Screening and isolation of PHB (Poly-β-hydroxybutyrate) producing bacteria as an alternative material for disease prevention on the shrimp culture

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Abstract. The shrimp mortality cases in ponds are still dominated by the bacterial diseases caused by Vibrio pathogenic bacteria. Antibiotic application as a disease alternative treatment is not recommended because it can trigger the host immunity and cause residual contamination in the environment. The latest study stated that poly β-hydroxybutyrate (PHB) has bactericidal activity against the pathogenic bacteria and can be a source of C (carbon) atoms that are easily decomposed by the bacterial intestinal flora. This ability is obtained from the short-chained fatty acids (butyric acid) in PHB compounds. This study was aimed to screen and isolate the candidates of PHB-producing bacteria as an alternative to control environmentally friendly disease prevention in shrimp culture. Sampling was performed at the caming sugar factory located in Bone. Samples were taken in the form of kettle ash, molasses, the soil around the factory site, bagasse, and solid sugar factory waste. Samples were serially diluted using a sterile aquadest and grown on the nutrient agar media, then incubated for 48 hours. After 48 hours, the morphological observations were performed for each bacterial colony growing in each analyzed sample. The isolation of PHB-producing bacterial candidates was performed by isolating triplicate colonies and cultured on agar nutrient (NA) media added with 1% glucose, then incubated for 48 hours. The growing bacterial colony was recultured on a spot of NA media through plating divided into 4 equal parts and incubated for 48 hours. Each colony was given an ethanolic 0.02% Sudan Black B solution and stood for 30 minutes, then rinsed with 96% ethanol. The positive results of PHB-producing bacteria were shown in dark blue color absorbed by the bacterial candidate due to the staining performed. The study results showed that 73 isolates of PHB-producing bacterial candidates were obtained from 5 samples analyzed and after stained with Sudan Black obtained 14 bacterial isolates containing 10 isolates produced a clear zone (inhibition zone) and 4 isolates absorbed the dark blue color. After purification and retesting on the 14 isolates, 5 isolates were obtained as candidates for PHB-production bacteria. Keywords: Screening, isolation, bacteria, PHB production, alternative, disease, shrimp.

1. Introduction
The classic problems that cause low shrimp culture production are bacterial and viral disease (Lightner and Redman 1998). Vibriosis is a bacterial disease caused by Vibrio spp. bacteria, which can cause a high mortality rate on not only occur in hatcheries but they’re happened in growing out the pond in traditional, semi intensive or intensive culture system. Vibriosis is a combination of physical stress factors or primary infections, followed by other pathogenic infections [1]. This factor is one of the
causes of variations in the mortality rate of shrimps when attacked by *Vibrio* spp, in addition to changes in bacterial gene mutations becoming more violent. Although *Vibrio* spp are often considered as opportunistic pathogens on shrimp, *Vibrio* spp can become the main disease. This happens due to the abundant population of virulent bacterial strains [2].

Some efforts have been performed to overcome the bacterial disease problem, one of which is the use of antibiotics and pesticides to control the bacterial pathogens. In the early years, the use of antibiotics and pesticides is considered the best way-out to overcome the low survival rate of the cultured animal. However, after some years passed, this method caused a new problem. The bacterial pathogen inhibition effort is incapable of using the usual dozes, but it had to be increased or replaced with a higher antibiotic type. This condition will happen continuously, while the searching results for more potentially new antibiotics is not as fast as the bacterial resistance rate. Moreover, the antibiotic withdrawal time to be degraded in nature is an imbalance with the added antibiotic residual, which always increases along with its doze usage. An alternative that has to be done to overcome the bacterial disease problem is an environmentally friendly and unrisky solution that will not cause new bigger problems to keep the cultured animal health.

Some previous studies showed that short-chained fatty acids can inhibit (bacteriostatic) and kill (bactericidal) yeast and enterobacteria, such as *Salmonella typhmurium*, *Escheresia coli* and *Shigella flexneri* [3–5] and fix the animal digestive tract condition. One of the compounds that has a short-chained fatty acid is β-hydroxybutyrate (PHB) or 3-hydroxybutyric acid. PHB is a compound used by the bacterial group to store their energy and as carbon storage compounds [6]. Nhan et al. (2010) reported that the given feed to *Macrobrachium rosenbergii* using *Artemia* enriched with PHB had better survival rate and could suppress the *Vibrio* spp. bacterial population, while Defoirdt et al. (2007) [7] stated that PHB could give better protection for *Artemia franciscana* against *Vibrio campbellii* bacterial attack.

Commercial PHB usage in the aquaculture system may probably clash with high costs; nevertheless, PHB can be produced easily from *Bacillus* and *Lactobacillus* spp., besides materials obtained from nature easily. The source of isolation of PHB-producing bacteria is an environment that contains a lot of carbon (C) sources that are excess compared to other nutrients (nitrogen and phosphorus). The condition of confusion with a high source of C can be found in high-carbohydrate food waste processing (sago processing waste, cassava) and sugar processing Factory. Therefore, this study was aimed to perform screening and isolation of bacteria with the ability of producing PHB, which later can be used as environmentally friendly bacterial problem prevention alternative on the shrimp culture.

