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Isolation of environmental DNA from soil of oil-palm plantation and detection of prokaryotes employing dot blot hybridization

Isolasi DNA lingkungan tanah perkebunan kelapa sawit dan deteksi prokariot menggunakan hibridisasi dot blot

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Ringkasan

Keberadaan serta keragaman organisme prokariot tanah sangat berperan dalam menentukan tingkat kesuburan tanah. Oleh sebab itu kemampuan untuk menentukan distribusi dan keragaman prokariot pada suatu lingkungan tanah termasuk yang tidak dapat dikulturkan, merupakan hal yang diperlukan dalam usaha kita untuk memahami dinamika kesuburan tanah. Teknik yang dapat dimanfaatkan untuk keperluan tersebut adalah kombinasi antara cara yang tepat untuk mengisolasi DNA lingkungan dengan teknik hibridisasi dot blot. *Sebagai pelacak dapat digunakan gen yang bersifat universal untuk prokariot. Penelitian ini bertujuan untuk mendapatkan DNA prokariot yang diisolasi langsung dari lingkungan tanah perkebunan kelapa sawit dan mendeteksi distribusi populasi prokariot pada lingkungan tanah tersebut. DNA lingkungan diisolasi dengan metode* freeze-thaw *dalam bufer pengekstrak, dan untuk hibridisasi* dot blot *digunakan gen penyandi rRNA 16S yang berasal dari* Pseudomonas aeruginosa *sebagai pelacak. Analisis* dot blot *dilakukan terhadap DNA kasar (crude DNA). Hasil penelitian menunjukkan bahwa dengan metode isolasi yang digunakan, DNA yang diisolasi tidak bisa dipisahkan dengan substansi tanah yang berwarna cokelat sampai cokelat tua. Perlakuan tambahan dengan* Prep-A-Gene DNA Purification System *mampu menghilangkan warna dan DNA murni bisa diperoleh dengan rendemen 30% dari sebelum dimurnikan. Hibridisasi* dot blot *dapat menghasilkan sinyal, yang menunjukkan bahwa pelacak dapat mendeteksi keberadaan gen serupa pada DNA kasar organisme contoh tanah. Sinyal diperoleh dari hibridisasi antara rRNA 16S dengan semua contoh tanah yang digunakan dalam penelitian ini. Namun diameter beberapa sinyal berbeda. Disimpulkan bahwa prokariot terdistribusi pada lingkungan tanah tersebut namun dengan populasi yang berbeda.*

Summary

The presence and diversity of prokaryotic organisms play a role in determining the level of soil fertility. Therefore, the ability to define prokaryotes distribution and diversity in a soil environment, including the "unculturable" prokaryotes, is an essential component in our effort to understand the dynamics of soil fertility. An applicable technique for this purpose is a combination of environmental DNA isolation and dot blot hybridization. A universal gene can be used as a probe of the prokaryotes. This research aims to isolate prokaryotic DNA, directly from the soil environment, and to detect the distribution of prokaryotes in the soil of oil-palm plantation. Environmental DNA was isolated by freeze-thaw treatment in extraction buffer, and for dot-blot hybridization the gene encoded for 16S-rRNA derived from *Pseudomonas aeruginosa* was employed as a probe. The dot blot analysis was conducted using crude DNA. The result showed that by using current DNA isolation method, the isolated DNA could not be separated from the brown to dark brown colored substances. Additional treatment with Prep-A-Gene DNA

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Purification System could separate the colored substances although the yield of purified DNA was below 30%. Dot blot hybridization yielded signals indicating that the probe could detect the presence of similar genes in the crude DNA of soil samples. The signals were resulted from hybridization between 16S-rRNA and each of the soil samples used in this study. However the diameter of some signals varied significantly. We conclude that there is a spatial distribution of prokaryotes in the soil environment with different population density.

