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Molecular Characterization of Bacterial Isolates from the Coastal Region of South Jember - Indonesia: Diversity and Carbon Substrates Utilization

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ABSTRACT

Bacteria play an important role in aquatic ecosystems decomposing organic matter which is a key process in the microbial food web i.e. nutrient and Carbon cycles. Furthermore, this decomposition process, which is basically depending on bacterial ability to hydrolyze nutrient in aquatic environment, is very important for the bioremediation process of polluted water. Indonesia is the largest archipelago in the world with sea area about 81% of the total area of the country. Many other water areas which are not included in this sea area flow throughout the country i.e. rivers, lakes and lagoons. Consequently, research on aquatic ecology and environmental protection is of utmost importance to Indonesia. Considering the important of bacteria in microbial aquatic food web, the analysis of natural bacterial assemblages and their activities would improve this area of research in indonesian microbial (aquatic) ecology which is very limited so far. This research is based on following hypothesis i.e. Carbon utilization of bacteria depend on their diversity (1), and therefore influence bacterial activity especially their hydrolytic activities (2). The objective of this research is therefore to investigate bacterial diversity related to its substrate utilization profile (Carbon substrates) in the coastal region of South Jember. Molecular diversity of isolates was studied by observing DNA profile of isolates after BOX-Polymerase Chain Reaction (BOX-PCR). The substrate utilization pattern kit BIOLOG GN2 was used to test the ability of isolates to respire in 95 substrates, incl. carbohydrates, amino acids, organic acids and selected polymers simultaneously producing a kind of metabolic fingerprint. 126 bacteria were isolated from the coastal region of South Jember Indonesia. 4 of 8 bacteria with diversity in genetic profiles showed diversity in their metabolic fingerprint as well. These 4 isolates were able to use polymer substrate in the BIOLOG GN indicating its potential used to produce hydrolytic enzymes which are important for material decomposition in the bioremediation process in aquatic ecosystem.

Introduction

Indonesia is the largest archipelago in the world with the total number of 18,108 islands according to the latest information from Indonesian Naval Hydro-Oceanographic office. The

Indonesian sea area is four times greater than its land area, which is about 1.9 million km^2. The sea area is about 7.9 million km^2 (including an exclusive economic zone) and constitutes about 81% of the total area of the country. Many other water areas which are not included in this sea area flow throughout the country i.e. rivers, lakes and lagoons. Research on aquatic ecology and environmental protection is of utmost importance to Indonesia as an agrarian country with the major product of rice (Van Bodegom et al. 2002) as well as aquaculture farming of macrophyte and shrimps. All these agricultural activities still introduce large amounts of nutrients, pesticides and antibiotics into the environment, esp. aquatic systems. The poor infrastructure of the country further promotes environmental pollution, e.g. potentially pathogenic bacteria even in drinking water resources (Kromoredjo and Fujioka 1991). Biodeterioration is also a major problem of the country, e.g. in housing due to climatological circumstances as well as architecture and poverty (Josodipoero 2003). Consequently, the analysis of natural bacterial assemblages and their activities would improve this area of research in Indonesian microbial (aquatic) ecology which is very limited so far.

Bacteria play an important role as decomposer for organic matter in aquatic environment. This process is a key process in aquatic microbial food webs as well as its application in the water bioremediation process (Hoppe *et al.* 2002, Biddle & Azam 1999). A bacterioplankton community constitutes bacterial cells with different physiological states, viable as well as non-viable, defect, cells. As viable cells may be active or inactive with respect to many different activities, e.g. substrate uptake, respiration, hydrolysis, and cell deviation. Modern microbial diagnostics are very important to recognize the existence of the invisibly small bacteria and the transparent biofilms on material surfaces as early as possible. Classical microbiological detection methods often include an isolation and cultivation steps that is not only time consuming (up to several days), but also missing most individuals or even species. Only up to 6% of all bacteria from water samples were found to grow colonies on agar plates, most often they don't exceed 1% of total number. Molecular genetic methods, esp. genomic profiling protocols, were introduced into environmental research, esp. into marine microbiology, several years ago with great success (Muyzer *et al.*, 1993). The repetitive extragenic palindromic-PCR (rep-PCR) DNA fingerprinting technique uses the PCR and primers based on highly conserved and repetitive nucleotide sequences to amplify specific portions of the microbial genome. The resulting banding patterns of PCR product display a "fingerprint" unique to each strain. The rep-PCR technique has proven to be a valuable tool to identify and track medically and environmentally important microorganisms (e.g. Versalovic 1991). The main objective of this research is to investigate bacterial community structure in relation to its activities as decomposer in the coastal region of South Jember, Indonesia. As a preliminary investigation, molecular characterization of bacterial isolates as well as its substrate utilization profile (Carbon substrates) have been carried out and will be discussed in this paper.

