Effects of polysaccharides-crude extract from Candida sp. OCL1 on hematological parameters of Aeromonas hydrophila-infected catfish (Pangasius pangasius)

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Abstract. Fishery processing waste is an environmental problem. Fish waste, such as the head of Nile tilapia fish (Oreochromis niloticus), is underutilized and has a low economic value, even though the waste can be re-processed. The Pangasius pangasius is an important economic fish species in Indonesia. The production usually decreased due to the infection of the bacteria, Aeromonas hydrophila. To overcome the infection, the addition of immunostimulant agents is a substantial effort. One of the well-known agents is β-glucan, which can be found in the yeast cell wall. This experiment evaluated the effect of polysaccharides-crude extract from Candida sp. OCL1 (PCEC) with four different concentrations (0.0, 0.5, 1.0, and 1.5%) on nonspecific immune parameters of catfish P. pangasius. Results indicated that diet supplementation with PCEC gave a non-significant difference (P > 0.05) in all blood cells parameter (total white blood cell, neutrophil, monocyte, lymphocyte, macrophage) and phagocytosis of macrophage activity on day 0. PCEC diet was significant (P < 0.05) affected all hematological parameters. We conclude that the PCEC diet in the feed improves the non-specific immune system of Catfish (P. pangasius).

1. Introduction
Microorganisms are among the most sensitive causing agent of fish diseases. Pathogenic organisms, such as bacteria, have the most significant problem [1]. In fish farming, A. hydrophila constitutes natural microbiota in both the aquatic environment and fish, but are also one of the most frequently diagnosed epizootic risk factors affecting fish around the world [2]. They are ubiquitous in aquatic environments, and they are capable of decomposing various organic compounds due to a broad range of enzymatic activities. A. hydrophila can spread from aquacultures and induce changes in the total populations of bacteria in aquatic ecosystems [3].

The spread of disease, which negatively impacts the development of the aquaculture sector, should be controlled. Antibiotic-based controls is not a best choice. Antibiotic, such as enrofloxacin reduce
the survival rate of fish due to the damaging effect on the tissue [4]. The residue in the product will harm the human health.

Hematological parameters in disease diagnosis and nutritional status of fish represent an essential tool for aquaculture. The immune system comprises a set of cellular and humoral components that act to defend the body against foreign substances, such as microorganisms, toxins, or malignant cells, responding to many factors, both exogenous and endogenous, that stimulate the components of this system [5]. Hematological parameters are used to provide information about the health and physiological status of fish, feeding conditions, and water quality in which they live.

Hematological parameters will contribute to more specific, convenient, and effective disease treatments in the future. The use of immunostimulants, as dietary supplements, can improve the natural defense of animals providing resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer, and vaccination [6]. Hence, a potential method for improving larval survival increases an innate response of the developing animals through immunomodulation.

A polysaccharide is a complex carbohydrate polymer. It consists of more than two monosaccharides, which covalently linked by glycosidic linkages in a condensation reaction. Various types of polysaccharides include cellulose, β-glucans, pullulans, glycogens, and various starches. The most common and abundant polysaccharide in nature is cellulose [7]. Structural differences can affect the extraction process of some β-glucans, and in turn, this can affect their immunostimulatory activity. The higher molecular weight of glucans can activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activities [8]. The objective of the research was to investigate the immunostimulatory effect of crude extract of polysaccharide from marine yeast on non-specific immunity of Catfish (P. pangasius).

2. Material and methods
2.1. Fish sample
Catfish (P. pangasius) with an approximate size of 10-12 cm full length were obtained from the local fish farm, located in Sumberpasir, Malang District. Fish transported into plastic containers and acclimated to the laboratory conditions for two weeks in a pond containing 500 L of continuously aerated freshwater. Fish were fed with commercial fish food twice a day during the acclimation period.

2.2. Isolation of Candida sp. OCL1
Candida sp. OCL1 were obtained from our previous work. The isolate was deposited on Lab. Microbiology, Faculty of Fisheries and Marine Science. The Candida sp. were previously isolated from Ocean water in Malang Regency, East Java, Indonesia.

