

Isolation and identification of lactic acid bacteria from abalone (*Haliotis asinina*) as a potential candidate of probiotic

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Abstract. Sarkono, Faturrahman, Sofyan Y. 2010. Isolation and identification of lactic acid bacteria from abalone (*Haliotis asinina*) as a potential candidate of probiotic. *Nusantara Bioscience* 2: 38-42. The purpose of this study was to isolate, select and characterize lactic acid bacteria (LAB) from abalone as a potential candidate probiotic in abalone cultivation system. Selective isolation of LAB performed using de Man Rogosa Sharpe medium. LAB isolate that potential as probiotics were screened. Selection was based on its ability to suppress the growth of pathogenic bacteria, bacterial resistance to acidic conditions and bacterial resistance to bile salts (bile). Further characterization and identification conducted to determine the species. The results showed that two of the ten isolates potential to be developed as probiotic bacteria that have the ability to inhibit several pathogenic bacteria such as *Escherichia coli*, *Bacillus cereus* dan *Staphylococcus aureus*, able to grow at acidic condition and bile tolerance during the incubation for 24 hours. Based on the API test kit, both isolate identified as members of the species *Lactobacillus paracasei* ssp. *paracasei*.

Keywords: lactic acid bacteria, isolation, identification, *Lactobacillus paracasei* ssp. *paracasei*.

Abstrak. Sarkono, Faturrahman, Sofyan Y. 2010. Isolasi dan identifikasi bakteri asam laktat dari induk abalon (*Haliotis asinina*) yang berpotensi sebagai kandidat probiotik. *Nusantara Bioscience* 2: 38-42. Tujuan penelitian ini adalah untuk mengisolasi, menyeleksi dan mengkarakterisasi Bakteri Asam Laktat (BAL) dari induk abalon yang berpotensi sebagai kandidat probiotik pada sistem budidaya abalon. Isolasi selektif BAL dilakukan menggunakan media de Man Rogosa Sharpe Agar. Isolat BAL yang berpotensi sebagai probiotik diskriming. Pemilihan ini didasarkan atas kemampuannya dalam menekan pertumbuhan bakteri patogen, resistensi terhadap kondisi asam, resistensi terhadap *bile salt* (empedu). Selanjutnya dilakukan karakterisasi dan identifikasi untuk mengetahui spesiesnya. Hasil penelitian menunjukkan bahwa 2 di antara 10 isolat yang berhasil diisolasi dari abalon berpotensi untuk dikembangkan menjadi bakteri probiotik karena mempunyai kemampuan menghambat beberapa bakteri patogen yaitu *Escherichia coli*, *Bacillus cereus* dan *Staphylococcus aureus*, mampu tumbuh pada kondisi asam dan toleran terhadap cairan empedu selama inkubasi 24 jam. Berdasarkan uji API Kit, kedua isolat teridentifikasi sebagai anggota spesies *Lactobacillus paracasei* ssp. *paracasei*.

Kata kunci: bakteri asam laktat, isolasi, identifikasi, *Lactobacillus paracasei* ssp. *paracasei*.

INTRODUCTION

One type of shellfish that has the potential and economic value is the seven eyes shellfish. Seven eyes shellfish (*Haliotis asinina*) is also called abalone, awabi, mutton fish, sea ear and in local language (Sasak, Lombok) it is called *medau*. Abalone is included in univalve shellfish species (Cholik et al. 2005) which the meat has high nutritional value with protein content of 71.99% and 3.20% fat. Its shell also has an aesthetic value that can be used for jewelry, the manufacture of buttons and various other forms of handicraft goods (Imai 1997). Abalone is mostly found in Eastern Indonesia (Bali, Lombok, Sumbawa, Sulawesi, Maluku, and Papua). On the island of Lombok abalone is often found in southern coast of central Lombok called Kute Beach and surrounding areas. During this time abalone have been exploited by local residents without any proper selection, resulting reduction in the catch and in the long term may threaten its sustainability.