Materials and Methods

Origin of samples and isolation procedure

Isolation of bacteria was carried out on June of 2009 from samples obtained from the coastal region of South Jember i.e. Watu Ulo and Bande Alit, East Java Indonesia. 200 µl subsamples were taken immediately upon return to the lab from water and plated onto M1 medium (DMSZ) agar plates. If high concentrations of soluble particles were found, samples were diluted up to a final concentration of 10% using sterile distilled water. Plates were then incubated at room temperature (ca. 21°C) for 24-48 hours. Single colonies were isolated and tested for its ability to growth at a temperature >40°C. Only isolates that were not able to grow at this temperature were then used for further experiments considering that they might be not pathogen. Name of isolates described the original location of isolation followed with bacterial isolate number (xx) i.e. BAxx for strains that were isolated from Bande Alit and WUxx from Watu Ulo. About 126 isolates from that area were collected. Isolates were conserved after incubating colonies in M1 liquid medium at room temperature (21°C) for 12 hours. These cultures were conserved by adding 200 µl of sterile glycerol to 800 µl of liquid culture and stored in 1.5 ml Eppendorf tubes at -80°C.

Molecular diversity by BOX-PCR

Molecular diversity of isolates was studied by observing DNA profile of isolates after BOX-Polymerase Chain Reaction (BOX-PCR). The difference between this PCR and conventional PCR is the use of only one single primer instead of a pair of primers (forward and reverse). The single primer binds to a specific repetitive sequence of the bacterial genome and this strand is then polymerised multiple times. This repetitive sequence of the bacterial genome is conserved and species specific (Oda *et al*. 2002). DNA was prepared from pellets by centrifugation (5 min, 13,000 rpm) of 2 x 1 ml liquid culture of respective isolates. The pellet was washed with 3 x 1 ml PBS (Phosphat Buffer Saline, in 1 l ddH_2O: 8 g NaCl, 0.2 g KCl, 1.44 g, $Na_2HPO_4 \cdot 12\ H_2O$, 0.2 g KH_2PO4). The last washing step was done by vortexing the mixture and final centrifugation for 5 minute at 13,000 rpm. The supernatant was discarded whereas the precipitate was frozen and ready for DNA extraction. DNA was extracted by boiling frozen pellets for 4 minutes. The pellets were then dissolved in 50 µl of sterile dH_2O and resuspended by vortexing. The supernatant obtained from the following centrifugation contained the genetic material and was stored at -20°C if not used immediately for BOX-PCR ("Freeze and Thaw" modified from Tsai and Olson 1991).

A BOX-AIR-Primer (5'>CTA CGG CAA GGC GAC GCT GAC G<3', Oda *et al.* 2002) was used to amplify the bacterial genome (synthesis of primer by Carl Roth GmbH + Co. KG). PCR was carried out in 25 µl reaction mixture containing 8.0 µl water (Qiagen Kit), 12.5 µl PCR Mastermix

(Fermentas Kit), 2.5 µl primer BOX-AIR (10 pmol/µl, final concentration of 1 pmol/µl), and 2.0 µl DNA extract. The temperature gradient was as follow: initial denaturation at 95°C for 6 min, 35 cycles at 94°C for 1 min, 53°C for 1 min, 65°C for 8 min, and final extension at 65°C for 16 minute (BIOMETRA PCR-maschine). DNA fragments were separated by electrophoresis on 1.5% agarose-gel at 72 mV for 1,5 h nd was stained using ethidiumbromid. 1 KB DNA ladder was used as marker. The gel was visualised under exposure of uv light. Similarity between band tracks of different isolates was compared. Samples with similarity of band tracks were then considered to be the same strains and *vice versa*.

