ISOLATION AND MOLECULAR ANALYSIS OF HUMIC ACID-TOLERANT BACTERIA THAT ARE RESISTANT TO PESTICIDES

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Isolation and Molecular Analysis of Humic Acid-Tolerant Bacteria that are Resistant to Pesticides

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ABSTRACT

Twenty two bacterial strains isolated from peat soils in a pineapple plantation were analyzed. Fourteen of the Gram-negative strains were tentatively classified into 12 genera that included *Thiobacillus, Acinetobacter, Acidiphilium, Enterobacte*r, and *Pseudomonas.* 91% of the isolates were able to tolerate 2,4-dimethylamine with concentrations of up to 10,000 ppm, while 60% of the isolates were resistant to malathion and chlorpyrifos with concentrations of up to 50,000 ppm. As for antibiotics, 82% of the isolates able to survive in carbenicillin agar with concentrations of 1000 ug/ml. 70% of the isolates can survive in both tetracycline and streptomycin media. Four bacterial isolates, C105 (*Acidiphilium cryptum*), C102 (*Sulpholobus spp*), C108 (*Leptospirillum spp*) and Y104 (*Pseudomonas flouresc/putida*) that were pesticide resistant were analyzed for the presence of plasmid. No plasmid was detected in antibiotic resistant isolates except for strains designated as Y103 and C105. The sizes of the isolated plasmids were estimated ranging from 6 kb to 54 kb. Further studies are required to investigate the genes which confer resistance properties in the acidophiles.

Key word: Acidophiles, Pesticide resistance, Antibiotic resistance, Plasmid

ABSTRAK

Dua puluh dua strain bakteria yang dipencil daripada tanah yang berasal dari ladang nenas telah dianalisis. Empat belas Gram negatif bakteria telah diklasifikasikan dalam 12 genera, termasuk Thiobacillus, Acinetobacter, Acidiphilium, Enterobacter, *and* Pseudomonas. *91% daripada strain tersebut adalah rintang kepada 2,4-dimethylamine dengan kepekatan setinggi 10,000 ppm. Sementara, 60% daripada bakteria adalah rintang kepada malathion dan chlorpyrifos masing-masing dengan kepekatan setinggi 50,000 ppm. Di samping itu, 82% daripada strain tersebut adalah rintang terhadap carbenicillin dengan kepekatan setinggi 1000 ug/ml. Sebanyak 70% bakteria pula rintang kepada tetracycline dan streptomycin. Empat strain bakteria, C105 (Acidiphilium cryptum), C102 (Sulpholobus spp), C108 (Leptospirillum spp) and Y104 (Pseudomonas flouresc/putida) yang rintang terhadap racun perosak telah dianalisis untuk menentukan kehadiran plasmid. Tiada strain yang rintang terhadap antibiotic menunjukan kehadiran plasmid kecuali strain Y103 (Aeromonas salmonicida) and C105. Strain-strain tersebut menunjukan kehadiran plasmid dengan saiz antara 6 kb hingga 54 kb. Kajian lanjutan perlu diadakan bagi mengenalpasti gene yang terlibat dalam ciri-ciri rintangan bakteria asidofilik tersebut.*

Kata kunci: Acidophiles, Antibiotik,Racun perosak, Plasmid

INTRODUCTION

Humic acid is one of the components of humic substances in soil, other than fulvic acid and humin (Tate, 1944). Humic acid is not soluble in water under acidic conditions (pH <2) but is soluble at higher pH values. The humic acid/fulvic acid ratio in humus occurs differently according to type of soil. For example, peat soils have higher content of humic acid compared with the humus of forest soils. Because of its complex aromatic macromolecule structure, it provides numerous benefits to crop production. It assists in transferring micronutrients from the soil to the plant, enhances water retention, increases seed germination rates, penetration, and stimulates development of microflora populations in soils.

The surface litters of the peat soils easily generate acidity of the soils and this is the usual habitat for acid-loving microbes, acidophilic organisms. Acidophiles are extremophiles that can survive in a pH lower than five. Acidophiles cannot tolerate acid internally as acids cause damage to the cell. Thus, they secrete special enzymes to protect themselves in acidic environment (Ingledew, 1990). Such enzymes may contribute to commercial applications in many areas. Primarily, acidophilic are occupied by chemolithotropic autotrophs that obtain energy by oxidizing inorganic chemical compounds and use CO_2 as major carbon source. Chemolithotrophs can be found widely in soil and water, which comprised of genera such as *Thiobacillus*, *Sulfolobus, Leptospirillum, Sulfobacillus* and *Acidianus* (Ingledew and Norris, 1992).

The genetic characteristic of selected acidophilic bacteria was investigated by Quentmeier and Friedrich (1994) who found that the antibiotic resistance plasmids in neutrophilic bacteria were transferred into acidophiles, *Acidiphilium cryptum*. Furthermore, Waters and Davies (1997) had found a high incidence of fluoroquinolone antibiotics

resistance in random bacterial isolates cultured from soil. Nevertheless, little is known about the resistance ability of acidophilic organisms to antibiotics and pesticides.

