Conference Paper

Characterization of *Bacillus megaterium* and *Bacillus mycoides* Bacteria as Probiotic Bacteria in Fish and Shrimp Feed

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Abstract

This study was aimedto identify probiotic characteristics and to test the cellulolytic ability of *Bacillus megaterium* and *Bacillus mycoides* bacteria for probiotic microbe candidates in fish and shrimp feed. The description of the cellulolytic and amylolytic abilities of these bacteriawas obtained by non-experimental method and descriptive analysis. Probiotic characteristic identification includes growth curve was obtained through total plate count method, cellular and colony morphology, and cellulase and amylase enzyme activity test using DNS method. Results indicated that the maximum growth of *B. megaterium*was observed after six hours at 35.62×10^{10} (CFU), while *B. mycoides* was after 30 hoursat 42.6×10^{10}(CFU). The macroscopic observation showed that the colony of *B. megaterium*was concave and smooth,while *B. mycoides* was flat, relatively rough, with silken threads around the colony.Both bacteria had milky white color, bacillus shape, Gram positive, and sporous. The activity of cellulose and amylase enzymes in *B. megaterium*were 3,974 units/ml and 1,831 units/ml, respectively. The activity of cellulose and amylase enzymes in *B. mycoides*were 3,506 units/ml and 3,730 units/ml, respectively. It can be concluded that both bacteria could be proposed as probiotic bacteria in fish feed.

Keywords: Characterization, Bacillus megaterium, Bacillus mycoides, probiotic microbes, feed.

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1. Introduction

The application of probiotics in fish and shrimp culturing has been utilized as a means of controlling diseases, increasing response of physical immunity, contributing nutrients and enzymes to the hatcher's digestive system and improving water quality [1]. Supplementation of probiotics may reduce epidemy of disease by improving the immune system of the fish and shrimp [2] and may further reduce the cost of culturing by increasing the growth and efficiency of fish feed [3]. Effective utilization of microbes

in fish and shrimp culturing is more advantageous since it leaves no residue in the shrimp's body and is more environmentally friendly and economical.

Selection of microbes as elements of probiotics is an important step since the microbes have to meet certain criteria as candidates of probiotic microbes. Aquatic animals are quite different from the land animals and a consequence of the specificity of aquatic microbiota is that the most efficient probiotics for aquaculture may be different from those of terrestrial species [4]. Certain basic criteria that the candidates must possess are rapid growth and adequate extracellular enzyme [5]. Furthermore, these probiotic bacteria should have excellent viability in order to survive the production, storage and preservation processes, as well as proven health benefits, resistance to acidity (low pH) and bile salts. These characteristics are essential in order for them to colonize and balance the microflora in the digestive system.

Bacillus megaterium and *Bacillus mycoides*are bacteria of the *Bacillus* genus that have potentials as probiotic bacteria.Most probiotics used in aquaculture belong to the lactic acid bacteria, of the genus *Bacillus*, to the photosynthetic bacteria or to the yeast, although other genera or species [6]. Their utilization as probotic bacteria has to go through preliminary selection which comprises macroscopic and microscopic observations as well as observations of growth pattern and enzyme activity, in order for them to meet the criteria of probiotic bacteria in fish and shrimp feed.

2. Method and Materials

2.1. Tools and Materials

The tools used in this study include centrifugation device along with the centrifugation test tube, aluminum foil, autoclave, stirrer, blender, sprayer bottle, bunsen burner, petri dish, erlenmeyer flask, grinder, hot plate, incubator, jara, object glass, muslin, filter paper, weighing paper, laminar air flow, refrigerator, micropipettes (5-50 μl, 20-200 μl dan 200-1000 μl), microscopes, analytic balance, Ohauss balance, ose, oven, water vaoprizer, ice vaporizer, pH MettlerDelat 350, *pippetier ball*, drop pipette, volume pipette (1 ml, 5 ml, 10ml), spectrophotometer UV-160A SHIMADZU with cuvtte and vortex. The materials used in this study are fuchsin water, aquades, alcohol 75%, alcohol 96%, alcoholic acid, rice, acetate buffer pH 4,4, acetate buffer pH 5,4, phosphate buffer pH 6, *Bacillus megaterium* and *Bacillus mycoides* bacteria isolate, CMC (Carboxymethyl Cellulose), ice cubes, NaCl salt, sugars (galactose, glucose, maltose, lactose, sucrose), HCl 0,1 N, HCl 10N, H_2SO_4 1%, red phenol indicator, Iodine, fuchsin carbolic acid, gentian violet carbolic acid, NaCl 0,9% solution, tetramethyl-p-phenylenediaminesolution, Simmon's Citrate medium, Semi Solid medium, TsiA medium, Urea medium, NA (nutrient agar), methylene blue, methyl red, immersion oil, NaOH 0,1N, NB (nutrient broth),

