

Isolation and Characterization *Bacillus* sp. Producing Cellulase Enzymes from Hanura Mangrove

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ABSTRACT

In the mangrove ecosystem, there are cellulolytic bacteria that act to decompose cellulose in nature. This study aims to obtain cellulolytic bacteria from mangrove forests in Hanura village. Bacterial isolation was carried out using Sea Water Complete Agar (SWCA) media which added Carboxymethyl Cellulose 0.5% (CMC). Characterization of isolates included colony and cell morphology, pH and NaCl stress test, pathogenicity test, and metal influence test on cellulolytic activity. The results of the study obtained 38 bacterial isolates. 16 isolates among them were cellulolytic bacteria and were able to grow well on pH (7 and 10) and NaCl (0%, 3% and 6%) stress media. IBK3, ID2K1 and IA2K3 isolates are non-pathogenic bacteria with rod-positive Gram-positive properties. IBK3 had the highest cellulolytic index of 7.36 and grew well on the addition of Fe, Al, Pb and Cu metals.

Keywords: mangrove ecosystem, Bacterial isolation, cellulolytic bacteria, cellulolytic index

1. INTRODUCTION

Hanura Mangrove Forest is a forest area bordering the coast. Lots of diversity of organisms that live in it. Hanura Village is an important location for the distribution of Lampung Province's mangrove forests. On the floor of the Mangrove Forest there is litter and weathered wood on mangroves containing cellulose. Wood has a cellulose content of about 50%, while in plants it is around 33% [1]. Cellulose is a glucose polymer with linear chains that are connected by glycosidic β -1.4 bonds which cause the cellulose structure to be crystalline and difficult to dissolve in water. Cellulose in nature is associated with other polysaccharides such as hemicellulose or lignin which form cell walls of plants [2]. With the abundance of cellulose it will support the presence of cellulose decomposing bacteria. Some bacteria in the mangrove forest include *Bacillus megaterium*, *Nitrococcus* sp., *Bacillus subtilis*, *Planococcuscitreus*, *Bacillus mycoides*,

Lactobacillus plantarum, *Bacillus pumilus*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Micrococcus* sp., *Staphylococcus* sp., *Micrococcus luteus*, *Vibrio* sp. [3]. The *Bacillus* genus is known to be capable of producing cellulase. Based on Sumardi's research [4], *Bacillus* sp. isolates UJ132 from Mangrove forest has cellulolytic ability.

Microorganisms that produce cellulase enzymes besides being used for cellulose decomposition can also be used as probiotics. Cellulose which is a polymer is found in the composition of fish feed. With the addition of probiotics producing cellulase enzymes can help in feed digestibility so that it can boost fish productivity. Based on the study of Widanarni [5], probiotic 1Ub isolates, SK and Ua effectively inhibited *V. harvey* growth, and significantly increased the survival and growth of tiger shrimp larvae.

The requirement for a bacterium to be used as a probiotic includes non-pathogens, tolerance to acids and bile salts, has the ability to survive in the preservation process and can survive in its storage and has the ability to provide proven health effects [6]. This study aims to obtain cellulolytic bacteria from mangrove forests in Hanura village, which can be used as probiotics and can help digestion of shrimp/fish against cellulose.

2. MATERIAL AND METHODS

2.1. Isolation and Selection of Cellulolytic *Bacillus*

Samples taken from the mangrove forest area of Hanura village include: water, mud, roots, flowers and leaves from mangroves and snails. The sample was heated at 80 °C for 15 minutes. Then the sample was enriched in liquid SWC media for 24 hours. Then the culture is diluted to 10⁻⁶. 10⁻⁴ dilutions of up to 10⁻⁶ were inoculated with the pour plate method on Sea Water Complete Agar (SWCA) media. The SWCA composition consists of; bacto peptone 5 g, yeast extract 1 g, glycerol 3 ml, sea water 750 ml, aquades 250 ml, bacto so that 15 g of CMC 0.5% is added. The growing colonies were colored with 0.1% congo red and rinsed with 1 M NaCl. The colonies with clear zones around it showed cellulolytic activity. Cellulolytic bacterial colonies were purified on oblique media as pure isolates.