Carbon sources turnover (BIOLOG)

Most aquatic bacteria are Gram negative. To identify and characterise bacterial ability to utilise or oxidise compounds from different carbon sources, The BIOLOG GN2 MicroPlate were applied (Invitrogen). With this method 95 discrete tests could be performed simultaneously resulting in a characteristic reaction pattern called a "metabolic fingerprint". The reaction based on reduction of tetrazolium, responds to the process of metabolism (i.e. respiration) rather than to metabolic by-products (e.g. acid). The test yields a characteristic pattern of purple wells, indicating the utilisation of carbon source. Precultures grown in 10 ml liquid M1 medium at 21°C for 12 h were prepared from single colonies. and harvested by centrifugation at 6000 rpm, 20°C for 5 min. The precipitate was washed 3 times using 0.9% physiological salt solution (Ringer solution) and repeated centrifugation. The last wash-buffer was discarded and the pellet dissolved in Ringer solution. 150 µl of this suspension was then added to each well of The BIOLOG GN2 MicroPlate and incubated at 21°C for a maximum of 48 h.

Results

126 bacteria were isolated from the coastal region of South Jember i.e. Watu Ulo and Bande Alit brackish water. Genetic profiles of 8 isolates were observed using BOX-A1R primer to investigate their diversity. BOX-PCR of 8 chosen isolates resulted products that had different band tracks. Thus, 8 chosen isolates are considered as different strains (Figure 1). 4 from these 8 isolates were chosen as model species i.e. BA698, WU9918, WU6950 and WU6962. The 4 selected strains were observed microscopically concerning their morphology and Gram staining. They are all Gram negative bacteria, however, in agar plate growth media, they have different colony form. Single colony of BA698 displayed circulate small colony having white opaque colour. The same shape with yellowish white colour was observed in WU9918 and WU6962, but the colony was larger than BA698. Single colony of WU6950 had creaming opaque colour and formed colony as large as WU9918 and WU6962. All these isolates were microscopically cocci. The difference band tracks of

WU9918 and WU6962 indicated that they are likely to be difference strains though they have similarity in their cell shape, morphology of colony and Gram staining.

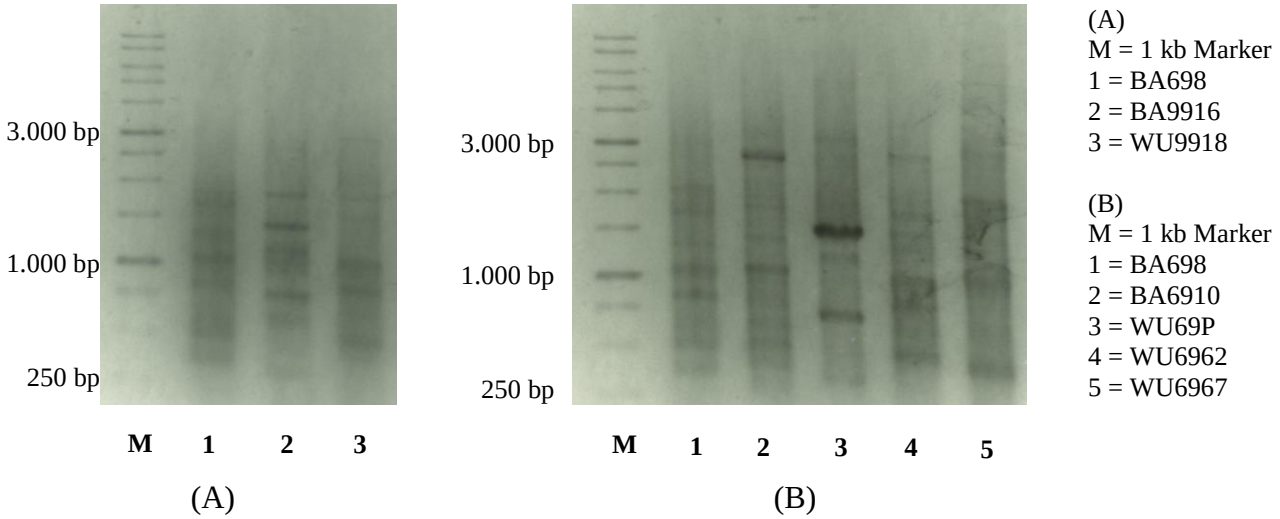

Figure 1. Fingerprint patterns of bacterial strains, obtained by REP-PCR with the BOX-A1R primer. The 8 isolates were collected from the coastal region of South Jember, Indonesia.

