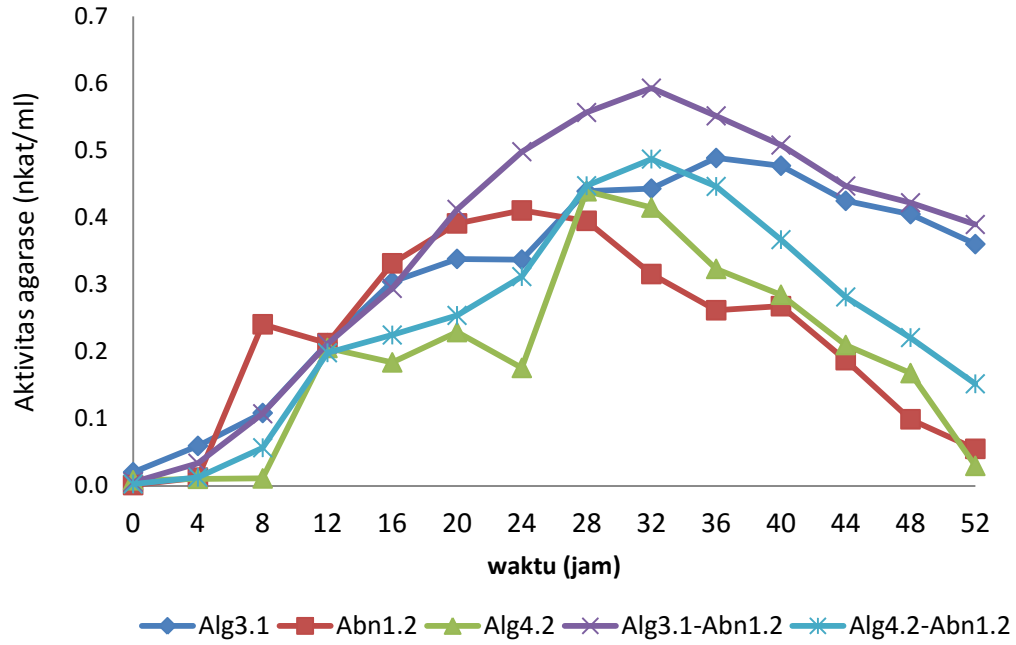

Figure 3. Cell growth and agarase enzyme activity of bacterial isolates were grown on media agarolitik BSM-B plus for 52 hours at a temperature of 29^{o}C. Data are presented as an average of two replications (±S.E)

DISCUSSION

In order to obtain optimum enzyme activity, it is necessary to have growth and production agarase medium that right. Selection of the best media based on the growth of cells (cfu/ml) and a clear zone formed after 24 hours of incubation. Therefore, the three isolates from sea waters and the same genus, then strain Alg3.1 used as test bacteria in assessing growth and optimum agarase production in various media. Agarase qualitative growth and activity are presented in Table 1.

Medium MB and SWM is a complex medium that is rich in nutrients that can support the growth of most bacteria. MB contains yeast extract, amino acids and minerals, while SWM made from meat extract, peptone and seawater as a mineral source. For many heterotrophic bacteria such as Vibrio, a nitrogen source and growth factors can already be met by meat extract or yeast extract and peptone.

Medium BSM-B is basically only made up of mineral salts, without the carbon source and growth factors, as a result of this medium are not able to support the growth of heterotrophic bacteria. The addition of yeast extract and peptone provide growth factors for bacteria so that the growth of the bacteria are getting better and allow bacteria to produce enzymes agarase in larger quantities. Growth factor is an organic compound as an essential cellular components that must be present in the cell, but can not be synthesized by the organisms of the basic sources of carbon and nitrogen (Madigan *et al*. 2009). Growth factors include vitamins, amino acids, purine and pyrimidine (Madigan *et al*. 2009).

Supplementation of probiotic bacteria in the host's role to help hydrolyze feed components like carbohydrates and protein that are available in the form of simple molecules ready for

absorption. This study has been conducted test capabilities agarolitik candidate probiotic bacteria in hydrolyzing starch, agar and casein. Results are presented in Figure 1.

