Journal of Coastal Development ISSN: 1410-5217
Volume 6, Number 3, June 2003 : 127-133 Accredited: 69/Dikti/Kep/2000

Original paper

CHARACTERIZATION AND IDENTIFICATION OF STRAIN KM221, A NOVEL MCPA HERBICIDE-DEGRADING BACTERIUM ISOLATED FROM CORAL SURFACE, MENJANGAN KECIL ISLAND, KARIMUNJAWA

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Received: April, 1, 2003 ; Accepted: May, 12, 2003

ABSTRACT

In this study, bacterial strain KM221 was isolated from coral tissue in Menjangan Kecil Island, Karimunjawa, Indonesia. This strain is facultative anaerobic with MCPA (2-methyl-4-chlorophenoxy acetic acid) serving as the only known energy sources. Microscopy of isolate revealed that strain KM221 is gram-positive, catalase-positive, rod, spore-forming bacterium, motile, opaque, hair-like outgrowth and unpigmented colonies. The bacterium could not be identified on the basis of its carbon-source-utilization pattern, but a partial sequencing of the 16S rDNA analysis suggest that this strain is closely related to Bacillus iodinum.The ability to degrade MCPA herbicide was examined qualitatively in EMBA indicator medium. This bacterium grew exponentially with MCPA as the sole source of energy and carbon. The maximum growth rate (μ_{max}) and the saturated concentration on MCPA (C_s) were determined to be 0.8024 h^{-1} and 5.10 mg/l MCPA, respectively.

Key words: MCPA, 16S rDNA, *Bacillus iodinum*, coral

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INTRODUCTION

The use of pesticides, herbicides and fungicides in Indonesia began when the government launched intensification programme in the 1960's. Consequently, large-scale application of these toxic mate-rials in agriculture areas can contribute to the presence of those compounds in surface and ground water, lakes, estuary and ultimately in the coastal areas. Most of these compounds are recalcitrant to biodegradation, and their entry into the sea, might be, posing many challenges to the existing coral

reefs and even to live in this vicinity in general. There is a lack of information available on pesticide effects to the coral reefs. Glynn *et al.* (1984) reported that herbicides can have a deleterious effect on corals, at relatively low concentrations and for short term.

Phenoxyalkanoic acids such as 4-chloro-2-methylphenoxyacetic acid (MCPA) are widely used in agriculture and forestry as herbicides against dicotyle-donous weeds. Trade names for some products containing MCPA are Agritox, Agroxone, Chiptox, Rhonox and Weed-Rhap (Anoname, 1993). Like many other xenobiotic compounds, MCPA is not readily decomposed in nature. The decomposition of MCPA in soil has been reported to be slow (Thorstensen and Lode, 2001). Since these toxic chemicals are made and used every year in large quantities, effective handling of their production wastes and the contaminated environment are required. Phenoxyal-kanoic acids-degrading bacteria from different genera, such as, *Alcaligenes* (Don and Pemberton, 1985), *Flavobacterium* (Chaudry and Huang, 1988) and *Pseudomonas* (Bhat *et al*, 1994) have been reported. However, the majority of those genera were isolated from soils, sludge, waste water and lake water. No reports were made on MCPA-degrading bacteria isolated from coral surfaces. Mineralization of MCPA involved side chain removal, hydroxylation of the resulting 4-chloro-2-methylphenol, opening of the 2-methyl-4-chlorocatechol ring, and subsequent conversion of 4-chloro-2-methylmuconate to succinate, which then metabolized by the cells (Bachmann, 1999).

Although it is evident from several studies that MCPA can be degraded by bacteria, little is known about the kinetic reaction and the environmental conditions needed for the reactions to occur. So, MCPA degradation in different systems, such as soil and marine, are difficult to predict and control. The aim of the present investigation was to isolate, characterize and identify a bacterium able to degrade MCPA as well as the growth rate and substrate depletion of this bacterium when growing on MCPA.