McFarland nephometer, Lugol's solution, peptone, CO_2pills, DNS reagent, catalase reagent 3% (H_2O_2), rubbing alcohol, Acid 3,5-Dinitrosalisilat (DNS) reagent, API Test CHB 50 stripes and xylol.

2.2. Method

Initial screening of probiotic candidates is performed by preliminary selection which comprises macroscopic and microscopic observations as well as observations of growth pattern and enzyme activity.

2.3. Macroscopic and microscopic observations

This study was conducted using a non-experimental design and analyze using descriptive methods. The processes included identification of *B. megaterium* and *B. mycoides* bacteria isolates using API Test CHB 50. The bacteria are grown using nutrient agar added with CMC, which are then incubated for 72 hours at 37 °C. After the colony is formed, macroscopic (on colony shape and color) and microscopic (on gram coloring, spore, capsule and resistance to acid) tests and biochemical test were conducted. In the nutrient soup, the bacteria growth curve and cell population were calculated using the TPC method every six hours.

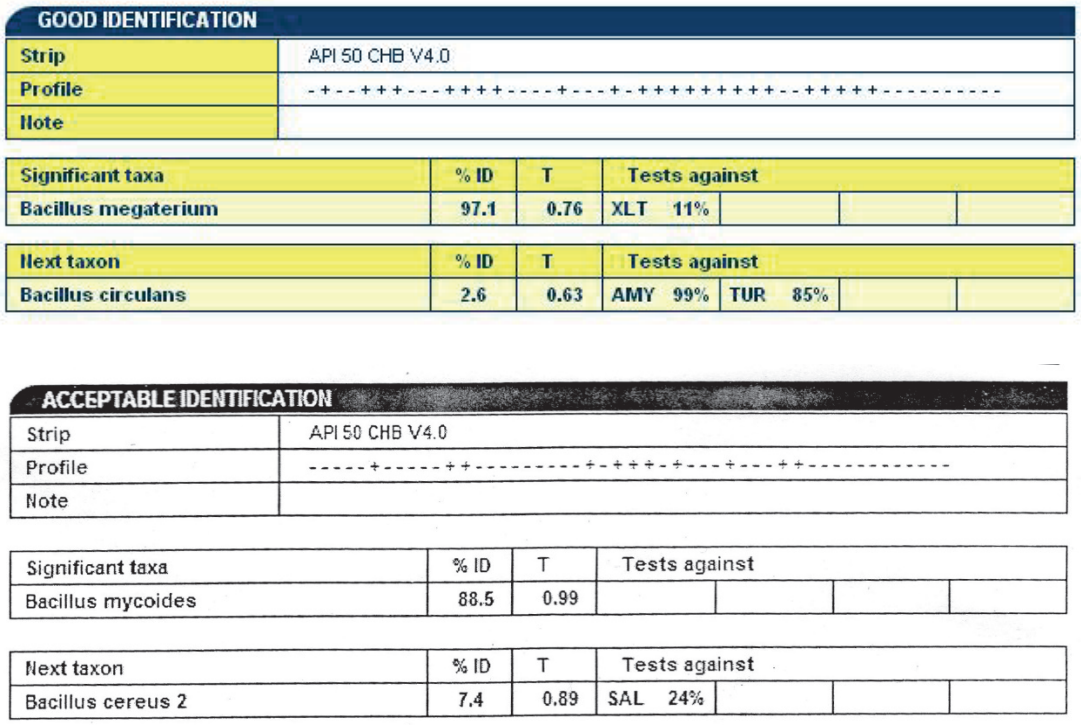
2.4. Determination of cellulose/amylase enzyme activity