[*Keywords*: Rapid detection, dot blot hybridization, 16S-rRNA, soil prokaryotes]

Introduction

The presence and diversity of soil microorganisms is an important factor to maintain the soil fertility. Those microorganisms are involved in the biological process of soil formation and in the cycle of some elements such as carbon, nitrogen, phosphorous, and others (Brock & Madigan, 1991). Environmental stresses can influence microbial populations and therefore threaten soil health. It was reported that in soil contaminated by pesticides or herbicides, the population and diversity of soil microorganisms decreased (Atlas, 1984; Atlas *et al.*, 1991).

Prokaryote is a member of microorganisms. The presence of this organism in the environment, including soil environment, could be detected by using molecular approaches. The combination of DNA extraction methods in environmental samples and DNA hybridization technique is one of molecular techniques that can be done. The application of DNA extraction directly from environment can obviate the need for cell cultivation. It was known that cell cultivation has the disadvantage of obtaining a very small proportion of the total microbial community. According to Torsvik *et al.* (1990), 99.5-99.9% of the soil bacteria observed in the fluorescence microscope cannot be isolated and cultured on laboratory media. Besides, the combination of the two techniques is very useful to detect the presence of microorganisms as a result of genetic manipulation in an environment (Steffan & Atlas, 1988; Chaudry *et al.*, 1989) as well as to detect the presence of resistant bacteria in contaminated soils (Diels & Mergeay, 1990). This method can be applied because of the availability of various specific genes or synthetic oligonucleotides to be used as probes in the hybridization process.

The presence of soil prokaryote could be determined by using a universal probe available in all prokaryotes. The RNA ribosome (rRNA) is a suitable molecule for this purpose because it has identical functions, e.g. protein synthesis. There are three kinds of rRNA e.g. 5S-rRNA (± 120 nucleotides), 16S-rRNA (± 1500 nucleotides) and 23S-rRNA (± 3000 nucleotides). Among these three rRNA, 16S-rRNA is more used in molecular research because it gives more information compared with the 5S-rRNA and its application is more practical than the 23S-rRNA (Woese, 1987). The 16S-rRNA of *P. aeruginosa* (± 2.2 kb) has been cloned into *E. coli*, producing pHF 1.1 plasmid (Schleifer *et al.*, 1985). This gene can be used as a heterologous probe to detect the presence of prokaryotes in environmental samples.

The general problem in DNA extraction directly from soil is the contamination of DNA with humic compounds or clay soil particles. Those contaminants usually give negative impact in molecular analysis, such as inhibit the activity of *Taq* DNA polymerase in PCR reaction, obstruct the restriction enzyme in DNA digestion process and influence the success in Southern hybridization (Tsai & Olson, 1991; Steffan & Atlas, 1988). The use of minicolumn and gel combination with minicolumn could produced the purify DNA which was directly isolated from the soil (Zhou *et al.*, 1996). However, the DNA purification phase is more costly and time consuming. Besides, certain DNA organisms in small quantities in the tested soil samples could disappear.

Dot blot hybridization is one of the DNA hybridization techniques based on the Southern blot. In this technique, the mix DNA directly drop on the surface of membrane and the digestion of DNA sample with restriction enzyme as in Southern hybridization is unnecessary. This technique, therefore, has an advantage, especially for DNA directly isolated from the soil environment which usually contaminated by humic compounds. Another advantage of dot blot hybridization is the quick implementation, semi quantitatively, and can be applied to detect simultaneously the presence of specific nucleic acid in various samples on the same membrane (Keller & Manak, 1989). However, the weakness of this method is that it can only be used to determine certain DNA fragments present or not present in the DNA sample or DNA target.

The aim of this research is to extract DNA directly from the soil organisms of oil palm plantation, to determine the ability of the 16S-rRNA gene in probing the presence of prokaryotes by using environmental DNA which is not yet pure and to detect the prokaryotes distribution in mentioned plantation soil.