Since 1950s, soon after the introduction of antibiotics into the field of human medicine, the potential for these "miracle drugs" to control plant diseases was recognized. Many kinds of antibiotics such as streptomycin, actidione, griseofulvin and blasticidin have been used for disease treatment and prevention. The extensive usage of antibiotics has encouraged the growth of resistant strains. Bacteria acquire resistance abilities either by mutation or plasmid acquisition. The efficient transfer of R plasmid between bacteria of diverse origin under simulated natural conditions by transformation and transduction indicated that R plasmid can spread among different bacteria strains of human, animal and fish (Hardy, 1981).

According to Salyers and McManus (2001), antibiotic use in the agroecosystem presents unique circumstances that could strongly impact the buildup and persistence of resistance genes in the environment. EPA's National Exposure Research Laboratory stated that in some cases as much as 80 percent of antibiotics administered orally pass through the animal unchanged into bacteria-rich waste lagoons and is then spread on croplands as fertilizer leaving the antibiotics available for entry into ground water and runoff into surface waters carrying both the drugs and resistant bacteria or genetic material to other bacteria in soils and waterways.

Apart from that, the discovery of dichlorodiphenyl trichloroethane (DDT) has convinced people that pest problems can be solved through recourse to extremely toxic chemicals. Subsequently, modern agricultural become seriously addicted to pesticides. There are millions of pounds are globally applied each year with the world market greater than 20 billion dollars (Singh, 1990). The ability of microorganisms to adapt for rapid catabolism of

some soil pesticides has in some case resulted in economically significant pest control failures (Singh, 1990).

Recent findings suggest that people chronically exposed to high pesticide levels are more likely to develop heart disease, serious immunodeficiencies, and cancers of the immune system (non-Hodgkin's lymphoma, leukemia, and myeloma). The continue use of pesticides may develop a strong selection pressure for resistance. If the microorganisms owe their survival to genetic resistance, the whole population may show signs of resistance. As what was stated by Singh (1990), one consideration of the use of soil-applied pesticides is that they should persist long enough in soil to control the target pests, but not so long as to create environmental risks. Nowadays, there are about 1000 major pest species has been detected pesticide resistance.

Bacteria have very high adaptation ability where they can share their DNA with one another in exchanges unrelated to reproduction to allow them resist in toxin environments. Bacteria may acquire resistance plasmid due to the nature of selection pressure. The prolong exposure of the pressure can lead to the resistant form of bacterium become predominant (Hardy, 1981).

Thus, the objective of this study is to identify microorganisms that are tolerant to humic acid and to determine whether the acidophiles are resistant to antibiotics and pesticides used in agriculture. This study is part of large research aimed at determining the microbial profile of peat soils. Such a profile can lead to the identification of the roles played by some microbes in the growth of some agricultural crops.

MATERIALS AND METHODS

1. Materials

a. Antibiotics

Commercial antibiotics' powder tetracycline was obtained from Amresco Company, Malaysia. Its molecular weight is 982 mcg/mg. Streptomycin sulfate was obtained from Life Technologies Company, Malaysia. Its molecular weight is 752 mcg/mg. Carbenicillin disodium salt was obtained from ICN Biomedicals Company, Malaysia. Its molecular weight is 694 mcg/mg.

b. Pesticides

Commercial malathion, chlorpyrifos and 2,4-dimethylamine were obtained from Brighton chemical supplier company, Malaysia. The pesticides were registered by Zeenex (M) Sdn. Bhd., Malaysia under registration number LRMP.R1/4918, LRMP.R1/2503, and LRMP.R1/6893 respectively.

c. Stock Solution

Stock solutions of pesticides and antibiotics as antibacterial agent were prepared fresh. The stock solution of 57% malathion is 570,000 ppm. The stock solution of 21.2% chlopyrifos is 212,000 ppm. The stock solution of 48% 2,4-dimethylamine is 480,000 ppm. The final concentration on the selective media was prepared by pipetting the correct volume of the pesticide from stock solution and mix thoroughly with the MH agar.

The stock solutions for antibiotics were prepared fresh. 0.5 g of antibiotics was added to 100 ml water. Thus, the final concentrations of stock solution were 5 mg/ml. The selective

media was prepared by pipetting correct volume of the antibiotics from the stock solution and mix thoroughly with MH agar.

d. Media

Media	Composition
Nutrient agar (NA) manufactured by Oxoid	'Lab-Lemco' powder 1.0, Yeast extract 2.0, Peptone 5.0, Sodium chloride 5.0, Agar 15.0, pH 7.4
Luria Bertani broth manufactured by Fluka	Trytone 10.0, Yeast extract 5.0, Sodium chloride 5.0, Agar 10.0, pH 7.2
Mueller Hinton agar manufactured by Scharlau	Casein hydrolysate 17.5, Meat extract 4.0, Starch 1.5, Agar 15.0, pH 7.4

Table 1: Types of media used in this study

*All media was autoclaved at 121^{0}C for 20 minutes. For preparing media supplemented with pesticide or antibiotic, the pesticide and antibiotic stock solution was added to the autoclaved media after cooling to 55^{0}C. The mixture was mixed thoroughly before poured onto the sterile petri dish.