Tabel 1. Substrate utilization for respiration (selected substrates from BIOLOG GN2 substrate utilization pattern kit) in 4 isolates from coastal region (brackish water) of South Jember; WU = Watu Ulo and BA = Bande Alit compared to 2 isolates from freshwater of sungai Bedadung Jember; BD = Bedadung.

Group of substrate	Name of carbon sources	WU 9918	WU 6950	WU 6963	BA 698	BD71	BD 81
Carbohydrates:							
Monomer	α-D-Glucose	-	-	-	-	+	+
	β-Methyl-D-Glucoside	-	+	-	-	+	+
	D-Fructose	-	-	-	-	-	+
	D-Galactonic Acid Lactone	+	+	+	-	-	-
	D-Galacturonic Acid	-	-	-	-	+	-
	D-Galactose	-	-	-	-	+	+
	D-Gluconic Acid	-	+	+	-	+	-
	D-Glucosaminic Acid	-	-	+	-	-	-
	D-Mannose	-	-	-	-	+	+
	D-Psikose	-	-	-	-	+	+
	D-Saccharic Acid	-	+	-	-	+	+
	Glucuronamide	-	-	-	-	-	-
	L-Arabinosa	-	+	-	-	-	-
	L-Fucose	-	-	-	-	-	-
	L-Rhamnose	-	+	-	-	+	-

Continued in the next page...

Table continued...

	N-acetyl-D-galactosamine	-	-	-	-	+	+
	N-acetyl-D-glucosamine	-	+	-	-	+	+
dimer	α-D-Lactose	-	+	-	-	+	+
	D-Cellobiose	-	+	-	-	+	+
	Gentibiose	-	-	-	-	+	+
	D-Mellibiose	-	-	-	-	+	+
	D-Raffinose	-	-	-	-	+	-
	D-Trehalose	-	+	+	-	+	+
	Laktulose	-	+	-	-	+	+
	Maltose	-	+	-	-	+	+
	Sucrose	-	+	+	-	+	+
	Turanose	-	+	-	-	+	+
oligo-/polymer	α-cyclodextrin	-	+	-	-	-	-
	Dextrin	+	+	-	-	+	+
	Glycogen	+	+	-	-	+	+
Proteins:							
monomer	D-Alanine	-	+	-	-	+	+
	D-Serine	-	+	+	-	-	-
	Hydroxy-L-Proline	-	+	-	-	-	-
	L-Alaninamide	-	+	-	-	+	+
	L-Alanine	+	+	-	-	+	+
	L-Asparagine	-	+	-	-	+	+
	L-Aspartic Acid	-	+	-	-	+	+
	L-Glutamic Acid	-	+	-	-	+	+
	L-Histidine	-	-	-	-	-	+
	L-Leucine	-	+	-	-	+	+
	L-Phenyllalanine	-	-	-	-	-	-
	L-Proline	-	+	+	-	+	+
	L-Pyroglutamic acid	-	+	-	-	-	-
	L-Serine	-	+	+	-	+	+
	L-Threonine	-	-	-	-	+	+
	L-Alanyl-Glycine	+	+	-	-	+	+
dimmer	Glycyl-L-Aspartic Acid	-	-	-	-	+	+
	Glycyl-L-Glutamic Acid	-	-	-	-	+	+
Acids/Esters/							
Alkohols:							
monomer	Acetic Acid	-	+	-	-	+	+
	Citric Acid	-	-	-	-	+	+
	D,L-Lactic Acid	-	-	+	+	+	+
	D-Mannitol	-	+	-	-	+	-
	D-Sorbitol	-	+	-	-	+	+
	Formic Acid	+	+	-	-	-	-
	Malonic Acid	-	-	+	-	+	+
	Succinic Acid	-	+	-	-	+	+
polymer	Tween 40	+	+	+	+	+	+
	Tween 80	+	+	+	+	+	+
	Inositol	+	+	-	-	-	-
	Gliserol	-	+	+	-	-	-

To further elucidate their activities, substrate utilisation pattern for respiration (BIOLOG) were further characterized in 4 chosen isolates (Table 1). All isolates used Tween as a substrate for respiration, indicating their ability to produce hydrolases to respire this polymer. WU9918 and WU6950 were able to use dextrin and glycogen (polymer of carbohydrates) as substrates. The ability to respire certain polymer indicating that they should produce hydrolases to breakdown the polymer. These hydrolytic activities should therefore further investigate to understand their role in the decomposition process (remineralisation).