Materials and Methods

Soil samples

Samples were taken from the top 5 cm of soil from Experimental Garden Ciomas, Biotechnology Research Unit for Estate Crops, Bogor, pianted with oil palms. The samples were taken from several spots at distances of ± 0.5 m and ± 3 m from the trees, on April 1998. The soil was mixed well and kept in polyethylene bags at 4°C.

DNA isolation

The DNA of soil organisms were prepared from twelve 5-g samples of fresh soil. To increase the yield of DNA, the isolation procedure of Zhou *et al.* (1996) was somewhat modified. The freeze-thaw treatment which was done repeatedly was used to separate the bacterial cell from the soil particle. The freeze treatment was done by soaking the mixture of soil and extract buffer in liquid nitrogen until the whole mixture was frozen, whereas the thaw treatment was done through soaking in boiling water until the mixture becomes again a suspension. Besides, 100 µL Proteinase-K (10 mg/mL) and 3 mL SDS 10% were added to lysis the cell wall. The separation of DNA from protein and carbohydrate was done by adding chloroform, while DNA was precipitated with isopropanol.

Part of crude DNA from seven samples, showing a brown colouring when suspended in water or Tris-EDTA (TE) solution, were purified with Prep A Gene DNA Purification System (Bio-Rad, California, USA). This step was done to examine the ability of the method to recover DNA from the crude DNA. By this system DNA was attached to a matrix so that it was separated from the other contaminants. The separation of DNA from the matrix was carried out by heating the mixed suspension to 50°C. The DNA concentration and purity were determined by a Spectrophotometer UV according to the method of Sambrook *et al.* (1989).

Preparation of probe

The pHF 1.1 plasmid (6.6 kb) containing the 16S-rRNA gene (2.2 kb) from *P. aeruginosa* (Schleifer *et al.*, 1985) was iso-

Miniprep Kits (QIAGEN, Germany) based on the recommended procedure. This plasmid was then digested with restricted enzyme *Bst*EII at 60°C for 2 hours. The digested DNA was separated by 1.0% agarose gel electrophoresis in TAE buffer and stained with etidium bromide. The 2.2 kb fragment was then separated from agarose by elusion using Gene Clean Kit (BIO 101, La Jolla, USA) based on the recommended procedure. As standard molecule λDNA/*Bst*EII was used. The probe was labeled by ECL Direct Nucleic Acid Labeling Systems (Amersham, England) at the time of hybridization.

Hybridization of 16S-rRNA probe with soil organism's DNA

Nylon membrane Zeta Probe (Bio-Rad, California, USA) for dot blot analysis was used to transfer DNA. The crude DNA from all samples were mixed with 20x SSC (3 M NaCl, 0.3 M Na citrate) to the SSC final concentration of 6x. The following procedure was used : 2 µL of every mixture was dropped on the nylon membrane, which was repeated several times until the whole solution was finished off. The DNA on the membrane was denaturalized with a solution containing 0.5 M NaOH and 1.5 M NaCl and neutralized with a solution containing 1 M Tris-HCl and 1 M NaCl (pH 7.5). The membrane was baked in oven at 85°C for two hours to fix the DNA samples. The membrane can be stored at room temperature before using it in the hybridization process.

The hybridization between probe and samples, as well as washing and detection of the hybridization result were done by using ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham, England), according to the recommended procedure. Hybridization was done in a Hybritube (Gibco-BRL, USA) by incubation at 42°C for one night. After washing, the membrane was exposed to X-ray film and then processed in a Developer and Fixer solution.

Results and Discussion

Environmental DNA yield and efficiency of DNA recovery

The DNA isolation protocol involves extraction of total DNA from soil prokaryotes as well as from fungal mycelia and other organisms. The yielded DNA, therefore, reflected not only the contain of DNA prokaryotes, but also DNA from the others. By employing this protocol, the total DNA was obtained from all tested samples. The DNA pellet, if dissolved in aquades or Tris-EDTA solution produced various colors, ranging from clear white to dark brown. It was assumed that the color was a contaminant originated from soil and could not completely be separated by using this procedure.