*All the cultures were grown at 30^{0}C for 24 hours.

e. Working culture

The working culture of microbial isolates was prepared on slant agar in universal bottle with MH. Single colony of microbial isolates was taken from agar plate and streaked onto slant agar. The cultures were incubated overnight at 30^{0}C and stored at 4^{0}C.

f. Stock Culture

The stock culture of microbial isolates was prepared in Luria Bertani (LB) broth containing 200 ul of pre-sterilised grycerol. The cultures were grown overnight before storage at -20^{0}C or -80^{0}C.

g. Laboratory Strain

Two laboratory strains were used in the experiment, *E.coli* V517 and *E.coli* JM109. *E.coli* V517 was used as a marker for bacterial plasmids. *E.coli* JM109 was used as negative control agents in this study.

h. Sources of sample

Soil samples were collected from a pineapple farm in the Kota Samarahan Division, Sarawak, Malaysia. Samples were taken from soil surface in air-tight containers to avoid the loss of moisture. The samples were stored in 4°C.

2. Methods

a. Isolation of bacteria from peat soil

1g of soil sample was suspended in 9 ml of distilled water and mixed gently. Serial dilution was conducted in 1:10 diluents to reduce the concentration of microorganisms in the sample. 0.1 ml of 10^{-2}, 10^{-3}....10^{-7} dilution were pipetted out and spread on nutrient agar in three replicates. The cultures were incubated at 30°C for 24 hours. The densities of bacteria in 1 gm of soil were calculated by using the appropriate multiplication factor.

A standard curve was drawn to determine the relation of number of cells/ml with optical density. It was used to measure the number of cells/ml of bacteria that grow under different pH from the optical density readings. Laboratory strain *E.coli* JM109 grow overnight on nutrient agar and single colonies were inoculate in LB broth, incubated overnight at 30°C. 0.1 ml of LB broth pipette out and serially diluted in 10^{-1}, 10^{-2}, 10^{-3}.....10^{-8}. 0.1 ml of the dilutions was spread on nutrient agar, incubated overnight for colonies count. The density of the cells in the dilutions was detected by spectrophotometer in 525 nm.

The bacteria which were acid-tolerant were isolated on MH agar with pH adjusted to 4.5. Few colonies were picked randomly from MH (pH 4.5) and inoculated in LB broth with pH adjusted (pH 1-8). The LB broths were incubated overnight to determine the optimal pH for the growth of acidophiles. The density of the cells was detected by spectrophotometer.

Subsequently, 22 colonies were picked randomly from the MH (pH 4.5) to prepared stock culture and working culture for further investigation.

b. Identification of selected bacteria

The isolated bacteria were characterized by examining their morphology, Gram staining, microscopic examination, catalase test and also oxidase test. After tested the resistance properties of the isolated bacteria, API 20E test kit is used for identification.

c. Detection of bacterial resistance properties

The identified bacteria were further analyzed to determine the antibiotics and pesticides resistance properties. Before that, laboratory strain JM109 used as a control in MIC test to determined the minimal concentration of antibiotics and pesticides that inhibit the growth of the laboratory strain.

Lab strain	**Pesticides (ppm)**		
	Malathion	Chlorpyrifos	2,4 –Dimethylamine
	5000	1000	2500
***E.coli* JM 109**	**Antibiotics (ug/ml)**		
	Tetracycline	Streptomycin	Carbenicillin
	250	500	50

Table 2: The MIC level of lab strain

The resistance patterns of isolated bacteria were screened by subculturing the bacteria onto media that supplemented with different concentration of antibiotics and pesticides respectively. The screening was done in triplicate.

d. Detection and Isolation of bacteria plasmid

Alkaline Lysis Miniprep described by Birnboim, *et al* (1979) is the most commonly used of laboratory technique to isolate plasmid from bacteria. This method was employed in this experiment with some modifications. Single colonies from the MH agar (pH 4.5) were picked and inoculate in LB broth. The cultures were grown at 30°C on rotary shaker operating at 200 rpm overnight. The lab strain *E.coli* V517 was cultured in LB broth and shake overnight at 200 rpm at 37°C.

e. Gel Electrophoresis

The method described by Sambrook, *et al* (1989) was used in this experiment with some modifications. 0.5% agarose gel was used and 80 volts was set for electrophoresis in 2 hours. 10 ul of plasmid marker was loaded in the first well with 2 ul 6x loading dye. The subsequent wells were loaded with 15 ul of plasmid sample with 3 ul 6x loading dye. After the electrophoresis, the gel was stained with 1 ug/ml of ethidium bromide solution for 15 minutes. Then the gel destained with sterile water. The gel was visualized under Fisherbrand UV transilluminator. The gel was photographed with photodocumentation camera.

RESULTS

a. Isolation of bacteria from peat soil

There were 22 strains isolated from two soil samples after screening on MH agar with pH adjusted to 4.5. The 22 strains were designated as C100 to C110, and Y100 to Y110. All of the colonies produce in white yellowish color on MH agar. They appeared in entire and circular forms.