2.3. Polysaccharides-crude extract of Candida sp. OCL1 (PCEC)
Each yeast-like microbe isolate was grown at 25 °C for 65 h in 200 ml of a modified YPD culture solution. The cells grown were collected by centrifugation at 5.000 rpm for 15 min at 4 °C. They were then suspended in 100 ml of sterile distilled water and centrifuged again, as described above. The cells thus obtained were used in the preparation of yeast extracts. The yeast solution was adjusted to pH 8.0 with a 2N NaOH solution, then mixed. Heat it at 115 °C for 45 min, centrifuged for 15 min, then washed three times with dH2O, add 0.6 L HCl and mixed. An aliquot was heated at 85 °C for 45 min. Pellets were collected by centrifugation three times washed and dried [9].

2.4. Challenge test using A. hydrophila Bacteria
Healthy P. pangasius was acclimatized in the pond for two weeks. The experiment consisted of four treatments:
(A) pellet feed + 0.0% PCEC
(B) pellet feed + 0.5% PCEC
(C) feed pellet + 1.5% PCEC
(D) pellet feed + 1.0% PCEC
The experiment used fifteen aquaria (0.5 x 1.0 m), and each aquarium contained ten fish. The fish were cultivated for fifteen days. Fish were fed with different doses of PCEC following the groups, at 3% of body weight. Water was changed every two days. On the seven days, A. hydrophila (density of $10^6$ cells/ml) infected to the water for 24 hours. Fish were cultured for fifteen days. Hematological parameters are determined before and after the challenge test.

2.5. Hematological parameters
Blood samples were taken on before the challenge test (day 0) and after infection on the fifteenth day (day 15). Blood samples were collected using 0.1 cc sterile plastic syringes, which contained EDTA as anticoagulation. As much as 0.2-0.3 cc, blood was taken randomly picked from the control and experimental group through the dorsal artery. Blood was stored in the box. The temperature of the box was maintained on 4° C. Blood samples were collected to measure the total number of White Blood Cell (WBC), differential count of leukocytes, macrophage, and macrophage phagocytosis activity. WBC was counted by using a Neubauer hemocytometer [10]. Blood was diluted 1:20 with Turk’s diluting fluid (1% glacial acetic acid solution and Gentian violet 0.3 percent w/v dissolved in distilled water). Four large (1 square mm) corner squares of the hemocytometer were counted on a microscope. Differential count of leukocytes (monocyte, lymphocyte, and neutrophil) and macrophage phagocytosis activity were determined using the Giemsa staining method and detected blood smears under the light microscope.

2.6. Data analyses
Data are presented as mean ± SD of samples. ANOVA (Analysis of Variance) technique, using MINITAB 13 program, was used to test the significance of differences between three crude extract of Candida sp. polysaccharides. If the result showed a real difference, it would be continued with the test of the Least Significant Differences (LSD) 5%.

3. Results and discussion
3.1. Hematology parameters
The result of the hematological test on catfish (P. pangasius) experienced PCEC diet prior to and during infection of A. hydrophila was resumed in Table 1. The results revealed significant differences among various concentrations of PCEC toward blood parameters after infection. Before the infection, all doses of the diet did not affect all parameters of hematology.

| Blood Factor | Time (Day) | A  | B  | C  | D  |
|--------------|------------|----|----|----|----|
| Total WBC    | day 0      | 55,500.00±3031.09a | 58,583.33±404.51a | 69,166.67±629.15a | 64,583.33±8427.39a |
| (cells per µL Blood) | day 15 | 62,916.67±7783.53a | 88,000.0±6805.33b | 115,083.33±5735.49c | 85,166.67±40018.19bc |
| Neutrophil (%) | day 0 | 30.67±1.15a | 36.33±1.53a | 39.00±1.00a | 34.00±1.00a |
|               | day 15 | 31.33±0.58a | 40.00±1.00c | 49.33±1.15d | 36.67±1.53b |
| Monocyte (%) | day 0 | 4.33±0.58a | 6.00±1.00a | 9.00±1.00a | 4.33±1.53a |
|              | day 15 | 6.00±1.00a | 8.67±0.58c | 13.00±1.00d | 7.00±1.00a |
Before the infection, total WBC varied from 55,500.00±3031.09 to 69,166.67±629.15. Even though there are no significant differences among all treatments, the lowest WBC was found in A treatment without PCEC. Its highest value for WBC before infection was in 1.0% diet of PCEC. After the infection, significantly, the highest WBC was found in the 1.0% diet of PCEC. Meanwhile, treatment A (0.0 % PCEC) gave the lowest WBC. In almost all treatments, for neutrophil, monocyte, lymphocyte, macrophage, and phagocytosis of macrophage activity, a similar trend was also found. All the treatments failed to give a significant difference in all parameters before infection (0 days).