Data results showed that isolates Abn1.2 has the highest starch hydrolysis capability, 89.20 and 99.16%, while the lowest degree of starch hydrolysis is Alg3.1 amounted to 76.05 and 90.20% at 48 hours of incubation. All three candidates agarolitik probiotic bacterial isolates showed a high capacity to hydrolyze casein. The degree of hydrolysis of carbohydrates and protein increased with increasing time of incubation to a certain extent. The results of the analysis of the t-test showed that the degree of hydrolysis of starch and casein isolates Alg3.1, Alg4.2 and Abn1.2 not significantly different ($P < 0.05$) at 24 and 48 hours of observation. Instead agar hydrolysis highest achieved by Alg3.1 isolates, followed by Alg4.2 and the lowest is Abn1.2. The results of the analysis of the t test showed that the degree of hydrolysis of agar from Alg3.1 isolates were significantly different ($P < 0.05$) with Alg4.2 and Abn1.2 at 24 and 48 hours of observation.

The ability of the isolates to degrade a variety of organic macromolecules would be very useful for the physiological function of the digestive host because it can enrich the number and type of 'pool' of digestive enzymes in the digestive system of the host and expand the types of feed components that can be digested host. Aslamyah (2006) introducing a mixed culture of bacteria amylolytic, proteolytic and lipolytic isolated from the digestive tract in fish and may increase the activity of the enzyme amylase, protease and lipase in the digestive tract, increase feed efficiency and at the same time can improve the growth rate of fish.

According to Erasmus *et al*. (1997) enteric bacteria probiotics that are candidates for abalone (herbivores) must have a high capacity to hydrolyze the complex polysaccharide component of macroalgae into simpler molecules that are readily absorbed by abalone. *Vibrio halioticoli* isolated from the stomach of *H. discus hannae* can produce acetic acid and formic acid in large quantities, which is allegedly used as an energy source or precursor protein synthesis by abalone (Thompson *et al*. 2003; 2004) thus considered that there is a mutual relationship between *V. halioticoli* with abalone. Besides that, Tanaka *et al*. (2002) reported that *V. halioticoli* produce enzymes polyguluronate lyase, an enzyme that catalyzes the degradation of the alginate dominant component of brown seaweed which is the main food of Haliotis.

Feed abalone aquaculture in many countries still rely on the use of natural food in the form of seaweed, although major companies have implemented artificial feed. Study of the use of seaweed as a single feed abalone suggest that growth is very slow (Setyono 2008). Gracilaria is a seaweed that has a lower protein content, but rich in carbohydrates such as agar-agar, carrageenan, floridean starch, and cellulose. Applications polisakarase extracellular bacteria is expected to help improve digestion host through the provision of exogenous digestive enzymes. Data from the study isolates the ability to hydrolyze carbohydrates Gracilaria in Figure 2.

The type and number of bacteria inoculum effect on the degree of hydrolysis of carbohydrates *Gracilaria* sp, both in the incubation period of 24 or 48 hours. The highest degree of hydrolysis is consistently demonstrated by a combination of isolates Abn1.2 and Alg3.1, ie 52.90% and 59.32% in the amount of inoculum 10^8 and 10^{10} cfu / mL with a 48-hour incubation period. These results are consistent with the data in Figure 3, showing activity qualitatively agarolitik Alg3.1 mixed culture with Abn1.2 higher than single or mixed culture isolates others. The results of the analysis of the ***t***-test showed that the degree of hydrolysis of carbohydrates gracilaria Alg3.1-Abn1.2 mixed cultures were significantly

different (P <0.05) with single or mixed culture isolates the other after 48 hours of observation.