The density of bacteria in 1 gram of soils growing on NA agar and MH agar (pH 4.5) were compared. The density of bacteria grown on MH agar (pH 4.5) was much less than on NA agar. This suggested that there were only particular bacteria from the peat soil which could survive on pH lower than 5.

The standard curve below showed the correlation between the numbers cells/ml (log) to absorbance (OD) which produce a linear plot (Figure 1). Thus, the number of colonies increases with the increase of OD. 5 replicates were done on the acid tolerance bacteria and the optimal pH for the growth of bacteria was from pH 4 to 7.

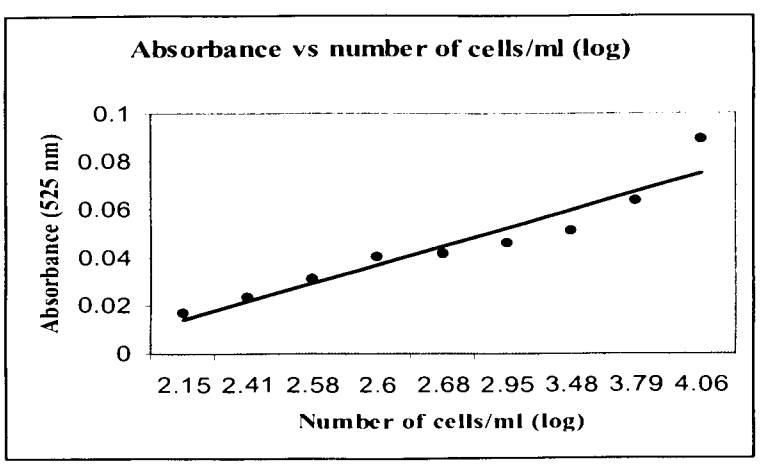

Figure 1: The relationship of number of cells/ml (log) and absorbance

b. Identification of selected bacterial strains

The morphology of the isolates was examined, only 14 out of 22 isolates were gram-negative. Four appeared as cocci form and ten were in rod form. Eight of the other isolates were Gram-positive. Most of the Gram-negative strains were oxidase positive, except for strain C100, C101, C102, C108, C109 and Y106 were oxidase negative. 6 of the Gram-negative strains C105, Y100, Y101, Y103, Y105, and Y109 were catalase negative while the others 8 strains were catalase positive.

The identification of the isolates was tentatively determined by API 20E kit. The results are shown in Table 3. Some strains were not able to be identified because API 20E kit only can identify Gram-negative bacteria.

Designation	**Bacteria strain**
Y100	*Sphmon. paucimobilis*
Y101	*Thiobacillus intermedius*
Y103	*Aeromonas salmonicida*
Y104, Y106	*Pseudomonas flouresc/putida*
Y105	*Flavi.oryzihabitans*
Y109	*Enterobacter spp*
C100	*Acinetobacter spp*
C101	*Chryseomonas luteola*
C102	*Sulpholobus spp*
C105	*Acidiphilium cryptum*
C108	*Leptospirillum spp*
C110	*Serratia rubidaea*

Table 3: The results of API 20E test

c. Detection of resistance properties

The concentrations of pesticides and antibiotics used in the screening were showed in Table 4 and 5.

Pesticide	Concentration in ppm
Malathion	5000-10,000-20,000-30,000-50,000
Chlorpyrifos	1000-5000-10,000-20,000-50,000
2,4- Dimethylamine	2500-3500-4500-5000-10,000

Table 4: Concentration of pesticides (ppm) that were applied in the study

Antibiotic	Concentration in ug/ml
Tetracycline	250 – 500 –1000 – 2000 – 3000 –5000
Streptomycin	500 – 1000 – 2000 – 3000 – 5000 – 7500
Carbenicillin	50 – 100 – 250 – 500 – 1000 - 2000

Table 5: Concentration of antibiotics (ug/ml) that were applied in the study

The number of isolates that are resistant to pesticides and antibiotics are showed in percentage in Figure 2:

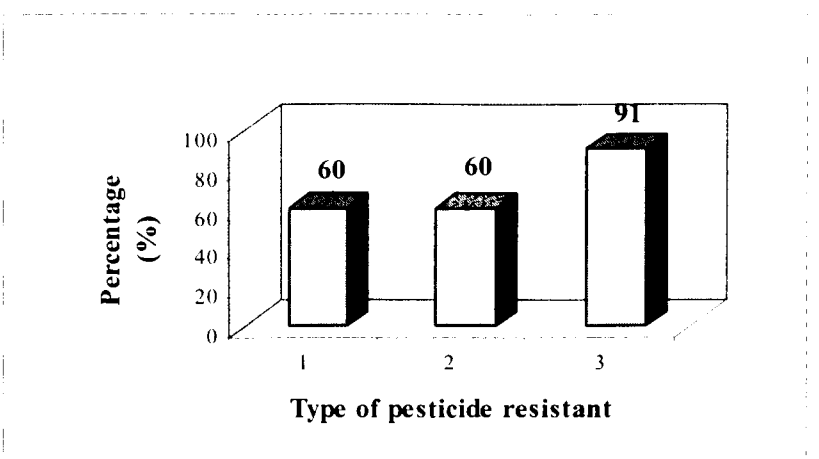

Figure 2: Percentage of pesticide resistant

*M^R = Malathion resistant, C^R = Chlorpyrifos resistant, D^R = 2,4-Dimethylamine resistant

Figure 2 showed that 91% of the isolates were able to survive in 2,4-dimethylamine. 60% of the isolates resistant to malathion and chlorpyrifos. The highest concentration of malathion and chlorpyrifos that the isolates can survive is 50,000 ppm. While for 2, 4-dimethylamine it is only 10,000 ppm.