The effort of Abalone cultivation technology ranging from domestication, a trial of gonad maturation in a controlled basin, spawning, larval rearing, and larval food preparation have been done (Sofyan et al. 2005), but these activities do not give a satisfactory result. Survival rate of abalone larvae in larval rearing tanks until this time is still very low at around 1.0%. Mortality was happening a lot on planktonic stage until attachment to the substrate (first weeks). Low larval survival rate is among others due to water filtration systems that are poor which resulted in the emergence of protozoa, worms and various types of pathogenic microorganisms that can cause death of larvae.

One effort to prevent the occurrence of population shifts (split population) and also suppress the growth of pathogenic microorganisms is to maintain the natural balance of microorganisms in the larval rearing system (Haryanti et al. 1997) through the addition of probiotic microorganisms (Fuller 1989). Prevention of disease was

taking place by controlling the growth of potentially pathogenic microbes in the gastrointestinal tract (Strompfova et al. 2005; Iñiguez-Palomares et al. 2007) and a number of positive effects of probiotic bacteria including immunomodulation (Wallace et al. 2003). Development of probiotics for the cultivation of abalone would be better if the probiotic microbes are indigenous abalone itself, to avoid the problem of microbial adaptation on larval rearing tanks and seven channels of the body of this seven eyes shellfish when applied. It is therefore important to do research on indigenous bacteria isolation and identification of potentially probiotic abalone.

MATERIALS AND METHODS

Isolation of LAB strains from abalone

A total of 20 examples of healthy male and female seven eyes shellfishes are obtained from the Institute for Marine Aquaculture Lombok. Then the fluid from the digestive tract is taken in a sterile way as much as 1-10 g. Selective isolation of Lactic Acid Bacteria (LAB) is performed with Spread plate method developed by Brashear et al. (2003) and Ray et al. (1997). A total of 1 g sample added into 10 mL of fever Rogosa Sharpe (MRS) broth sterile and mixed until homogeneous. The suspension is then spread on MRS medium pH 5.5 plus 0.1% Na-azide, each in-trade with calcium carbonate 1%. Furthermore, the Petri plates were incubated at 37°C for 48 hours in an incubator in a microaerophilic atmosphere. Single colonies that grew were taken from each plate and transferred into test tubes containing 10 mL MRS broth. Then they were incubated at 37°C for 18-72 hours to obtain maximum growth cultures. Culture isolates were streaked again on MRS media for the Petri plates and incubated at 37°C for 48 hours to obtain a single colony/pure culture. In pure culture Gram stain is done for initial identification. Lactic acid bacteria culture obtained is stored by freezing the temperatures. Stock to be used was prepared by growing the isolated bacteria in MRS liquid medium and incubated at 37°C for 24-48 hours (Rahayu et al. 2004).

Test of LAB strains antibacterial power

LAB isolates were tested their ability to inhibit the growth of pathogenic bacteria namely *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus* by using well diffusion assay. Each isolate was treated in the form of fermentation result supernatant containing the extracellular metabolites, which are obtained by inoculating liquid culture of isolate lactic acid bacteria as much as 2% into the liquid media fever Rogosa Sharpe (pH 6.5) and then incubated at 37°C for 96 hours (Bar et al. 1987). After incubation pH measurements were taken, subsequently centrifugation was done upon liquid culture using a centrifuge with a speed of 3500 rpm for 20 minutes. Supernatant obtained was sterilized with bacterial filter (porous diameter of 0.2 µm, Whatman) in order to obtain sterile extracellular metabolites.

Antibacterial test was conducted using well diffusion assay developed by Djaafaru et al. (1996) and modified by

Sarkono et al. (2006), by plating test bacterium *E. coli*, *B. cereus* and *S. aureus* in Petri disk with Nutrient Agar solid medium, then added by Nutrient Agar soft medium on it. After being cooled for 1 hour in a refrigerator room, a well was made with a diameter of 0.7 mm and then isolates of bacterial supernatant was inserted and incubated at 37°C for 24-48 hours. The diameter of each isolate contained clear zone is measured.