Figure 2 also indicates that the degree of hydrolysis of carbohydrates gracilaria increases with incubation time and the increased concentration of inoculum. Analysis by *t*-test showed that the concentration of inoculum 10^8 cfu / ml were significantly different (P <0.05) with 10^6 cfu/ml. Only an increase in the concentration of the inoculum up to 10^{10} cfu / ml has little effect on the degree of degradation. The bacteria efficiently utilize substrates in accordance with their needs, when nutrients are still available in sufficient quantities for the growth of the bacteria will stop the production of enzymes that degrade macromolecules to avoid wasting energy. That is why the addition of the amount of inoculum to 10^{10} cfu / ml not significant (*t*-test P <0.05) on the degree of hydrolysis of carbohydrates *Gracilaria*.

The capacity of bacteria to degrade certain substrates or organic macromolecules can be measured based on the activity of crude extract and purified enzyme extracted from the bacteria produced in liquid culture. Values enzyme activity can be plotted in the form of curves enzyme based on time or to do with the growth of cells. Agarase enzyme activity curves with the growth of cells in this study conducted for 52 hours with the observation period every four hours. Test result data agarase enzyme activity of crude extract is shown in Figure 3.

Agarase enzyme activity of crude extract became visible during 4 h after inoculation at Alg3.1 (0036 nkat / ml), 8 hours on Abn1.2 (0.241 nkat / ml) and 12 hours at Alg4.2 (0.206 nkat/ml). This activity continues to increase until it reaches maximum activity at 36 hours after inoculation (0.489 nkat/ml) at Alg3.1, 28 hours after inoculation (0.439 nkat/ml) at Alg4.2 and 24 hours after inoculation (0.411 nkat/ml) at Abn1.2. Maximum activity is achieved by a mixed culture Alg3.1-Abn1.2 was 0.593 nkat/mL at 32 hours and Alg4.2-Abn1.2 was 0.487 nkat/mL at 32 hours (Figure 3). Increased activity of agarase of mixed cultures can occur because two different agarase isolates produce enzymes that work complementary. Agarase enzyme is a trimeric enzyme complexes that work in sequence, in which the first enzyme product (agarase I) be the substrate for a second enzyme (agarase II) and a second enzyme product is a substrate for the enzyme third (agarobiosa hydrolase). Identification of hydrolysis products both isolates is necessary to know the type of enzyme produced.

Agarase enzyme activity Alg3.1 crude extract is 2.9 times higher than *P. antartica* N-1 (Vera *et al*. 1998). Some reports indicate that the agarase produced by genus Vibrio has a specific activity lower, eg strains of *Vibrio* PO303 activity 7.54 U/mg (Dong *et al*. 2006) and 6.3 U/mg of strains of *Vibrio* JT0107 (Sugano *et al*. 1993) , Agarase of the genus *Agarivoran* medium has a specific activity, such activity HZ105 strain was 57.45 U/mg (Hu *et al*., 2009) and 25.54 U/mg of strain YKW-34 (Fu *et al*., 2008). Agarase of the genus *Alteromonas* showed high specific activity, eg activity of strains of *Alteromonas* sp. SY37-12 was 83.5 U/mg (Wang *et al*. 2006) and 234 U/mg of *Alteromonas* sp. C-1 (Leon *et al.* 1992).

Based on data isolates the ability to degrade the substrate, hydrolysis of carbohydrates in *Gracilaria* and agarase enzyme activity of all three isolates then have Abn1.2 and Alg3.1 as a candidate probiotic provider agarase exogenous enzyme for abalone.

CONCLUSIONS

Vibrio natriegens showed a high capacity to hydrolyze casein. The highest degree of hydrolysis of carbohydrates gracilaria consistently demonstrated by a combination of isolates Abn1.2 and Alg3.1, ie 52.90% and 59.32% in the amount of inoculum 10^8 and 10^{10} cfu / mL with a 48-hour incubation period. The highest agarase activity by mixed culture Alg3.1-Abn1.2 ie 0593 nkat / mL at hour 32. Thus, the mixed culture strains of *Vibrio natriegens* Alg3.1 and Abn1.2 potential as a candidate probiotic for abalone..

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