Separation of the contaminant from soil was accomplished by using an additional treatment with Prep A Gene DNA Purification System (Bio-Rad, California, USA) to a part of DNA which has a brown to dark brown color. This treatment could remove the colors, resulting in a clear DNA solution. However, the purity of the DNA did not improve, it was shown by the A_{260}/A_{280} ratio, approximately 1.2 (Tabel 1).

The efficiency of recovery of the soil organisms was determined by comparing the concentration of DNA prior and after treatment with Prep A Gene DNA Purification Systems. By using Spectrophotometer UV, it was known that the DNA concentration prior to additional treatment was quite high, i.e. between 16 – 350 µg/g soil. While DNA concentration of the same soil sample after additional treatment was between 12 – 33 µg/g soil. This result showed that the yield of

Table 1. Comparison of concentration and purity of DNA isolated from soil samples, prior and after purification using Prep A Gene DNA Purification System

Sample code	Origin of soil sample	DNA prior to treatment		DNA after treatment		DNA recovery
		Concentration µg/g	Purity A_{260}/A_{280}	Concentration µg/g	Purity A_{260}/A_{280}	%
S1	± 0.5 m from	75.0	1.10	-	-	-
S2	the oil palm tree	100.4	1.18	13.8	1.29	14
S3		197.3	1.18	15.4	1.27	8
S4		16.4	0.68	-	-	-
S5		37.5	0.97	-	-	-
S6		128.5	1.19	19.0	1.21	15
S7	± 3 m from the	47.6	1.01	-	-	-
S8	oil palm tree	100.6	1.17	12.6	1.23	13
S9		349.5	1.19	33.6	1.16	10
S10		149.5	1.20	-	-	-
S11		85.0	1.14	21.3	1.24	25
S12		141.6	1.21	41.0	1.17	29

Note: -) Not treated with Prep A Gene DNA Purification System

purified DNA which could be recovered was low, i.e. under 30%. Thus, approximately 70% of the total DNA present in the crude DNA was not recovered by this method.

DNA concentration of soil organisms derived from this experiment was higher than that which has been previously reported. It was noted, however, that the DNA concentration was not a reflection of the DNA prokaryotes. Zhou *et al.* (1996) reported that the concentration of DNA isolated directly from some soil environment were 2.5 – 26.9 µg/g soil. While Tsai & Olson (1991) reported that from soil contaminated by aromatic hydrocarbon compound and mercury, DNA concentration of 12 µg/g soil was obtained. Jacobsen & Rasmussen (1992) obtained 1 µg DNA/g soil by isolating directly the soil sample using Cation-exchange resin method. These reports showed that DNA concentrations which have been directly isolated from soil organisms were very much influenced by the soil environment and the isolation method. The environmental condition of the soil was closely related to the capability of microorganisms to live in that environment. In the soil with balance of nutrients, the numbers and activity of soil microorganisms were usually high. The nutrient status of a soil is the major factor affecting the activity of microorganisms, beside water and oxygen (Brock & Madigan, 1991). On the contrary, in polluted soil environment caused by heavy metal, for example, only microorganisms resistant to that contaminant could be found.

Soil samples used in this experiment originated from agricultural soil, thus it could be assumed that the nutritional condition of the soil was quite high. The existence of grasses or cover crops aside from oil palm, could enrich the microflora of the environment. This was assumed to share the role in nutrition supply for soil microorganisms so that their population in the environment was quite high. Beside soil environment and DNA isolation method, the higher concentration of the DNA in this experiment could be due to the presence of eukaryotic organisms or other microorganisms which were not enumerated during the experiment. DNA isolation from a soil sample which contains a high population of organisms will produce DNA with a higher concentration too, because these organisms as a source of DNA are available in large quantity.