Test of tolerance towards acid and bile

The tolerance Isolates LAB which inhibits the growth of pathogenic bacteria extensively was screened towards acids and bile. Tolerance test towards the acid uses the method of Brashear et al. (2003). LAB fresh culture harvested from MRS broth by centrifugation and the pellet obtained was washed and suspended with sterile phosphate buffer saline (PBS). Each strain was added by 4 mL of sterile PBS and pH was adjusted to pH 2, 4, 5 and 7 (control) and incubated for 2, 4 and 24 hours in a water bath at a temperature of 37°C. After each incubation period, the growth of strains can be identified by measuring the absorbance at 620 nm. Bile tolerance test was using the method of Gilliland et al. (1984). Fresh cultures of selected LAB isolates were inoculated into tubes containing 10 mL MRS broth with levels 0 (control), 0:05, 0:15 and 0.3% ox-gall. Inoculated tubes were incubated at 37°C in a water bath. Growth of isolates was observed at 2, 4, 6, and 24 hours by measuring absorbance at 600 nm.

Early identification of isolates with the API

Initial identification made to isolated LAB with inhibitory activity on the growth of *E. coli*, *B. cereus* and *S. aureus* and their tolerance to acid and bile. LAB isolates were identified through fermentation patterns with index of profile analysis standard test with 50CHL API Kit (Biomerieux 2009).

RESULTS AND DISCUSSION

Selective isolation of Lactic Acid Bacteria from abalone

Isolation process yields 10 colonies which were suspected as isolates Lactic Acid Bacteria (LAB) because they produce a clear zone in isolation medium (Figure 1), then a strengthened test was conducted by growing on solid MRS medium plus CaCO₃ 1%. From this confirmation test by re-growing process showed that all 10 isolates LAB could grow well and produce clear zones around colonies. The characterization results further prove that the 10 isolates allegedly a member of the lactic acid bacteria (Table 1).

Results of identification at the genus level confirm that the four isolates that were characterized are members of the genus *Lactobacillus*. These isolates have phenotypic characters among others the forms of stem cell are long, the structure resembles a fence and row of cells singly scattered, Gram-positive reaction, not motile and does not form endospores (Sneath et al. 1986). Images of each isolated cell can be seen in Figure 2.

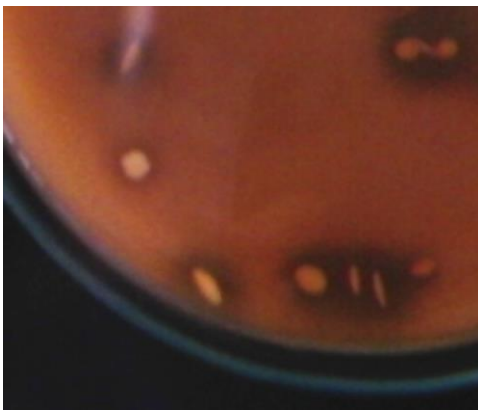


Figure 1. Colonies are indicated as LAB with clear zones around colonies

Test of antibacterial power LAB strains against pathogenic bacteria *E. coli*, *B. cereus* and *S. aureus*

The result of bacterial growth inhibition test with the indicator diffusion method showed that seven among ten isolates showed the ability to inhibit the growth of bacteria, characterized by the formation of clear zones around the wells with varied sizes. Three isolates had the ability to inhibit the three bacterial indicators as well as the isolates OPA3, OPA4 and AL1. Three isolates could inhibit the growth of two indicator bacteria namely OPA5, OPA6, and OPA7.

Table 1. Test results that characterized the feature of Lactic Acid Bacteria isolated from abalone

| Isolates | Resources | Feature that characterized Lactic Acid Bacteria | | | | |
|----------|------------------|---|---------------|---------|----------|-----------|
| | | Cell shape | Gram reaction | Kataase | Motility | Endospora |
| OPA1 | Digestive organs | Stem | + | - | - | - |
| OPA2 | Digestive organs | Stem | + | - | - | - |
| OPA3 | Digestive organs | Stem | + | - | - | - |
| OPA4 | Digestive organs | Stem | + | - | - | - |
| OPA5 | Digestive organs | Stem | + | - | - | - |
| OPA6 | Digestive organs | Stem | + | - | - | - |
| OPA7 | Digestive organs | Stem | + | - | - | - |
| AL1 | Sea water | Stem | + | - | - | - |
| RL1 | Seaweed | Stem | + | - | - | - |
| KA1 | Abalone feces | Stem | + | - | - | - |