The determination of cellulose/amylase enzyme activity (FP-ase) is conducted through culturing bacteria and taking samples from the first through the fifth hour. Activity of cellulose/amylase value was performed through mixing 0.5 ml of enzyme using a piece of 50 mg filter paper with 1 ml 0.05 M citrate phosphate buffer with pH 4,8. The mixture was then incubated at 50°C for 1 hour. The reaction was stopped by adding 3 ml DNS (3,5 dinitrosalicylic acid). It was then heated in boiling water for 5 minutes. After cooling off, it underwent centrifugationt 3,000 rpm for 15 minutes.Sugar reduction was then performed using spectrophotometer at wavelength 575 nm.

3. Results and Discussion

3.1. Growth Curve

Bacteria identification is conducted using API Test CHB 50. Using bacteria isolate *Bacillus megaterium* and *Bacillus mycoides*, the results after 48 hours of API test are the following (Figure 1).



GOOD IDENTIFICATION	
Strip	API 50 CHB V4.0
Profile	-+--++++---++++----+---+-+++++++++--++++-----------
Note	

Significant taxa	% ID	T	Tests against			
Bacillus megaterium	97.1	0.76	XLT 11%			

Next taxon	% ID	T	Tests against			
Bacillus circulans	2.6	0.63	AMY 99%	TUR 85%		

ACCEPTABLE IDENTIFICATION	
Strip	API 50 CHB V4.0
Profile	-----+-----++----------+-+++-+---+---++----------
Note	

Significant taxa	% ID	T	Tests against			
Bacillus mycoides	88.5	0.99				

Next taxon	% ID	T	Tests against			
Bacillus cereus 2	7.4	0.89	SAL 24%			

Figure 1: API Test CHB 50 results on isolate of *B. megaterium* and *B. mycoides*.

A way of determining probiotic effectiveness, among others, is by observing the ability of the microorganism to live in its substrate or in its environment. Calculation of microbe cells at every time unit is important in the selection of probiotic microbe candidates, since the calculation informs the growth ability of the bacteria in inoculum substrate and the exponential phase (log phase) of said bacteria. According to [7], the log phase is the most important phase since it is the period when the microbes experience accelerated growth. *B. megaterium* was observed after six hours at 35.62×10^{10} (CFU), while *B. mycoides* experienced its peak growth at 30 hours (Figure 2). Other researchers showed that in liquid medium plus biotin *(20* pg/l) the growth become heavy after 40 h; namely 4×10^4 organisms/ml [8], which needed longer growth time compared to ours. The normal log phase of *B. mycoides* occured after 40 h culture [9].

3.2. Macroscopic and microscopic observations

From macroscopic and microscopic observations and biochemical test, it is shown that *B. megaterium* are concave, smooth and milk white, while *B. mycoides* looks white, coarser with fine threads around the colony and the color is milk white. The cell morphology (Figure 3 and Table 1) shows that the cell is rod-shaped, gram positive and sporous. The Table 2 shows further characteristics of the two isolates.

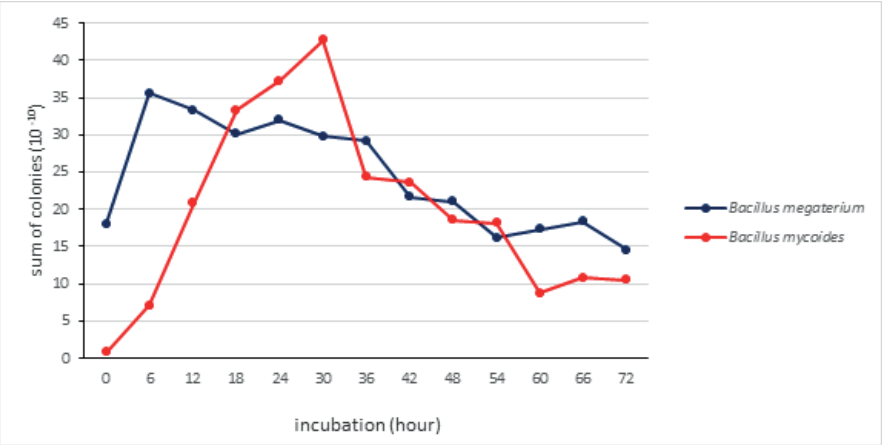


Figure 2: *Bacillus megaterium* and *Bacillus mycoides* growth curves.

