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Culturable Cellulolytic Bacteria From Mangrove With *Anadara Granosa* Cultivation In Sukal, West Bangka

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***Abstract* - Sukal, West Bangka district became one of the centers of *Anadara sp* culture. Not yet known the effect of shellfish cultivation on the ability of decomposition in mangroves. Cellulolytic bacteria are part of degradation in mangroves. Cellulolytic bacteria have cellulose degradation ability and it made carbohydrates are easier about digested for livestock. This research aims to obtained cellulolytic bacteria in soil, decayed wood and leaf from mangrove with *Anadara sp* culture in Sukal, West Bangka. The research was conducted from March until June 2018. Isolation was done using agar media with 1% Carboxymethyl Cellulose (CMC). An isolate of bacteria grew in agar with 1% CMC and given the Lugol and Congo Red at 72 hours to test cellulose degradation ability. Isolates from Sukal Mangrove that result the largest cellulose degradation had their species identification using 16S rRNA gene sequenced and microbact analysis. The results of biochemical tests using Mikrobact for bacterial isolates cellulolytic show selected lead to species *Bacillus megaterium, Citrobacter freundii,* and *Vibrio alginoliticus*. DNA sequences of isolate bacteria showed close kinship with *Pseudomonas psychotolerans*.**

Keywords–Phylogeny, Cellulolytic Bacteria, Mangrove, Anadara sp culture

I. INTRODUCTION

Blood clams (*Anadara granosa*) are one of the potential natural fishery commodities in the Bangka Belitung Islands [1]. The increasing demand for consumption of blood clams makes the market needs blood clam culture. Sukal and Tanjung Punai, Belo Laut Village, Muntok Subdistrict of West Bangka Regency developed into a area of blood clams cultivation because of the condition of the waters that support the development of the business.

Mangroves are an area with a high decomposition of organic matter. This decomposition process allows mangroves to become a source of food and habitat for various fishery commodities such as fish, crabs, shrimp, and shellfish. The process of overhauling organic matter with cellulose content allows the role of microorganisms that produce extracellular enzymes as well as cellulolytic bacteria [2]. Microbes in mangrove play an important a source of cellulolytic enzymes as Prihanto and Wakayama argue that oceans and coastal regions are the main sources of unexplored enzymes [3]. Identified cellulolytic bacteria were found in weathered logs [4, 5], leaf litter [6, 7] and mangrove mud [8]. Bacteria with cellulose enzymes have the ability to remodel complex cellulose into easily digestible sugars in sediments [8]. The ability of cellulolytic bacteria obtained from nature is expected to be useful to degrade cellulose to increase its utilization [9]. About 70% of the plants in the world are bound to 5- and 6-sugar identified in the lignocellulose section as the main part of cellulose [10].

Reduction of crude fiber, producing simple sugars, lignin production, application in the manufacture of food and waste decomposition can take advantage of applications cellulolytic bacteria. Waste of palm oil industry can be used to carbohydrate and protein source with some problems about cellulose. The results of research on culturable cellulolytic bacteria from mangroves that are used for *Anadara granosa* aquaculture provide an opportunity to use them to increase the digestibility of cellulose materials.

II. METHODS

A. Sediment, weathered wood and leaf litter sampling

Samples of mangrove soil, weathered wood, and leaf litter were taken in Sukal Mangrove area, Muntok Sub-district, West Bangka Regency, Province of Bangka Belitung Archipelago, Indonesia. Soil sampling is done with a depth of 10-20 cm. Leaf litter sampling is done by taking the leaves that have undergone weathering. Weathered wood sampling was taken by cutting the wood that experienced the most weathering. All sample stored in the sterile container. Each sampling location was taken 2

samples and composite into a combined sample. Location of sampling of mangrove soil, leaf litter and weathered wood in Sukal is shown in Figure 1.

B. Bacterial Isolation

Bacterial isolation was done by sampling from mangrove soil. Mangrove soil samples were weighed as much as 1g and cultured on nutrient broth medium. Culture is done with a shaker for 24 hours. The results of culture on nutrient broth are diluted to 10^{-7} dilutions. 10^{-4} to 10^{-7} dilutions were grown on agar media plus 1% CMC (Carboxy Methyl Cellulose) and incubated at 30 ° -37 ° C for 72 hours.The cultivation results were isolated with the same medium to obtain the pure isolate using to scratch plate method.