Pesticides resistance patterns	Isolates
$M^RC^RD^R$	C105,Y100,Y101,Y103,Y105,Y106,Y107,Y108,Y109
M^RD^R	C101,C104,C108,C109
C^RD^R	C106,Y104
C^R	C107,Y102
D^R	C100,C102,C103,C110,Y110

Table 6: The pesticides resistance patterns of the isolates

*M^R = Malathion resistant, C^R = Chlorpyrifos resistant, D^R = 2,4-Dimethylamine resistant

The pesticides resistance patterns of all the isolates were showed in Table 6. The strains that can survive in the highest concentration of the three pesticides were C105 and Y109. While for strain Y110 was only resistant to 2, 4-dimethylamine at 5000 ppm. Strain C107 was only resistant to chlorpyrifos at 5000 ppm.

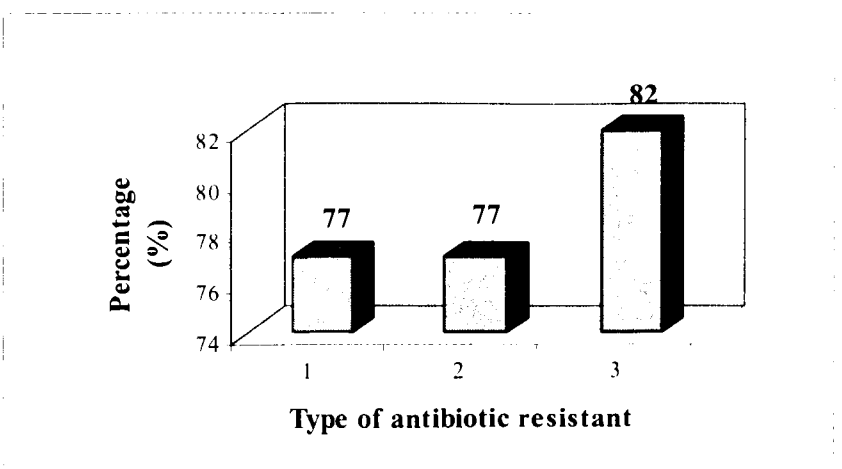

Figure 3: Percentage of antibiotic resistant

T^R = Tetracycline resistant, S^R = Streptomycin resistant, C^R = Carbenicillin resistant

Figure 3 also shows that 82% of the isolates were able to survive in carbenicillin medium. 77% of the isolates survived on both tetracycline and streptomycin. The highest concentration of tetracycline that the isolates can survive in was 5000 ug/ml. For streptomycin it was up to 10,000 ug/ml while for carbenicillin it was only 1000 ug/ml.

Antibiotics resistance patterns	**Isolates**
$T^RS^RC^R$	C101,C103,C105,C108,C110,Y100,Y101,Y103,Y104, Y106,Y109
T^RS^R	Y110
T^RC^R	C104,Y107,Y108
S^RC^R	C100,C102,C109,Y105
T^R	C107,Y102
S^R	C106

Table 7: The antibiotics resistance patterns of the isolates

* T^R = Tetracycline resistant, S^R = Streptomycin resistant, C^R = Carbenicillin resistant

The antibiotic resistance patterns of all the isolates are shown in Table 7 .The strains that can survive in all three antibiotics in high concentration were Y100, Y101 and Y104. Strain C106 was resistant only to streptomycin in low concentration, 250 ug/ml. While, strains Y107 and Y102 was resistant to tetracycline in 250 ug/ml.

d. Detection and Isolation of bacteria plasmid

The screening of resistant bacterial strains for extrachromosomal DNA was conducted through the alkaline lyses technique (Birnboim *et al*, 1979) with some modifications. The presence of four plasmids was demonstrated in pesticide resistant strains designated as C105, C102, C108 and Y104 (Figure 4). Strains C105 was resistant to three of the pesticides in high concentration, while strain C102 is only resistant to 2,4-dimethylamine. Strain C108 was resistant to malathion and 2,4-dimethylamine in high concentration with 20,000 ppm and

4500 ppm respectively. Strain Y104 was resistant to chlorpyrifos and 2,4-dimethylamine with 10,000 ppm and 4500 ppm respectively.