2. Data and Methods

2.1. Screening and Isolation Bacterial from Some Samples

Bacterial screening and isolation were performed on the taken samples as the results of the by-product remaining from the Camming sugar factory located in Bone Regency, South Sulawesi. Samples taken contained molasses (sample code 1), bagasse (sample code 2), ash kettle (sample code 3), solid sugar factory waste (sample code 4), and from soil around the factory as the solid sugar factory waste dump location (sample code 5). The bacterial isolation from the samples was performed through a serial dilution using a sterile aquadest. Each serial dilution on each sample was grown on the nutrient agar (NA) media in the Petri dish and incubated at 37°C for 48 hours. After 48-hour incubation, the colonies formed on each serial dilution were observed their growth, counted their density based on total plate count (TPC), and characterized morphologically.
2.2. *PHB Producing Bacterial Selection*

2.2.1. Culture Preparation

Bacterial pure culture obtained for each colony formed, which could be seen morphologically on each sample, was isolated with replications to produce 3 same isolates and grown on NA medium added with 1% glucose, then incubated for 48 hours at 37°C.

2.2.2. PHB Production Test by Bacterial Isolates

Each bacterial isolate was recultured on NA+1% glucose media by spotting on the Petri dish divided into the five equal parts, then incubated for 24 hours at 37°C. PHB production test by the bacterial isolates was performed through a staining method using Sudan black stain. The Suddan black stain was made by mixing 60 mg Sudan Black into 200 ml ethanol 70% (0.02% ethanolic solution [8,9], then incubated for 24 hours with a magnetic stirrer. The Sudan black stain was performed with a spreading method (spreaded stain volume of ± 100µL) onto each colony grown in each Petri dish and stood for 30 minutes, then rinsed with ethanol 96% to remove the stain on the colony [10].

2.2.3. Gram Staining

Gram staining was also performed to identify the characteristics and shape of isolated bacteria by streaking each bacterial isolate on the object-glass and dried for approximately 1 hour, then fixed using Bunsen flame. Crystal violet stain was given and stood for a minute and rinsed with the flowing water, then given iodium and stood for 2 minutes. After 2 minutes, the isolate was cleaned using PA ethanol by dipping-pulling until clean, then dried and given safranin stain, then rinsed. Sample was stood until dry and observed under the microscope with 40X magnification.

3. Result and Discussion

The first step performed to obtain the candidate of PHB producing bacteria was by conducting screening and isolation of bacteria from the samples taken from the substrates containing sufficiently high carbons as one of which took the samples from the by-product of the sugar factory. From 5 samples analyzed, 68 bacterial isolates were obtained, then screened using a Sudan black stain and obtained 14 PHB producing isolate candidates with positive reactions (absorbing blue color of Sudan black stain). Besides showing positive results, some isolates also produced clear zones on the media. These bacterial isolates were purified and performed a restaining, which obtained 5 isolates as the candidate of PHB producing bacteria. The bacterial population on each sample can be seen on the following Table 1:

| Sample type and code | Density (CFU/mL) |
|----------------------|------------------|
| Mollase (1)          | $7 \times 10^4$  |
| Bagasse (2)          | $5 \times 10^6$  |
| Kettle ash (3)       | $1.48 \times 10^7$ |
| Solid sugar factory waste (4) | $2.58 \times 10^7$ |
| Soil (5)             | $1.3 \times 10^8$ |

Table 1 shows that the bacterial population from each sample is varied as the highest was found from the soil sample with $10^8$ CFU/mL density, while the lowest was obtained from the molasses sample. The high bacterial population in the soil sample may be because soil is the collected materials containing minerals, organic matters, other nutrients, water, even air, therefore becoming a good living site for organisms—the more microorganisms on land, then the higher microorganism activities on the
land. Moreover, the microorganism population in the soil is affected by the region's climate condition, grown vegetation, ongoing reaction, and humidity level.

From 5 isolated samples, 29 colony types had different morphological characteristics, i.e. 7 types from molasse sample (1), 2 types from bagasse sample (2), 7 types from kettle ash samples (3), 6 types from solid sugar factory waste, and 7 types from a soil sample. The isolation on each colony type was made with 2 or 3 isolates depending on the colony types existed; therefore the initially analyzed isolates were 73 isolates comprising 17 isolates from molasse sample (with the isolate code of 1a–1n), 4 isolates from bagasse sample (isolate code 2a–2d), 18 isolates from Kette ash (isolate code 3a–3r), 16 isolates from solid sugar factory waste (isolate code 4a–4q), and 18 isolates from soil sample (isolate code 5a–5r).