C. Bacterial selection of cellulase degradation ability

Selection of bacteria with the ability to degrade cellulosewas done by the cellulose hydrolysis test method. Pure bacterial isolates were recycled on Carboxy Methyl Cellulose enriched agar medium (CMC) by a streak method. One loop of bacteria is scratched on the media to form a straight line about 1 centimeter. Streaking results are stored at 30-37°C for 72 hours.Qualitative test uses two methods. In the first method, Lugol solution which is a mixture of 2g of potassium iodine and 1g iodine in 300 ml of aquadest is dripped and shaken to cover the media and let stand 1-3 minutes. On the second method, Congored 0,1% (0,1 g of Congored in 100 ml Aquadest) is dripped to cover medium until 15 minutes and rinsed with NaCl 1M. The clear zone formed around bacterial colonies was observed and identified as isolating of cellulose-degrading bacteria. Isolates identified to have cellulolytic activity were selected for further identification. Isolates showing the largest clear zone were identified by sequencing DNA analysis.

D. Biochemical Characterization of Bacteria

Biochemical characterization using Microbact™ according to company manual procedures. Determination of bacterial identity is done by comparing the results of biochemical characterization with Bergey's Manual of Determinative Bacteriology.

E. Molecular Identification of bacteria.

The procedure used to identify the best cellulose degradation was to use the Prihanto and Wakayama methods [3] with some modifications. DNA isolation using a DNA Isolation Kit from Promega. PCR used a total volume of 20 µl / tube consisting of 6 µl ddH2O, PCR kit 10 µl GoTaq® Green Master Mix (10 x taq polymerase buffer, dNTP, MgCl2, primer, Taq DNA polymerase, ddH2O), 1 µl primary forward, primary reversing 1 µl and 2 µl of the DNA sample isolated. Primers for this analysis were 20F (52 – GTAATCGTCGGCCA GTAGAGTTTGATCCTGGCTC-32) and 1510R (52 – CAGGAAACAGCTATGACCGGCTACCTTGTTACGA CT-32). DNA sequences were analyzed using BLAST (Basic Local Alignment Search Tool) at NCBI. Comparative sequences were taken from the NCBI bank genes and displayed their kinship in phylogenetic trees built using Phylogeny.fr.

III. RESULTS AND DISCUSSION

The results of screening and isolation of bacteria with 1% CMC enrichment of selective media showed that there were 16 bacterial isolates found with details of 5 bacterial isolates in leaf litter samples, 8 isolates in weathered wood samples and 3 isolates in mud samples. All bacterial isolates that were able to live with media with cellulose enrichment were tested for their cellulose degradation ability qualitatively using Lugol and congored. Isolates with the largest seed zone in gram staining test. The results of the qualitative cellulolytic test with Lugol and congored are found in Figure 2-3 and Table 1.

Bacterial isolates showed a clear zone were tested to determine the gram staining included in gram-positive bacteria or gram-negative. There were 7 isolates that were identified as having the ability to degrade cellulose with the appearance of clear zones in both Lugol and congored methods. The results of gram staining testing showed that there were 4 isolates namely SKL 5.1, SKK 5.1, SKK 5.2 and SKK 7.1 which were gram-positive bacteria and continued their identification.

TABLE 1. RESULTS OF QUALITATIVE TESTS FOR CELLULOLYTIC BACTERIA WITH LUGOL AND CONGORED.

Code Number	Isolate Code	Diameter of clear zone (mm)		Gram staining
		Lugol	Congored	
2	SKK 5.2	0	1	-
6	SKK 5.3	13	0	Negative
9	SKL 6.1	0	0	-
11	SKL 5.1	13	5	Positive
14	SKS 6.1	0	0	-
15	SKS 5.1	12	0	Negative
16	SKK 5.2	10	0	Positive
19	SKK 7.3	3	0	-
23	SKS 5.2	0	0	-
24	SKK 7.2	0	2	-
25	SKK 7.1	13	0	Positive
26	SKK 5.1	14	0	Positive
27	SKS 6.2	0	3	-
28	SKK 6	0	0	-
48	SKK 6.1	19	12	Negative