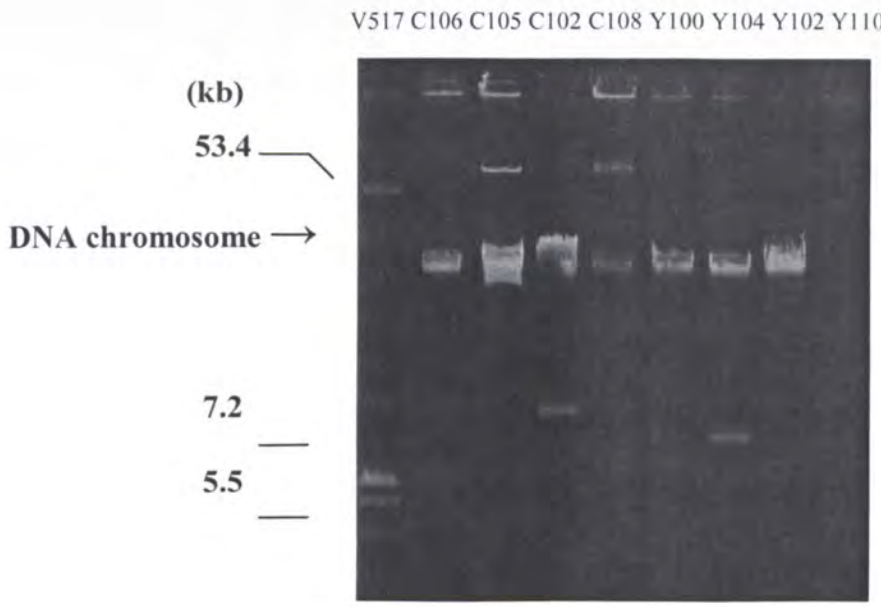

Figure 4: Agarose gel electrophoresis of plasmid DNA isolated from pesticides resistant isolates.

As for isolates that were resistant to antibiotics, two of them had shown the presence of plasmids. The strains were Y103, and C105. Strains C105 and Y103 were resistant to three of the antibiotics with the same concentrations i.e. tetracycline (1000 ug/ml), streptomycin (10,000 ug/ml) and carbenicillin (500 ug/ml).

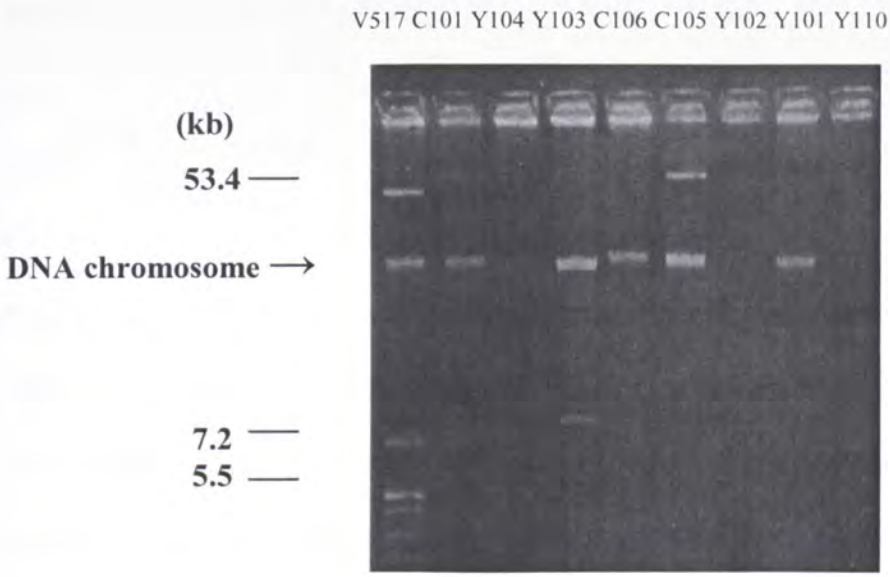

Figure 5: Agarose gel electrophoresis of plasmid DNA isolated from antibiotics resistant isolates.

Designation	Bacterial strains	Pesticides resistance patterns	Antibiotics resistance patterns
C102	*Sulpholobus spp*	D^R	S^RC^R
C105	*Acidiphilium cryptum*	$M^RC^RD^R$	$T^RS^RC^R$
C108	*Leptospirillum spp*	M^RD^R	$T^RS^RC^R$
Y104	*Pseudomonas flouresc/putida*	C^RD^R	$T^RS^RC^R$
Y103	*Aeromonas salmonicida*	$M^RC^RD^R$	$T^RS^RC^R$

Table 7: Resistance patterns of bacterial strains that showed the presence of plasmid.

*M^R = Malathion resistant, C^R = Chlorpyrifos resistant, D^R = 2,4-Dimethylamine resistant

* T^R = Tetracycline resistant, S^R = Streptomycin resistant, C^R = Carbenicillin resistant

DISCUSSION

The aim of this study was to isolate and characterize bacterial isolates from peat soils, which contained humic acid. Most of the bacterial isolates were oxidase and catalase positive. Catalase positive indicated that the bacterial isolates were aerobes or facultative anaerobes as well as microaerophiles. While oxidase positive indicated that the bacterial isolates were among the members of genera *Neisseria* and *Pseudomonas* (Cappucino and Sherman, 1996). For instance, strainY104 was identified as *Pseudomonas flouresc/putida* by API 20E kit was oxidase and catalase positive.