2.2. *Bacillus* Characterization

Characterization of *Bacillus* includes morphological and physiological characters. Morphological characters are carried out by observing the morphology of colonies and cells. Observation Colony morphology includes shape, edge, elevation and color, while observations of cell morphology are carried out by Gram staining.

2.3. Pressure Test for pH

Bacillus isolates on liquid media were inoculated on agar SWC media with variations in pH 4, 7 and 10. *Bacillus* culture was incubated for 24 hours at room temperature. After incubation observed growth of bacterial colonies.

2.4. Pressure Test for NaCl

Bacillus isolates on liquid media were inoculated on agar SWC media modified by the addition of 0%, 3% and 6% NaCl (salt). *Bacillus* culture was incubated for 24 hours at room temperature. After incubation observed growth of bacterial colonies.

2.5. Pathogenicity Test

Pathogenicity tests were carried out by observing the nature of hemolysis on blood agar media. *Bacillus* isolates were inoculated on SWC media so that blood was then incubated for 24 hours at room temperature. Observations were made by looking at the color changes that occur in the media so that the blood around the isolate shows the nature of hemolysis. There are 3 types of hemolysis properties. α-hemolysis with the formation of yellow to greenish color around the colony, β-hemolysis with the formation of clear zones in the media around the colonies which indicate total lysis and γ-hemolysis does not occur lysis on the media with no change in the media color.

2.6. Test of The Effect of Metals on The Ability of Cellulolytic Bacteria

Metal influence tests were carried out using 4 types of metals and 3 different concentrations, namely FeCl₃ (10, 20 and 40 mM), AlCl₃ (1.5, 3 and 6 mM), CuCl₂ (0.015, 0.03 and 0.06 mM) and PbCl₂ (0.015, 0.03 and 0.06 mM). These metals are added to SWC + CMC 0.5% media. Isolates were inoculated and incubated at room temperature for 24 hours. After incubating the colony on the medium, the congo red 0.1% was colored for 15 minutes. Then the congo red is washed with 1 M NaCl. Clear zone formed around the colony and measured in diameter.

3. RESULTS AND DISCUSSION

3.1. Isolation and Selection of Cellulolytic Bacteria

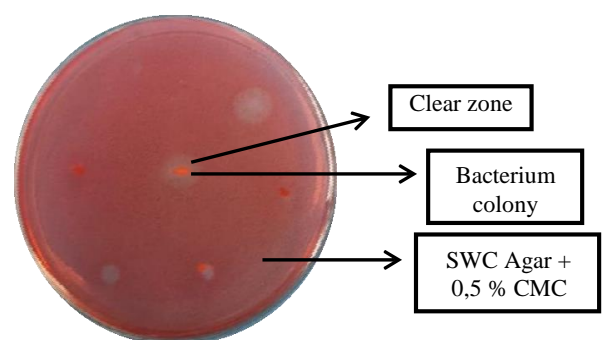


Figure 1 Qualitative Cellulolytic Test

The bacteria that were isolated from the total sample were 38 isolates with the most species coming from mud. Of the total isolates, 16 *Bacillus* sp. isolates were selected which has cellulolytic ability. Each isolate showed a different ability to hydrolyze cellulose in the form of carboxymethyl cellulose in the media shown in the results of the calculation of

the cellulolytic index value (Table 1). The ability of isolates to hydrolyze cellulose was shown by the clear zone area produced around the isolate colonies (Figure 1).

3.2. *Bacillus* Characterization

Bacillus isolates which showed positive ability to degrade cellulose amounted to 16 isolates. From the observation of colony and cell morphology, most of the colony forms are circular with various edges and elevations and colors.