TABLE 2. RESULT OF BIOCHEMISTRY TEST OF CELLULOLYTIC BACTERIA FROM SUKAL MANGROVE

Biochemistry Test	Result			
	SKL-51	SKK-51	SKK-52	SKK-71
Spore	-	-	-	-
Oksidase	-	-	-	+
Motility	-	+	+	+
Nitrate	+	+	+	+
Lysine	+	-	-	+
Ornithin	-	-	-	+
H_2S	-	+	-	-
Glukose	+	+	+	+
Mannitol	-	+	+	-
Xylose	+	+	+	+
ONPG	+	+	+	-
Indole	-	+	-	+
Urease	+	+	-	+
V-P	+	+	+	+
Sitrat		+	+	-
TDA	-	-	-	-
Gelatin	-	-	-	-
Malonat	-	-	-	-
Inositol	-	-	-	-
Rhamnose	-	-	-	-
Sukrose	-	-	+	-
Lactose	-	-	-	-
Arabinose	-	-	-	-
Adonitol	-	-	-	-
Raffinose	-	-	-	-
Salicin	-	-	+	-
Arginin	-	-	-	-
Catalase	+	-	+	+
Coagulase	-	-	-	-
Hemolise	beta	gama	gama	beta
Uji sensitive Novobiosin	Resisten	TDK	TDK	Resisten
Starch hydrolysis	-	+	+	-
Casein hydrolysis	+	-	+	-
	Bacillus megaterium	*Citrobacter freundii*		*Vibrio alginoliticus*

Figure 1. Sampling location in Sukal Mangrove

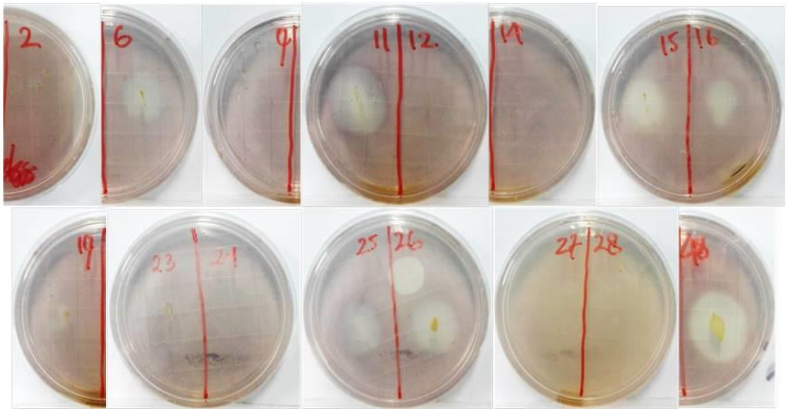

Figure 2. Qualitative test results of cellulolytic bacteria using lugol method

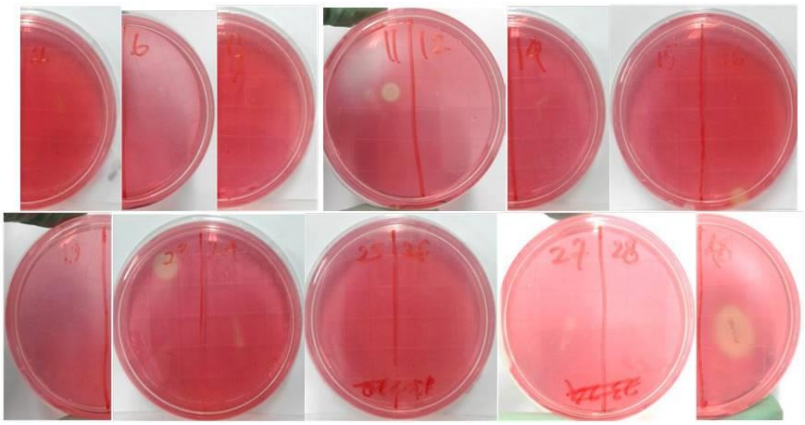

Figure 3. The results of cellulolytic bacteria qualitative test methods with congored

Isolate bacteria with the largest clear zone and included in gram-positive bacteria followed by identification testing through biochemical characterization and DNA sequencing with 16S rRNA encoding genes. Gram-negative bacteria more pathogenic rather than bacteria gram-positive bacteria [11]. Gram-negative bacteria are characterized by cell wall components called lipopolysaccharide (LPS), which are connected with substantial diseases in humans and marine organisms [12]. The selection of gram-positive bacteria in the hope that the identified cellulolytic bacterial isolates can be further applied to the overhaul of cellulose and fiber in natural materials that are used as raw material for fish feed which is useful to increase nutrient absorption.