The bacterial isolates were subsequently screened for their resistance properties to pesticides and antibiotics. According to Karns (1990), the increased number of resistant bacterial strains in soil was due to the elimination of the sensitive organism or the acquisition of plasmid after prolongs exposure to toxic chemicals. One of the key factors in the metabolic adaptability of microorganisms is related to the possession and utilization of plasmids (Singh, 1990).

There were four bacterial isolates, C105 (*Acidiphilium cryptum*), C102 (*Sulpholobus spp*), C108 (*Leptospirillum spp*) and Y104 (*Pseudomonas flouresc/putida*) that carried pesticide resistant genes although some of their resistance levels were not very high. This may be due to the fact that the stress factors were not the major reasons for the resistance or the plasmid frequency reduction (Wichkham *et al*, 1988). Strains C102, C105, C108 and Y104 were found to carry plasmids. Strain C105 has multi-resistant characteristics, being resistant to both pesticides and antibiotics. Another antibiotic resistant strain that showed the presence of plasmid was Y103 (*Aeromonas salmonicida*). The size of plasmid present in strains C105 and C108 were estimated to be more than 53.4 kb. The size plasmid for strain Y103 was

approximated 10 kb, while the plasmid for C102 was 7.2 kb and for Y104 was estimated at 6 kb. It is not known whether any of the pesticide or antibiotic resistance genes are located on the plasmids that have been identified.

Studies have shown that some wild-type strains of *Aeromonas salmonicida* isolated from diseased fish showed resistant to multiple low levels of antibiotics (Wood *et al*, 1986). MIC test showed that its resistance to streptomycin was more than 1,000 ug/ml and for tetracycline was more than 60 ug/ml. In this study, the MIC results for strain Y103 was 250 ug/ml for tetracycline and for streptomycin was 2,000 ug/ml which slightly higher than previous studies. This probably due to possibility that the soil bacteria were more exposed to antibiotics compared to the bacteria in fish.

From all the results that had presented here, most of the isolates showed resistance towards pesticides and antibiotics. The resistance characteristic may depend upon the stability of the degradative plasmids that carry the resistance gene. According to Stapleton *et al.* (1998), the microbial degradation of aromatic hydrocarbon compounds could occur in the extremely low pH of environments. This finding was supported by the involvement of degradation plasmids in the acidophiles. In addition, the study also suggested that the biodegradation in acidic environment may require complex interaction of the microbial community.

Although some bacterial strains showed high levels of resistance during the screening process, but no plasmid was obtained from the isolates. These negative results were probably due to the resistance gene being located on the chromosome instead of plasmid. Besides, some of the bacteria plasmids that are too big in size may not be able isolated by miniprep alkaline lyses method. The large plasmid are prone to shearing and lost during the isolation process.

Plasmids from Gram-negative bacteria especially *Pseudomonas* can have size more than 150 kb (Hardy, 1981).

Further investigation to obtain more detailed results cannot be carried out due to the limitation of time. Transformation can be conducted on the bacterial strains which showed the presence of plasmid. This is to determine whether the plasmids are transmissible or vice versa. Positive results may indicate that the resistance mechanisms of the strains are caused by gene presence in those plasmids. While negative results may be because of the plasmid not transmissible or the resistance gene that is located on the chromosomal DNA.

CONCLUSION

Nine out of the 22 bacterial isolates were resistant to three of the pesticides and 4 other strains (C101, C104, C108 and C109) were resistant to malathion and 2,4-dimethylamine. Strains that are resistant to chlorpyrifos and 2,4-dimethylamine were C106 and Y104. Strains C107 and Y102 were only resistant to chlorpyrifos at 5000 ppm and 1000 ppm respectively. While the last 5 strains (C100, C102, C103, C110 and Y110) were only resistant to 2,4-dimethylamine. Only isolates C105, C102, C108 and Y104 showed the presence of plasmids.

As for antibiotic resistance screening, 11 of the total 22 isolates were resistant to all of three antibiotics. The other 4 strains (C100, C102, C109 and Y105) were resistant to streptomycin and carbenicillin. Three isolates (C104, Y107 and Y108) were resistant to tetracycline and carbenicillin. Strains Y110 was resistant to only tetracycline. While the last two strains (C107, Y102) and C106 were resistant only to tetracycline and streptomycin respectively. Only two isolates, Y103 and C105 showed the presence of plasmid.

SUGGESTION

Further investigation should be conduct by using probe to detect the genetic factors that caused the resistance properties to the acidophiles. Probes for gene coding for enzyme which likely involved in the resistance gene should be useful for detecting the gene in the bacteria. These specific probes even could be utilized to trace the particular resistance gene that probably spread to other soil microbial community. Subsequently, the resistance genes could be utilized in biotechnological application.