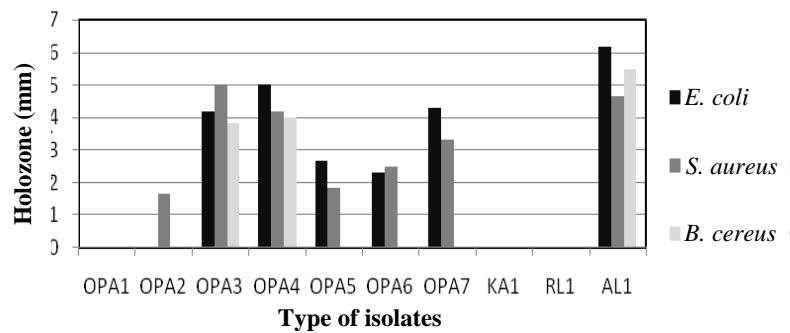


Figure 3. Antibacterial test isolates LAB supernatant against indicator bacteria *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* with well diffusion method

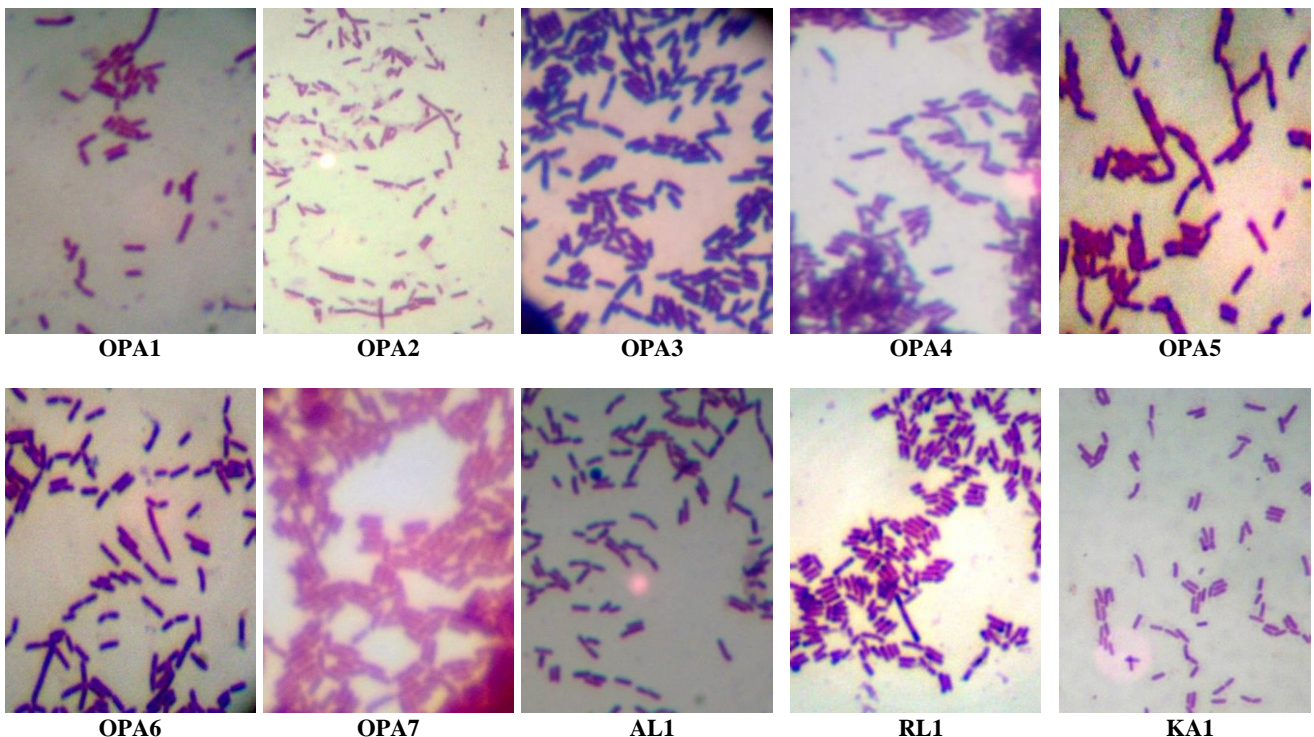


Figure 2. Gram reaction and cell shape of LAB isolates which were isolated by seven eye mussel (abalone) and their habitats

Meanwhile, only one isolate which is only able to inhibit the growth of one indicator bacteria namely OPA1 isolates, whereas three other isolates namely OPA1, RL1, and KA1 did not have the ability to inhibit any bacterial indicator (Figure 3).