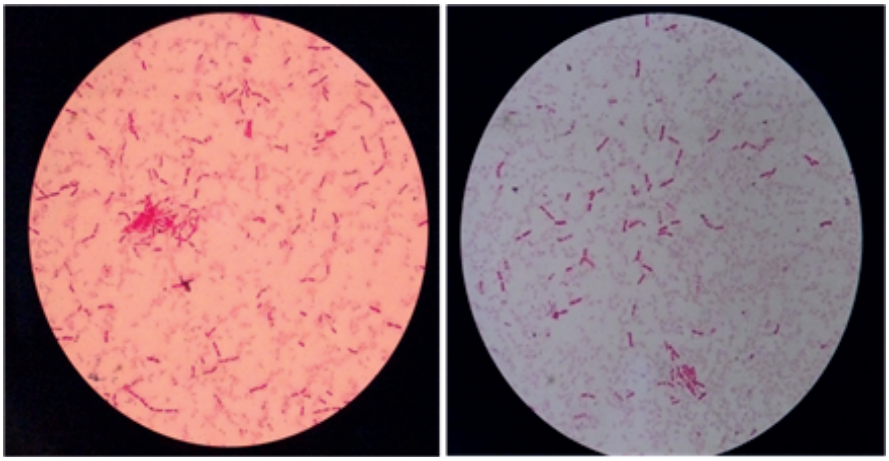


Figure 3: *Bacillus megaterium* (left) and *Bacillus mycoides* (right) 1000x magnification.

B. megaterium is a bacterium that can live in various kinds of substrate as carbon source, such as meat industry waste and petrochemical waste. In extreme conditions, these bacteria survive by producing spores [10]. On the other hand, *B. mycoides* is a non-motile bacterium that is able to extract acid from glucose and produce endospores. This bacterium can hydrolyse starch and does not produce toxin. Among probiotic bacteria for shrimp *Bacillus* spp. are more widely used and proved to enhance shrimp health with no visible side effects [11].

Table 1: Cell morphology of *Bacillus megaterium* and *Bacillusmycoides*.

Label	Shape	Gram	Spore	Capsule	Acid Resistant
Bacillus megaterium	Rod	Positive	Sporous	-	-
Bacillus mycoides	Rod	Positive	Sporous	-	-

Table 2: Biochemical Test Results of *B. megaterium* and *B. mycoides*.

Test	Bacteria species	
	B. megaterium	*B. mycoides*
Glucose	+	+
Lactose	+	-
Mannitol	+	-
Maltose	+	-
Saccarose	+	-
Indol	-	-
VP	+	+
MR	+	+
SC	+	-
TsiA	+	-
Urea	+	-

3.3. Cellulase-amylase enzyme activity test

Enzyme has a specific ability in degrading substance according to its type. This ability is measured through enzyme activity test. The enzymes produced by *B. megaterium* and *B. mycoides* are tested as well, in order to measure their ability in assisting the digestion of feed that contains high crude fiber, such as cellulose.

The results of the testing of cellulase and amylase enzymes in both *B. megaterium* and *B. mycoides*indicate certain activities of the enzymes. The highest cellulase enzyme activity in *B. megaterium* is 3,974 unit/ml, while amylase enzyme is at 1,831 unit/ml. In *B. mycoides* the highest cellulase enzyme activity is 3,506 unit/ml and amylase enzyme is 3,730 unit/ml (Figure 4). The highest production of cellulase in *B. megaterium* occurs during the fourth hour, while amylase is during the third hour. The highest production of cellulase in *B. mycoides*occurs during the third hour, while amylase is during the fourth hour. These results confirmed to those of [12] who concluded that *B. mycoides* isolated from Decayed Mangrove Stem Waste Product showed cellulose (55,026 IU/g) and amylase activity.