All bacterial isolates that grew on the growth medium were morphologically characterized and each of the three different colonies was taken as three isolates to be stained. Morphological characterization for each bacterial colony can be seen in table 2.

| Isolat Code | Morphological characteristics |
|-------------|------------------------------|
| Mollase (1)  |                              |
| a,b,c       | milky white, wide shape, spongy edge, dry |
| d,e,f       | white, dry, middle surface arises |
| g,h         | yellow, convex, glossy |
| i,j         | small round shape, milky white, convex, glossy |
| k,l         | flat, wide, jagged edges |
| m,n         | medium round, middle wet, sandy edge, creamy |
| o,p,q       | round, milky white, glossy |
| Bagasse (2)  |                              |
| a,b         | round shape, flat surface, shiny |
| c,d         | round shape, convex, shiny, milky white |
| Kettle ash (3)  |                              |
| a,b,c       | small round shape, white, convex surface, glossy |
| d,e         | Round shape, wide, dry, wavy edge |
| f,g,h       | Round shape, beige, glossy, embossed surface |
| i,j         | dry, wide shape, flat, spongy edge, |
| k,l,m       | Round shape, wet, flat edge, bubbling inward |
| n,o,p       | Round shape, convex surface, wet, glossy, beige |
| q,r         | Small shape, dry, beige, serrated |
| Solid sugar factory waste (4)  |                              |
| a,b,c       | orange, round shape, shiny |
| d,e         | small round shape, white, convex surface, glossy |
| g,h,i       | Widened shape, slightly wet, sandy edges |
| j,k,l       | small round shape, convex surface, glossy, flat edges |
| m,n         | small round shape, convex surface, glossy, jagged edges |
| o,p,q       | big round shape, shiny, middle embossed |
| Soil (5)  |                              |
| a, b, c     | small round shape, glossy, convex surface, milky white |
| d,e,f       | small round shape, convex surface, glossy, white |
| G           | Round shape, convex, shiny, pink |
| h,i,j       | Round shape, the middle is rather dry, sandy, beige |
| k,l,m       | Round shape, glossy, orange |
The selection of PHB producing bacteria was performed on the 73 bacterial isolates, after pured and stained using Sudan black repeatedly, 5 bacterial isolate candidates could accumulate the PHB compound on their cells. These bacteria were obtained from the by-product of sugar factory suspectively, who stated that bacteria live in a rich C source environment intend to accumulate certain reserved materials, such as lipid granules and PHB.

PHB producing bacteria are positive reacted bacteria by absorbing the dark blue color of Sudan black stain [10] as seen on the following Figure 1:

**Figure 1.** The absorption of the dark blue color of Sudan black stain by PHB producing bacterial isolates

Figure 1 presents that the absorption of dark blue color as the cause of Sudan black stain by PHB bacterial isolate candidates is different, although it has been cleaned using ethanol 96%. This condition may be due to the compound content and concentration difference of PHB compounds owned by the bacteria. This suspicion was based on Du et al. (2004) in Haedar (2015), who stated that bacteria live in a rich C source environment intend to accumulate certain reserved materials, such as lipid granules and PHB.

The morphological, Gram, and cell shape characteristics of 5 bacterial isolate candidates are presented in the following Table 2.

**Tabel 3.** Morphological, Gram, Cell Shape from Each PHB Producing Bacterial

| Isolate code | Morphology                        | Gram   | Cell Shape         |
|--------------|-----------------------------------|--------|--------------------|
| 3K           | Circular shape, wet, flat margin, creamy | Positive | Rod                |
| 4H           | Widened shape, rather wet, sandy margin, creamy | Positive | Chained coccus    |
| 4I           | Widened shape, rather wet, sandy margin, creamy | Positive | Chained coccus    |
| 5O           | Rather a flat shape, wet, bubbly margin, white | Positive | Chained coccus    |
| 5P           | Rather a flat shape, wet, bubbly margin, white | Positive | Chained coccus    |

The observation result of bacterial cells based on the Gram staining under the microscope indicated that all PHB producing bacterial isolate candidates isolated, as seen in Table 2 had positive characters with one bacteria had rod shape and four others had chained coccus shape. The positive characters from these bacteria were based on the explanation of Byrom (1987) that bacteria could produce PHB granules in their cells that can come from either Gram-positive or negative bacteria. Gram-positive bacteria are bacteria that can absorb the dark purple color from crystal violet stain, while Gram-negative bacteria are bacteria that can absorb the red color from safranin.
Sudan black is the specific stain to color various lipids, such as neutral lipids, phospholipids, and sterols, therefore can be used to select bacteria that have PHB compounds as a sudanophilic character (can be stained using a lipid staining).

4. Conclusion
Based on the study performed, it can be concluded that from the screening and isolation results of 73 bacterial isolates from 5 sample types, namely molasses, bagasse, kettel ash, solid sugar factory waste, and soil from Camming sugar factory in Bone Regency, South Sulawesi, 5 bacterial isolates could accumulate PHB compounds in their cells.

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