Discussion

Several studies indicated that rep-PCR done with Box A1R primers yielded more consistent and complex DNA fingerprints (e.g. Dombek *et al.* 2000). Figure 1 shows typical fingerprints for bacterial isolates generated by REP-PCR with primer BOX-A1R. The profiles of the 8 strains revealed multiple DNA bands corresponding to sizes ranging from approximately 0.2 to 3.5 kb. The banding patterns were diverse and strain specific. Data generated by the REP-PCR DNA fingerprint technique with the BOX-A1R primer in the present study show that genetic diversity exists among 8 bacterial isolates. However this data should further approve using genetic tool software to generate its phylogenetic tree.

Kits to estimate utilization patterns of various ecologically relevant organic substrates have been recently developed not only for the bacterial strain identification but also for the estimation the functional diversity of bacterial assemblage, e.g. soil bacterial communities. It is important to differentiate between the ability of bacterial cells to use substrates for their respiration and/or for their growth, because these are two major functions of heterotrophic bacteria in the transformation of organic matter: remineralisation and secondary production. Biolog microplates were introduced to identify bacterial strains assisting taxonomic description of bacteria based on respiration profiles (Bochner 1989). This test is based on the ability of living bacterial cell suspensions to oxidize up to 95 different carbon sources in micro-titre plates. The result constitutes a “metabolic fingerprint” of the organism. The classification of isolates is strongly supported by a sole-carbon-source utilization pattern. The rate of colour development in each well of the Biolog microplates depends on the cell density (Garland and Mills 1991, Winding 1993, Christian and Lind 2006).

Substrate utilization patterns were used to describe the “activity level” of three microbial communities in Biolog the GN microplates (Zak et al (1994). However, it was not described detailed enough, how many (active) bacteria are needed for this test. If log phase bacterial cultures were applied (this study), the widely varying rates of colour development were explained by different inoculum densities (Haack et al. 1995). Thus, for bacterial communities with bacteria in different physiological states, the rate of colour development may not indicate the activity level of

one bacterial community as described by Zak et al. (1994). Moreover, negative responses of bacterial cultures to certain substrates may be attributed to a complete loss of viability if the sole carbon source does not support sufficiently every requirement for proliferation. Thus, the substrate oxidation profiles of communities are not simple summations of the individual profiles of their members (Haack et al. 1995). However, the low cost and ease of use of Biolog microplates provides a valuable insight into bacterial substrate utilization patterns and metabolic functional potential in aquatic ecosystems.

Respiration is the prerequisite for growth. Both mechanisms are theoretically well coupled. However, the uncoupling of these two processes can provide bacteria with the metabolic flexibility necessary to cope with the instable conditions of a largely oligotrophic and ever-changing environment (del Giorgio and Cole 1998). Therefore, it is important to elucidate weather the cells do have to grow (biosynthesis) to consume the carbon substrate (energy production) or if the cells solely maintain their high-energy flux under conditions of severe growth restrictions. Many bacteria may need to maintain high a flow of energy to optimise microbial growth efficiency. Among all isolates, there was an obvious difference between their ability to use substrates for duplication or only for respiration. However, results of substrates utilization pattern for respiration in this study should consider several conditions which can interfere respiration signal (colour development) as discussed above. The ability to respire certain substrates (Biolog results) does not implicate that the isolate can use them as sole substrate for duplication (growth). This indicated the metabolic flexibility of mostly all isolates to utilize carbon substrate even in well supplied cultures, which is actually not as viccissitudes as aquatic systems as described by del Giorgio and Cole (1998). This hypothesis should therefore further investigate in these 4 model species by assessing their growth in certain substrates.

All isolates were able to use polymer of carbohydrates (dextrin and glycogen) as well as polymer of ester (Tween) to respire, indicated that they must have hydrolases activities. With high molecular weight of polymeric Carbon, they cannot therefore be transported across microbial cell membrane unless they are enzymatically hydrolyzed. Consequently, the 4 model species should be able to produce hydrolases in order to respire this sole Carbon sources in the BIOLOG well. This indicated their potential use for further activities investigation in remineralisation process in aquatic environments.

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