On the day of fifteen, the sample was investigated for its blood parameters. Blood parameters showed the different result on *A. hydrophila*-infected fish. A treatment exhibit the lowest neutrophil with a value of 31.33±0.58. One percent PCEC diet resulted in the highest neutrophil. Monocytes increased following the amount of PCEC in the diet feed. The increase was only on the 1% PCEC diet. While on a diet of 1.5%, monocytes have decreased. A similar trend was also found in Lymphocyte and Phagocytosis of Macrophage activity parameters. All of the figures on the blood parameters as an effect on the PCEC diet were depicted in Figure 1.

![Images](image1.jpg)
Marine yeast immunostimulants are those substances obtained from non-host structural materials. β-glucan is a major structural component of cell walls. It acts as storage carbohydrates, and they sometimes play a protective role by forming at specific sites in response to a particularly high molecular weight of glucan produced by the fungus [11]. Besides, the immunostimulatory activity of glucans derived from marine yeast has been observed to be even higher than that of Saccharomyces cerevisiae glucan [12].

Yeast β-glucan and nucleotide was significantly lower mortality during the first four weeks or 4 – 8 weeks post initial feeding. It has been proved that dietary oligosaccharides supplementation stimulated humoral and cellular innate immune responses and improved disease resistance of fish due to its positive effects, including activation and facilitation of antigen processing and regulation of intestinal microbiota [13]. Additionally, Yeast β-glucan administration at 0.1% in Asian catfish (Clarias batrachus) feed significantly raised the levels of serum MPO, lysozyme, and protection against A. hydrophila challenge, regardless of feeding periods (1, 2 and three weeks) [14]. The study showed that the growth of Persian sturgeon was significantly increased by dietary β-glucan supplementation. Growth-enhancing effects of β-glucan have been reported earlier in other fish species [8].

Glucan-specific receptors are present on phagocytic cell membranes of several species [15]. Increasing total leukocytes can be used as a sign of the initial phase of infection. The presence of infection will cause inflammation, leukocytes respond by reacting to interference with foreign objects or infections from bacteria by multiplying themselves. If there is an attack of a foreign object in the body, the leukocytes will respond by increasing the amount in defending the body from foreign body attacks. In this study, a hematological analysis was conducted to examine the effect of crude extract. Leucocyte was slightly increased during the study.

The percentage of the leucocyte cell types is different among fish species [13]. Fluctuations in leukocyte count after the test showed that P. pangasius defended itself from A. hydrophila infection. According to Uribe [16], the leukocyte is one part of the non-specific immune system. Leukocytes, which produced in high numbers, will occur when there is an infection in the body related to the immune system working against the infection. In the post-challenge test period, there was the effort of the fish to defend itself from infection by the phagocytic process. Phagocytosis is the first defense of the cellular responses made by monocytes (macrophages) and granulocytes (neutrophils).

Indicators of fish health are the number of lymphocytes. These blood cells are vital components of innate immune defense and are involved in the regulation of immunological function in the organism [17]. Changes in differential leukocyte count are recognized as a sensitive indicator of environmental stress [18]. The lymphocytes are reported to be responsible for immune response producing antibodies and chemical substances serving as a defense against infection [19].

A foreign object or cell infiltrates into the tissue, and the neutrophil cells will increase the amount in the conflict area or at the place where the foreign body is present. It is due to the presence of specific signals issued by tissue cells in the place of foreign matter (conflict) so that neutrophils in other places migrate to that place. The increase in the percentage of monocytes during the bacterial infection of A. hydrophila is caused by a foreign body, which causes monocytes to destroy foreign objects (A. hydrophila).

Lymphocytes were the most common cells found in fish leukocytes because lymphocytes served as the body defense system against infection. Changes in the number of leukocyte cells could be a symptom of stress resulted in the decline of the fish’s immune system, which made it susceptible to disease attack [20].