Based on the character of inhibition zone, ten isolates tested showed different characters of inhibitions, but in general, some of them showed inhibition zone with blurred edges (not firm) and others showed inhibition zone with firm edges. Blurred edges zone indicates that the active metabolite found in the supernatant is bacteriostatic, which only inhibit cell growth of indicator bacteria but not kill the cell. According to Rahayu (2004), inhibition with vague zone might be the action of acid and other antibacterial components which are only bacteriostatic, since most bacterial test (indicator) remains alive in the clear zone, although with very slow growth. Meanwhile, inhibition zone with a firm edge indicates that isolates can produce metabolites which are bactericidal, where metabolites can kill bacterial cells indicator. This is one of the expected ability of probiotic bacteria so it can control the growth of pathogenic bacteria in their applications.

Test of tolerance to acid and bile

Based on the results of testing the ability of inhibition on the growth of pathogenic bacteria in a previous study phase and then continued by selecting two isolates that had the best inhibitory then proceed with the test of isolates growth in an atmosphere of acid and bile. Data obtained from this test form absorbance data using a spectrophotometer. The addition of absorbance values in line with the addition of incubation time showed the growth of LAB isolates tested (Figure 4). Figure 4 shows that both of tested Lactic Acid Bacteria isolates showed the ability to grow in acidic environment which is relatively similar. Isolate OPA4 and AL1 have excellent adaptability to acid atmosphere, because of an increase in growth at 3 pH levels in 24-hour period. At pH 2 the two isolates did not grow, because the pH of 2 is a very extreme pH for growth of microorganisms, including lactic acid bacteria which are generally well adapted to living in habitats with a relatively low pH environment. At pH 4, 5 and 7 both isolates are able to grow well, the exponential increase in growth occurred in the observation at 24th hour because of the incubation period is long enough from the 4th hour up to the 24th hour resulting in significant cell division. Isolate OPA4 achieve the best growth at pH 7 whereas at pH 5 isolates AL1. Lactic acid bacteria

generally prefer the atmosphere of a pH slightly below a neutral pH for best growth (Axelsson 1998). The result of the endurance test isolates of bile showed that the four isolates had very, very good ability, because of an increase in growth in the overall level of concentration of bile (0.05%, 0.15%, and 0.30%) in 24-hour period (Figure 4).

The ability to grow of the two isolates namely AL1 OPA4 in the bile cannot be distinguished from each other. This is predicted caused by the very low concentration of bile that is used. The tests for resistance toward bile liquid used method that was developed by Gilliland et al. (1984) which uses bile concentration of 0.05%, 0.15%, and 0.30%. As a comparison, other researchers (Ljungh et al. 2002) tested the resistance of isolates *Lactobacillus paracasei* subsp. *paracasei* F19 in 20% bile and continues to show growth on incubation time of 2 hours.

Early identification of isolates with the API

API biochemical test kits are used to determine the biochemical characteristics of LAB isolates that are tested so that it can be used for identification purposes. Because the two LAB isolates tested are members of the genus *Lactobacillus*, so we only use the API Kit 50CHL content of which is 49 kinds of sugar and its derivatives, plus one negative control so in overall there are 50 types of test (Biomerieux 2009). Visually Kit API 50CHL represented by Figure 5.