In observing cell morphology as a result of Gram staining, Gram-positive stem cell forms were obtained. The isolates that were isolated were 38 isolates, and most isolates came from mud. 16 isolates were cellulolytic. The morphology of the colonies of the isolates obtained varied mostly with circular shapes with entire edges, raised elevations and white colors. All isolates are rod shaped and are Gram positive. This character shows *Bacillus* characteristics [7].

3.3. Pressure test for pH

Based on testing, all isolates were able to grow well at pH 7 and 10. However at pH 4 no isolates were able to grow (Table 1).

The pH stress test results for 16 isolates were able to grow well at pH 7 and 10 (Table 1). However, at pH 4 no isolates were able to grow. The absence of bacteria that grows at pH 4, this shows that bacteria cannot stand high acidic conditions because most aquatic environments have a pH between 5 to 9. Based on the research of Triyanto [8] marine bacteria can survive at a pH level of 5-9. According to Bergey [7], *Bacillus* is able to grow at a pH of 5-10 some are resistant to saline. In addition, bacteria unable to grow at acidic pH occur because of the inhibition of transport of essential ions because the membrane permeability to ion protons rises, so the cell cannot produce ATP (Adenosine Triphosphate) and the respiratory process fails, growth slows to death [9].

3.4. Pressure Test for NaCl

Based on the testing, all isolates were able to grow well in the media with 0%, 3% and 6% NaCl (salt), but 3 isolates were unable to grow at 6% saline (Table 1). In contrast to stresses for NaCl salts, the 16 isolates grow well on media with the addition of salt 0, 3 and 6%, only 3 isolates did not grow at 6% salinity.

Based on the research of Triyanto [8] marine bacteria can survive at technical NaCl or salt levels 0.5% -3.5%. *Bacillus* is able to grow on media given NaCl with a concentration of up to 20% [7]. The ability to grow isolates tested for various stresses is

thought to be related to the source of isolation. The ability to live in these extreme conditions is needed by probiotic candidates in order to function properly in various environmental conditions inside and outside the organism's body [10].

3.5. Pathogenicity Test

Most isolates have the properties of β (Beta) hemolysis in the presence of clear zones around the colony, and 5 isolates with the properties of α (Alfa) hemolysis with the occurrence of partial lysis on the media. Non-pathogenic bacteria obtained 3 isolates with the properties of γ (Gamma) hemolysis which did not demolish the media so that blood is shown in Table 1.

The results of pathogenicity tests on SWC so that blood obtained 3 isolates with hemolysis γ (gamma) properties, namely IA2K3, IBK3 and ID2K1. Hemolysis activity has three types namely α -hemolysis, β -hemolysis and γ -hemolysis [11]. α -hemolysis that occurs partially lysis on the media around the colony is marked by the change in color of the media to greenish and this green color comes from biliverdin which is a by-product of the breakdown of hemoglobin, β -hemolysis that is complete lysis of the media around the isolate marked with media color changes clear. and γ -hemolysis which is the absence of lysis in the media [11]. With this isolate can be used as a candidate for probiotics because it does not cause pathogenicity.

3.6. Test of The Effect of Metals on The Ability of Cellulolytic Bacteria

IBK3 isolate is able to grow on the media with the addition of metals. Addition of metal ions decreases cellulolytic activity. The cellulolytic index formed on media that uses 4 metals with 3 different concentrations shows different index values. In media with the addition of Cu metal ions from 0.015 to 0.06 mM, the cellulolytic index formed continues to decrease to 0.71 (Figure 2).

Heavy metal is one of the environmental pollutants. The presence of heavy metals in the waters can affect the lives of organisms in these waters. The ability of bacteria that can survive in this condition is needed so that these bacteria can live in an unfavorable environment. Based on tests conducted on IBK3 isolates. Bacteria inoculated on the media with the addition of metals continue to grow and are able to degrade CMC substrates on the media. This is because microorganisms are able to absorb or transfer metals through enzymatic reactions, the metal can then be utilized for metabolic processes in cells or converted into other chemical forms that are not toxic in soil, water or oil [12].