In spite of the brown color mixed into DNA could be removed, however, the result showed that DNA purity could not be increased after Prep A Gene DNA Purification treatment. It was assumed that the contaminant, except the color, was closely stuck to DNA and hard to be separated. This contaminant may influence the DNA purity. Steffan *et al.* (1988) and Zhou *et al.* (1996) reported that in direct isolation of DNA from the soil environment, the humic compounds are usually hard to be separated. Although, the addition of hexadecylmethyl-ammonium bromide (CTAB) or polyvinylpolypyrrolidone (PVPP) in buffer extract could decrease humic compound content, but could not remove all contaminants. The difficulty in separating humic compounds from DNA might be closely related to the structure of the compound.

Figure 1 illustrates the ethidium bromide-stained DNA extracted directly from soil. The largest DNA was approximately 23 kb, and most DNA was in the size between 9 – 23 kb. Thus, the environmental DNA isolation method proved to be quite effective to obtain the large size of DNA fragments. In general, direct DNA isolation method from soil which is currently available could result

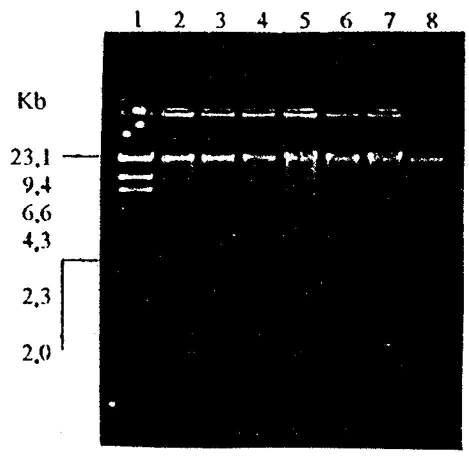

Figure 1. The electrophoresis of total DNA from soil samples. Column 1, DNA λ *Hind*III as molecular size marker;Column 2 - 8 Prokaryotic DNA in soil samples

in DNA sizes ranging between 6.5 – 23.0 kb (Tsai & Olson, 1991; Zhou *et al.*, 1996). Another researcher, however, reported that the size of DNA fragments could reach 48 kb when DNA was isolated directly from the soil environment (Holben *et al.*, 1988).

Probe for DNA hybridization

The result of pHF 1.1 plasmid digested with *Bst*EII restriction enzyme is shown in Figure 2. The 2.2 kb fragment contain the gene encoded 16S-rRNA and a part of 23S-rRNA from *P. aeruginosa* (Schleifer *et al.*, 1985). This fragment was used as a probe to examine the presence of prokaryotes in the soil samples. The sensitivity of 16S-rRNA as a probe could be expected, because rRNA are abundant in the cell, i.e. more than 80% of available total RNA (Griffiths *et al.*, 1993). Even though the rRNA sequence in a certain part of each organism could change its primary structure, however, it has been

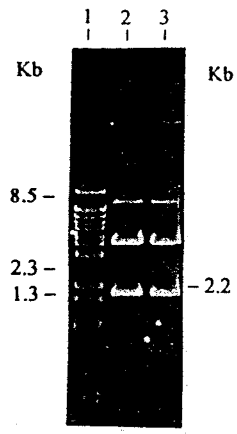

Figure 2. Restriction profile of pHF 1.1 plasmid, digested with *Bst*EII. (1) λ DNA/*Bst*EII as a molecular size standard (2) and (3). pHF 1.1 plasmid digested with *Bst*EII.

known that homologous secondary and tertiary structures were constantly maintained (Gutell *et al.*, 1994). Therefore, the sensitivity and specificity of 16S-rRNA as a heterologous probe could be expected.

Effects of impurities on DNA dot blot *and analysis*

To determine the ability of 16S-rRNA in detecting the presence of similar genes in the target DNA, crude DNA samples were subjected to dot blot analysis. The DNA isolated from a pure culture of *Rhodobacter sphaeroides* 2.4.1 and DNA from salmon sperm were used as positive and negative control respectively. The dot blot analysis allows crude DNA to be fixed onto nylon membrane and subsequently used in the hybridization experiment to detect the DNA sequence of interest. The result showed that the intensity of the hybridization signals between crude DNA samples and pure culture were not different. This result indicated that the crude DNA could be used as a target sequence in dot blot analysis without further treatment to purify DNA.