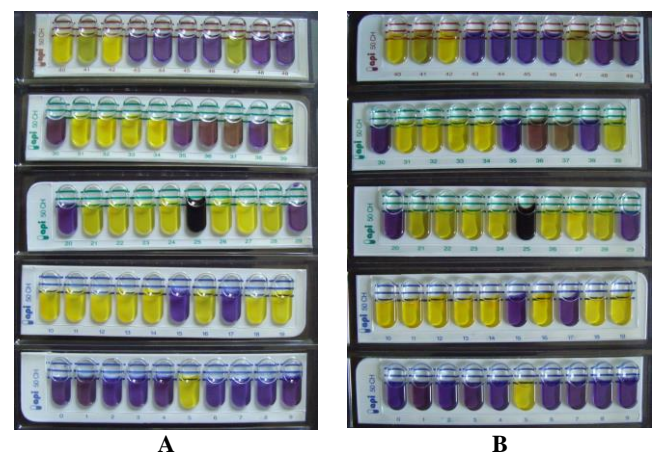


Figure 5. Visualization of the results of sugar fermentation test with API Kit 50CHL (a) isolates AL1 48 hours and (b) isolates OPA4 48 hours

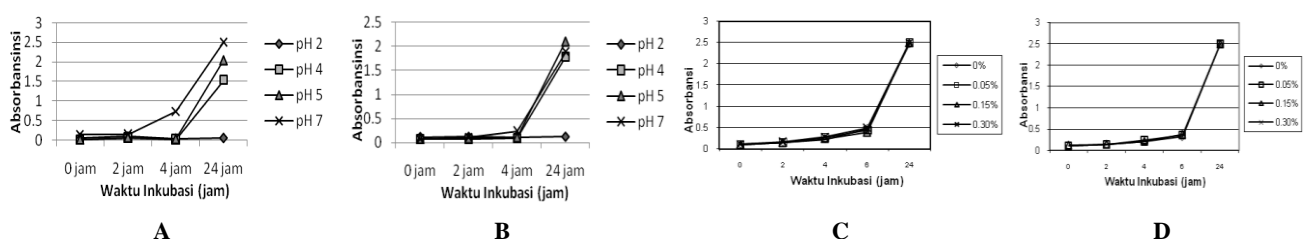


Figure 4. Selected isolate growth test during 24 hours incubation time. A. Isolates OPA4 in bile, B. Isolates AL1 in bile, C. Isolates OPA4 in the acid atmosphere, D. Isolates AL1 in the acid atmosphere

The test of sugar fermentation is a very important characterization process in the genus *Lactobacillus* to know the character to the identification of species diversity (Holt et al. 1994). The result of sugar test with the API kit toward 10 isolates of the isolated form of positive character (+) and negative (-) which in total amounted to 50 characters, then analyzed by a computer program Apiweb™ Version 1.2.1 to identify the species name.

The test results showed that after 48 hours incubation AL1 OPA4 isolates gave the same results, that are positive reactions on sugar numbers 5, 10, 11, 12, 13, 14, 16, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 31, 32, 34, 39, 40, 41, 42 and 47, the rest react negatively. This shows the level of characters with very high similarity between the two isolates, so it is possible that they are from the same strain, at least a member of the same species. The test results which is in the form of sugar fermentation profile was analyzed with the program Apiweb™ version 1.2.1, the result is that the two isolates tested is a member of the same species of *Lactobacillus paracasei* ssp. *paracasei*. This species has a very close relationship and even considered as neotype strain of *Lactobacillus lactic* species (Dellaglio et al. 2002). According to Vlieger et al. (2009) members of this species have been applied as probiotic bacteria in infant milk together with *Bifidobacterium*.

CONCLUSIONS AND SUGGESTIONS

A total of ten isolates of LAB can be isolated from gastrointestinal tract of abalone and their habitats. After the selection, there are two isolates obtained potentially to be the candidates for probiotic that is OPA4 and AL1. Both isolates have the ability to inhibit the growth of enteropathogenic bacteria namely *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* with inhibition zone varied widely, and able to grow in acidic conditions and tolerant of bile during 24 hours incubation. Based on the API test kit and analyzed with software 50CHL Apiweb™ Version 1.2.1, both isolates are identified as members of the species *Lactobacillus paracasei* ssp. *paracasei*. Isolates of this research which have the potential to be a candidate of probiotics in abalone larval rearing system of (*Haliotis asinina*) are expected to be studied further in order to know its potential in improving the survival ability of abalone larvae in vitro and in vivo, so it can be recommended as probiotic bacteria, especially in the abalone farming systems in the future.

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