A number of researchers have informed that the *Bacillus* bacteria possess high cellulolytic/amylolytic ability. *B. mycoides* and *B. megaterium* are multi-functional bacteria capable of producing cellulose, besides producing chitinase and protease as well as dissolving phosphate. Furthermore, it is also discovered that both bacteria are also capable of producing amylase [13-14]. *B. megaterium* has also the ability to produce a number of enzymes, such as mutarotase, glucose dehydrogenase, β-galactosidase, amylase and cellulase. The enzyme activity test has also been congruent to the results of study by [15] that states *B.mycoides* is a bacterium species that produces cellulase, protease and phosphatase enzymes.

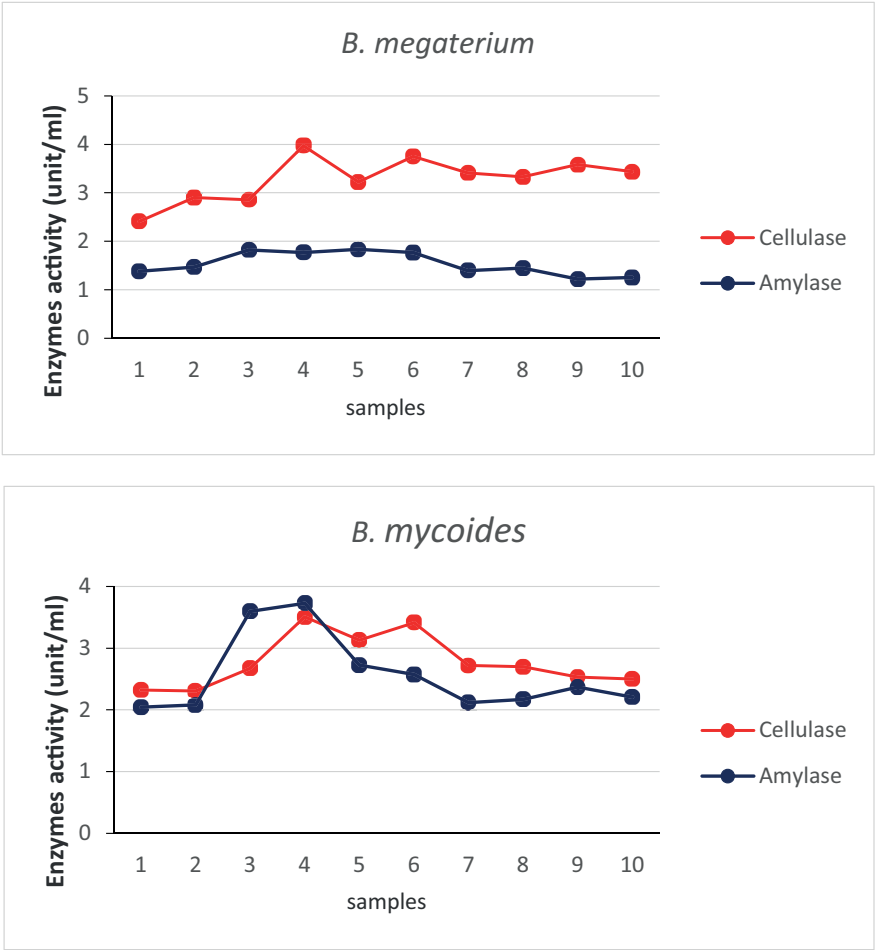


Figure 4: Enzyme activities of *B. megaterium* (upper) and *B. mycoides* (lower).

Our result showed that enzyimeactivityof *B. megaterium* and *B. mycoides* can be applied as probiotics bacteria for fish and shrimps feed. [16] state the ability of fish and shrimp to use feed nutrients depends on certain factors, such as the appropriate synthesis, adequate enzyme production and enzyme distribution in the digestive system.

4. Conclusion and Recommendation

Based on the growth curve results, macroscopic and microscopic observations, biochemical test and cellulase and amylase enzyme activity test on *B. megaterium* and *B. mycoides*, it is concluded that both bacteria are potential for use as probiotic bacteria in fish feed.

For further testing, it is recommended that a biological test be conducted on the usage of bacteria in probiotic form in fish feed with respect to immunity response and growth of shrimp and fish.

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