Table 1. Cellulotic index and characterization of bacteria

Sample Source	Isolat	Colony				Cellulolytic index	Cell		Growth in media with added pH			Growth in media with added NaCl			Hemolytic			
		Shape	Margin	Elevasi	Color		Gram reaction	Cell Shape	4	7	10	0%	3%	6%	α	β	γ	
Roots of mangrove	IAK1	Circular	Filamentous	Raised	White	7,24	+	Bacilli	-	++	++	+	+	+	+			
	IAK2	Circular	Entire	Raised	White	1,81	+	Bacilli	-	++	++	+	+	+	+			+
	IAK3	Irregular	Lobate	Raised	Yellow	6,13	+	Bacilli	-	+	++	+	+	+	+			+
	IAK4	Circular	Filamentous	Raised	White	7,29	+	Bacilli	-	++	++	+	+	+	+			+
	IA2K1	Circular	Entire	Raised	White	1,31	+	Bacilli	-	++	++	+	+	+	+			+
	IA2K2	Circular	Entire	Flat	Pink	-												
	IA2K3	Circular	Filamentous	Flat	Clear	4,94	+	Bacilli	-	++	++	+	+	+				+
Mud	ILK3	Circular	Entire	Raised	Orange	-												
	ILK5	Circular	Entire	Raised	Orange	-												
	ILK6	Circular	Undulate	Raised	Pink	4,50	+	Bacilli	-	++	++	+	+	-				+
	ILK9	Irregular	Lobate	Flat	Clear	-												
	IL2K1	Circular	Entire	Umbonate	White	-												
	IL2K3	Circular	Entire	Raised	Orange	-												
	IL2K5	Circular	Entire	Flat	Clear	6,31	+	Bacilli	-	++	++	+	+	-				+
	IL2K6	Circular	Filamentous	Raised	Clear	-												
	IL2K8	Irregular	Undulate	Flat	Clear	-												
	IL2K9	Circular	Entire	raised	White	-												
Snail	IKK1	Circular	Undulate	Raised	White	6,33	+	Bacilli	-	++	++	+	+	+	+			+
	IKK2	Circular	Entire	Raised	Pink	-												
	IKK3	Circular	Entire	Raised	White	3,67	+	Bacilli	-	++	++	+	+	-				+
Leaves of mangrove	IDK2	Circular	Entire	Raised	Pink	-												
	IDK3	Irregular	Lobate	Flat	Clear	-												
	IDK4	Circular	Entire	Flat	White	-												
	IDK5	Irregular	Undulate	Flat	Clear	-												
	IDK6	Circular	Entire	Raised	White	6,25	+	Bacilli	-	++	++	+	+	+	+			+
	ID2K1	Circular	Filamentous	Flat	Clear	6,93	+	Bacilli	-	++	++	+	+	+	+			+
	ID2K2	Circular	Undulate	Raised	White	4,33	+	Bacilli	-	++	++	+	+	+	+			+
	ID2K3	Circular	Entire	Raised	Cream	2,85	+	Bacilli	-	++	++	+	+	+	+			+
	ID2K4	Irregular	Undulate	Flat	Clear	-												
Flower of mangrove	IBK1	Circular	Filamentous	Flat	Clear	-												
	IBK2	Circular	Undulate	Raised	White	-												
	IBK3	Circular	Entire	Raised	Cream	7,36	+	Bacilli	-	++	++	+	+	+	+			+
	IBK4	Irregular	Undulate	Flat	Clear	-												
	IBK5	Circular	Undulate	Flat	White	-												
Mangrove Water	IPK1	Circular	Entire	Raised	Cream	-												
	IPK2	Circular	Undulate	Flat	Clear	-												
	IPK3	Circular	Irregular	Flat	Clear	3,54	+	Bacilli	-	++	++	+	+	+	+			+
Total	38					16		16	-	16	16	16	16	13	5	8	3	

Description: Cellulolytic index (- = none clear zone), NaCl and pH (+ = small colony, ++ = Large colony, - = none growth), hemolytic (+ = positive reaction).