Lymphocyte deficiency can reduce the concentration of endurance and cause an increase in disease attacks. According to Maftuch [21], an increase in the number of lymphocytes, monocytes, and
neutrophils was to be the reason for the increasing total number of leucocytes. Another reason that could increase the number of leucocytes was a bacterial infection.

Monocytes and neutrophils are important white blood cells to protect the body through their phagocytic activities against bacterial infection in damaged cells [19]. Monocytes transform into macrophages and may be involved in phagocytosis and killing of pathogens upon first recognition and subsequent infections. The monocytes population exhibited a significant decrease in all the infected fishes after 24 hours of infection after that fluctuated erratically in control feed fed fishes whereas recovered to normalcy in Immunostimulant fed fishes [22]. The more bacteria that will be phagocytosed by monocytes will encourage monocytes to divide and multiply themselves. Bacteria will be phagocytosed by the neutrophils, and then was inserted into the phagosome. In phagosome acidic hydrolase enzyme, myeloperoxidase and lysozyme will lyse and digest the cell of pathogenic bacteria [23].

Macrophages have protective properties carried out by phagocytic cells against the presence of foreign material /microorganism infection. In the 1% treatment, the crude extract of marine yeast polysaccharide increased the phagocytic activity of macrophages compared to other controls and treatments. Polysaccharides in marine yeast cell walls binding to molecular receptors on the surface of phagocytic cell membranes, these cells will be increasingly active in engulfing, destroying (killing), and digesting (digesting) pathogens.

The level of macrophage activity depends on the fish species, pathogen, and type of external stimuli, as seen from the responses to live or dead bacteria, and techniques used to measure this activity [24]. The phagocytic cells are involved in eliciting an inflammatory response, phagocytosis, and bactericidal activity, producing bactericidal reactive oxygen species (ROS) and nitric oxide (NO) for killing pathogens. The incubation time and temperature for optimal macrophage expression also differ between fish species. For striped catfish, the optimal time for macrophages adherence to the plates before assessing their respiratory burst and phagocytic activity was found to be 2 hours at 300 °C. In contrast, the optimal adherence time for Nile tilapia was one hour for peak macrophage phagocytic activity [25].

The study on Piaractus mesopotamicus gave a similar result. P. mesopotamicus fed using feed containing vitamin C and E, before being challenged by A. hydrophila. It was indicated that the numbers of total leukocyte counts, lymphocytes, and eosinophils decreased, while the numbers of neutrophils and monocytes increased [26].

3.2. Clinical Symptoms of Patin (P. pangasius) After Infection with Bacteria A. hydrophila

Changes in the clinical pathology of catfish tested with A. hydrophila bacteria include decreased appetite, dark body color, fish tend to be at the bottom of the tub. In very severe conditions, the sick catfish shows a wound on the skin, fin rot (fin rot), there are red spots on the base of the fin, and fish occasionally swim to the surface and hover with which indicates the fish has lost balance. In fish that nearly dead, they will swim in circles, the body faces upwards, necrosis in the skin tissue.

The effects of A. hydrophila on fish seems likely to vary between species. It is due to the difference in its resistance to the infection and the immune system. Furthermore, it could depend on bacterial virulence, the size of the animal, water quality, way of infection, and many other experimental conditions. In the present study, the results indicated that the first mortality was after injected 36 hours, and the LD50 of A. hydrophila to 50% of a population of P. bocourti was 2.24 x 108 CFU ml-1 of saline solution [27].

3.3. Fish Mortality

Feeding treatments that were added to the crude extract of polysaccharides from marine yeast at different doses in this study have provided different mortality or mortality rates in catfish. From the results of the study, the most catfish deaths were experienced in treatment D (1.5%) at an average of 88% and treatment A (0%) by 70.67%, treatment B (0.5%) by 66, 67% and treatment B (1%) of 34.00%.
Based on the data, treatment C (1.0%) is the least treatment of mortality when compared with other treatments. In treatment A (0%), mortality is also high because it is caused by immunostimulant doses that are too low so that the body's ability to fight bacterial attacks is also low and causes the high mortality of treated fish. Feeding of β-glucan in Asian catfish in the present study significantly reduced the percent mortality compared with controls at all periods. The degree of protection obtained by glucan administration is probably related to the stimulation of non-specific components of the immune system having antibacterial activity. Earlier reports also suggest that glucans incorporated in feed enhanced the resistance of several fish species against pathogenic bacteria [28].

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