The results of biochemical tests using Mikrobact for bacterial isolates cellulolytic show selected lead to species *Bacillus megaterium, Citrobacter freundii,* and *Vibrio alginoliticus.* Although in the initial selection character selected gram-positive bacteria, *Vibrio alginoliticus* dan *Citrobacter freundii* bacteria turns which is a gram-negative bacteria that became one of the identification results. This was possible presence of errors in the process of rejuvenation of bacterial culture or contamination in the testing process.

Bacillus megaterium is a bacterium that is often found in mangrove areas. *B. megaterium* is found in the mangroves of *Rhizophora apiculata* and *Sonneratia alba* from Mangrove Coastal Waters of Kraton, Pasuruan [13] and the mangroves of Tarakan City [14]. *B. megaterium*identified has high extra-cellular enzyme activity to break down the cellulose up to 216 U / ml [15]. *B. megaterium* cellulase enzyme has the potential to degrade talus sargassum and can be used for hydrolysis of seaweed which extracts bioactive molecules [16].

Citrobacter freundii is a one of the Enterobacteriaceae family and everpresent in soil, sewage and water. *Citrobacter*can also colonize in gastrointestinal ofhuman and animal. *C. freundii* is an liability pathogen [17] and identified in polluted waters [18]. *C. freundii* was added to one of the probiotic bacteria obtained from the digestive tract of *Anguilla bicolor bicolor* based on hydrolytic and antagonistic ability of *Aeromonas hydrophilla* [19]. *C. freundii* has an positive side about potentiality for marine sulfate reduction bacteria [20].

Vibrio genus is known as a vibriosis disease-causing bacteria such as *Vibrio anguillarum, Vibrio ordalii, Vibrio salmonicida,* and *Vibrio vulnificus.* The existence of bacterial species in this genus is a significant problem for aquaculture organisms [12]. *Vibrio alginolyticus* is a natural bacterium in the estuary and coastal waters that have the potential to be a threat to public health that consumes products from the region. *Vibrio alginolytic* is a halophilic associated to the causes of several diseases in aquatic commodities including aquaculture fish [21].

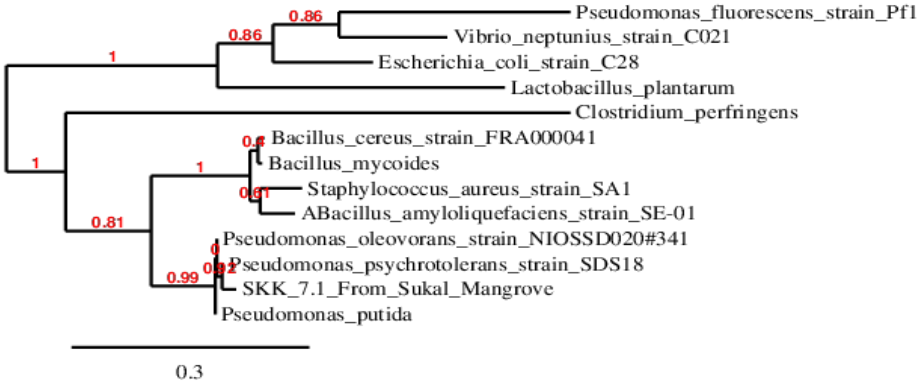

Figure 4. Phylogenetic of isolate SKK 7.1 from Sukal Mangrove

DNA sequences of SKK 7.1 isolate bacteria showed close kinship with *Pseudomonas psychotolerans.* Based on phylogenetic trees, *pseudomonas psychotolerans* has a close relationship with *P. oleovorans* and *P. putida.* Psedomonas genus was also identified in cellulolytic bacteria from mangrove sediments in Tukak Sadai, southern Bangka [22], mangrove Kraton Pasuruan [13], mangrove soil in the Gunung Anyar river estuary, Surabaya [23], mangrove sediments in the Hong Kong nature reserve [24], Bajul Mati Beach, Malang, East Java [25] and natural mangrove of Great Nicobar Island, India [26].

IV. CONCLUSION

Biochemical tests using Mikrobact™ show the selected cellulolytic bacterial isolates lead to *Bacillus megaterium, Citrobacter freundii,* and *Vibrio alginoliticus*. DNA sequences of SKK 7.1 isolate bacteria showed close kinship with *Pseudomonas psychotolerans. Bacillus megaterium* can be recommended to be tested as cellulose degrading bacteria in palm waste.

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