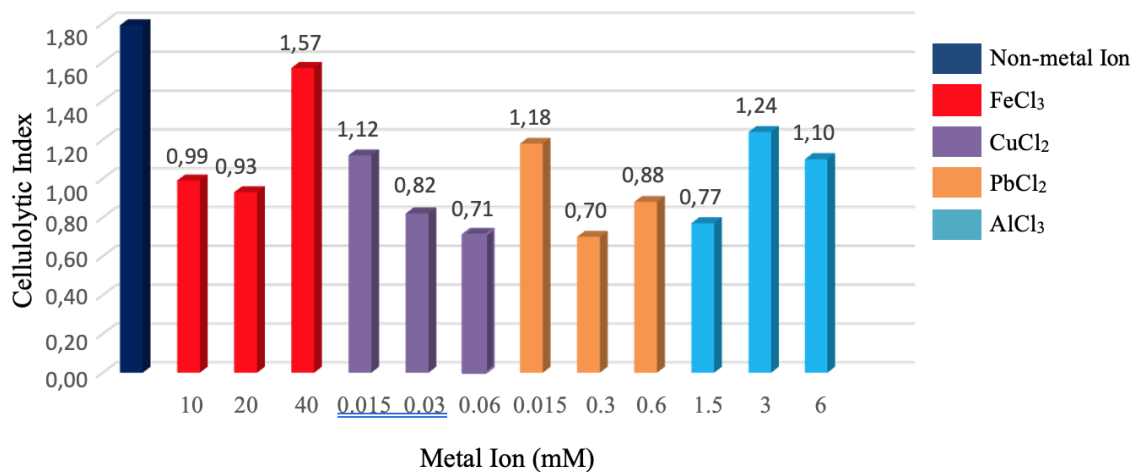


Figure 2 Test of Effect of Metals on Cellulose Degradation Process

The negative control test used without the addition of metals has the highest cellulolytic activity. This shows that metals added act as inhibitors. *Bacillus* is resistant to heavy metals Pb and Cu with concentrations up to 50 ppm [13]. The largest cellulolytic Fe metal index was used at a concentration of 40 mM. This is presumed because Fe

is needed for metabolic activity in almost all microorganisms because it is a cofactor for a number of enzymes [14]. Fe becomes an activator in the activity of protease enzymes [15].

At the highest concentration of Al, Cu and Pb metals, the cellulolytic index in the media decreases. This, because the concentration of metal that is too high can cause toxic microorganisms. The presence of metals that are too high can affect growth, morphology, biochemical activity, decrease biomass and diversity of microorganism populations [16]. Cu metal ions can suppress *Escherichia coli* microbial growth and act as inhibitors [17]. Cu metal was reported as well as being an inhibitor of cellulase, protease and amylase activity. The maximum Cu inhibitor in dehydrogenase activity. Dehydrogenase plays a role in the oxidation of organic compounds [18]. Pb metal ion can inhibit the work process of the enzyme [19]. Aluminum oxide nanoparticles (Al₂O₃) which are exposed for 30 minutes, reduce microorganism dehydrogenase activity from 66% to 0.39% [20].

4. CONCLUSION

This study obtained cellulolytic *Bacillus* bacterial isolates from the mangrove of Hanura Village, Pesawaran, namely IBK3 Isolate had the highest cellulolytic index of 7.36 and was a non-pathogenic bacterium with Gram-positive rod shape. IBK3 isolates are able to grow well on pH (7 and 10) and NaCl (0%, 3% and 6%) stress media and on the addition of metals (Fe, Al, Pb and Cu). IBK3 isolates can be used as probiotic candidates for shrimp feed.

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