Figure 3 shows the result of dot blot hybridization between crude DNA from soil samples as target DNA and 16S-rRNA gene as a probe. The intensity of some hybridization signals was quite strong, as strong as the signal resulting from positive control (+) where the DNA was isolated from the pure culture. It showed that the contaminant did not affect hybridization results. It is not clear whether this matter is due to DNA blotting method or to kind and concentration of contaminants. In dot blot hybridization, the crude DNA was directly spotted onto membrane. Fixation by heating caused DNA to crosslink with membrane, while the contaminant probably only weakly bound, thus in the washing process it was easily released from the membrane. Moreover, it is well known that the humic compound is a contaminant usually bounded to DNA directly isolated from the soil. Steffan *et al.* (1988) reported that 10 μg of humic acid interfered with the result of hybridization in dot blot analysis. However, if the amount of humic acid was less than 10 μg, it did not influence the hybridization results. Since in this research the hybridization signal could be detected, it was presumed that the amount of the contaminant, especially humic acid, was in the tolerable range.

The hybridization signals indicated the presence of prokaryotes in each soil sample because the 16S-rRNA probe was specific for the prokaryotes. It was observed from the Figure 3 that the diameter of some hybridization signals were different. Since each DNA sample applied to the membrane contained the same volume of DNA, the diameter of the hybridization signal reflected the amount of DNA which was also related to the concentration of DNA, while the DNA concentration was determined by the population density of prokaryote in each soil sample. Therefore, the diameter of hybridization signals reflects the number or the population of prokaryotes in the soil. The large diameter signal indicated that the population of pro-

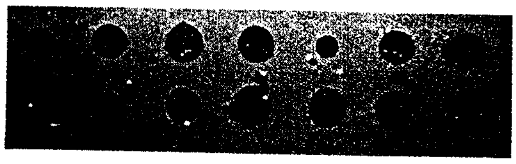

Figure 3. The results of dot blot hybridization of soil environment from oil-palm plantation using 16S rRNA probe. (+), control: DNA isolated from pure culture of *Rb. sphaeroides* 2.4.1 (-), control: DNA salmon sperm, S1 to S6: DNA of soil organism isolated ± 0.5 m from the tree, S7 to S12: DNA of soil organism isolated ± 3 m from the tree

karyote was higher compared with the small diameter signal. Because of the hybridization signals obtained from DNA of all soil samples, although some diameters were different, it was assumed that the prokaryotes were widely distributed in the soil environment with different populations. The presence of prokaryotes in each soil sample could be used as indicator that the analyzed soil contained enough nutrient to support the growth of prokaryotes which also required nutrients other than water and oxygen from the environment (Brock & Madigan, 1991).

Based on the hybridization signals obtained, there were no differences between areas of sampling. The signals could be obtained both at the sampling distances of 0.5 m and 3.0 m from the trees but the population density might be different. Aini (1998) reported that the population density of prokaryote in the soil environment was affected by fertility, texture and condition of the soil. In fertile soil with loose texture and not flooded with water, the activity of microorganism was higher than in clay flooded with water. Although the area at the distance of about 3.0 m from the tree was a relatively open area and received high intensity of light exposure, it did not seem to affect the population of prokaryotes.

Conclusions and Suggestions

1. Total DNA from oil-palm plantation could be obtained by direct DNA isolation. Concentration of crude DNA was approximately 16 – 350 μg/g soil.
2. Dot blot hybridization could be used to detect the existence of prokaryotes.
3. All soil samples used in this study contained prokaryotes, indicating that the prokaryotes were widely distributed but with different population density as shown by the hybridization signals. To determine the quantitative population density, it is necessary to calculate the relative signal's intensity using a Densitometer or Image Analyzer.

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