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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A, and Struhl, K. 1992. Vectors derived from plasmids. *In*: Short protocols in molecular biology, 2nd Edition. Ed: Brooklyn, NY. Greene Pub. Associates, New York. P 16-24

Birnboim,H.C., and Doly, J.. 1979. A rapid alkaline extraction method for screening recombinant plasmid DNA. *Nuclear Acids*.**7**:1513-1523

Chaube, H. S., and Singh, U. S. 1991. Chemical control. *In*: Plant disease management: Principles and practices. Ed: Boca Raton. CRC Press. P 293

Cappuccino, J. G., and Sherman, N. 1996. Biochemical activities of Microorganisms. *In*: Microbiology: A laboratory manual. The Benjamin/Cummings Publishing Company, Inc. P 129-183

Demaneche, S., Kay, E., Gourbiere, F., and Simonet P. 2001. Natural transformation of *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* in soil. *Applied Environmental Microbiology*. **67**:2617-2621

Eberhard W.G. 1990. Evolution in bacterial plasmids and level of selection. PubMed, National Library of Medicine. **65(1)**:3-22

Guha, A., Kumari, B., Bora, T.C., and Roy, M.K. 1997. Possible involvement of plasmids in degradation of malathion and chlorpyrifos by *Micrococcus sp. Folia Microbiology*. **42**: 574-576

Hardy, K.G. 1981. *Bacterial plasmid.* American Society for Microbiology. Washington, D.C.

Henis,Y. 1987. *Survival and dominancy of Microorganism.* John wiley & Sons, Inc, Canada. P32

Ingledew, W. J. 1990. Acidophiles. *In:* Microbiology of extreme environments. Ed: C. Edwards. Open University Press. P 33-54

Ingledew, W. J., and Norris, P. R. 1992. Acidophilic bacteria: adaptations and applications. *In*: Molecular biology and biotechnology of extremophiles. Ed: Glasgow. Blackie. P 115-14

Johnson, D.B. 1998. Biodiversity and ecology of acidophilic microorganisms. *Microbiology Ecology*. **27**: 307-317

Karn, J.S. 1990 Molecular genetics of pesticides degradation by soil bacteria. In *Enhanced biodegradation of pesticides in the environment*. Ed: Racke, K.D. and Coats J.R. Ameran Chemical Society, United Stated of America. P141-152

Kruse, H. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Applied Environmental Microbiology*. **60**: 4015-4021

Lipthay, J. R., Tuxen N., Johnsen, K., Hansen, L.H., Albrechtsen, H., Bjerg, P.L., and Aamand J. 2003. In situ exposure to low herbicide concentrations affects microbial population composition and catabolic gene frequency in an aerobic shallow aquifer. *Applied Environmental Microbiology*. **69**: 461-467

Mallick, K., Bharati, K., Banerji, A., Shaki, N.A., and Sethunathan, N. 1999. Bacterial degradation of chlorpyrifos in pure cultures and in soil. *Bulletin of Environmental Contamination and Toxicology*. **62**:48-54

Metting, F.B. 1993. *Soil microbiology ecology*. Jr. Marcel Dekker, Inc. New York. P66-68

Penn, C. 1991. Growth of microorganisms-theoretical aspects. *In:* Handling laboratory microorganisms. Ed: Milton Keynes. Open University Press. P 62-78

Quentmeier, A., and Friedrich, C. G. 1994. Transfer and expression of degradative and antibiotic resistance plasmids in acidophilic bacteria. *Applied Environmental Microbiology*. 973-978

Salyers, A. A., and McManus, P. 2001. Possible impact on antibiotic resistance in human pathogens due to agricultural use of antibiotics. *In*: Antibiotic Development and Resistance. Ed: D Hughes, and D Anderson. Taylor & Francis, London. P 137-154

Sambrook, J., Fritsch, E.F., and Maniatic, T. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor, Laboratory Press. P 252-262

Singh, R. S. 1990. Plant disease control. *In*: Plant Disease, 6th Edition. P 553-576

Singh, B. K., Walker, A., Morgan, A. W., and Wright, D. J. 2003. Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. *Applied Environmental Microbiology.* **69**: 5198-5206

Stapleton, R. D., Savage, D.C., Sayler, G.S., and Stacey G. 1998. Biodegradation of aromatic hydrocarbons in an extremely acidic environment. *Applied Environmental Microbiology.* **64**: 4180-4184

Sneath, P.H.A., Mair, N.S., Sharpe, M.E., and Holt, J.G. 1986. *Bergey's manual of systematic bacteriology.* Williams and Wilkins. Vol.2. P 23-67

Summers, D.K. 1996. *The biology of plasmids.* Hartnolls Ltd, Bodmin,Cornwall. P 2-7

Tate, R. L. 1944. *Soil microbiology. 2nd Edition.* John Wiley & Sons, Inc. P 20-24

Waters, B., and Davies, J. 1997. Amino acid variation in the GyrA subunit of bacteria potentially associated with natural resistance to fluoroquinolone antibiotics. *Antimicrobial Agents Chemotherapy.* **41**:2766-2769.

Wickham, G.S. and Atlas, R.M. 1988 Plasmid frequency fluctuation in bacterial populations from chemically stressed soil communities. *Applied Environmental Microbiology.* **54**: 2192-2196

Wood, S.,C., McCashion, R.,N., and Lynch, W., H. 1986. Multiple low-level antibiotic resistances in *Aeromonas salmonicida. Antimicrob Agents Chemotherapy.* **6**: 992–996