MATERIALS AND METHODS

Bacterial Isolation

Corals representing different life forms (massive, sub-massive, branching and foliose) were collected from Karimun-jawa islands, North Java Sea by using scuba diving, and were put on small sterile plastic bag (Whirl-Pak, USA) and stored on ice and immediately brought to laboratory at the Marine Station of Diponegoro University. Coral mucus were collected by scrapping coral surface and put into sterile beaker glass containing 90 ml sterile seawater. One ml of this dilution was transferred into a test tube containing 9 ml sterile seawater and shaken for homogeneity. Dilution series were then be prepared and 100 l of diluted sample spreaded onto Zobell 2216 E medium and incubated for 48 hours in room temperature**.** Based on the morphological features, each colony was streaked on to ZoBell 2216E solid medium and transferred several times until pure isolate is obtained.

Screening Test of MCPA Degradation

The coral associated-bacterial isolates were tested for their ability to qualitatively degrade pesticides using EMBA media indicator (Loos, 1975). Isolates were streaked on the surface of ZoBell medium containing 100 ppm MCPA and incubated for 24 hours and observed by the color change of colonies into red.

In order to assess quantitatively the effects of MCPA, the MCPA positive isolates were grown in a liquid Zobell 2216E that was amended with 0.02 % yeast extract, after which the pH was adjusted to 7.6. The sterilized stock solution of MCPA was added to the desired concentration (80

ISSN: 1410-5217
Accredited: 69/Dikti/Kep/2000

ppm) after autoclaving. Liquid media were inoculated with bacteria and were incubated at room temperature while shaking at 700 rpm. Degradation of the MCPA in representative acid-producing cultures was confirmed by diluting culture samples after centrifugation (1 : 2) with distilled water and determining their ultraviolet absorption spectra on a Beckman DB recording spectrophotometer over the wavelength range 200 to 310 nm. Acid production was invariably associated with partial or complete disappearance of the 228-nm MCPA absorption peak. Growth rate was measured with UV-vis spectrophotometer OD_{600}. The best isolate was selected and used for further study on kinetic growth and MCPA degradation.

Kinetic constants regarding the growth of microorganism on MCPA was determined by cultivating the bacterium in a 50 ml Erlenmeyer. Different concentrations of MCPA were directly added to the Erlenmeyer containing Zobell 2216E culture medium and the specific growth rate was determined by measuring the optical density of the culture during the exponential growth phase.

Microscopic and Biochemical Charac-terization

All cells used in microscopic characterization were grown in Zobell 2216E medium. The morphologies of selected isolate were determined from photomicrograph. Gram staining, motility and the presence of spores were performed using Cappuccino and Sherman method (1987). Biochemical characterizations were determined based on method of Atlas (1993).

Sequencing of PCR-amplified 16S rDNA

Primers (20 F; position 8 to 27 and 1500 R; position 1510 to 1492 of *E. coli* 16S rRNA numbering) described by Weisburg *et al.* (1991) were used for PCR amplification. PCR amplification was carried out in a thermal cycler (Mini Cycler TM; MJ Research Inc., Watertown, MA, USA) with the following temperature profile: an initial denaturation at 94 ^{o}C for 2 min; 25 cycles of denaturation (2 min at 94 ^{o}C), annealing (1.5 min at 45 ^{o}C), and extension (2 min at 72 ^{o}C), and final extension at 72 ^{o}C for 3 min. Amplified DNA was examined by horizontal electrophoresis on 1.0 % agarose gel in TAE electrophoresis buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA) with 1 l aliquots of PCR product.

Sequencing was conducted as previously described by Urakawa *et al.* (1999). The PCR product was purified and concentrated with Microcon-100 microconcentrators (Amicon, Beverly, MA, USA) according to manufacturer's instructions. Sequencing was carried out with a SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) and an automated sequencer (Pharmacia LKB Biotech, Uppsala, Sweden).

RESULTS AND DISCUSSION

Isolation and Screening of MCPA-Degrading Strains

One hundred seven bacterial isolates which utilize MCPA as sole source of carbon and energy were obtained from 337 enrichment cultures. Screening of MCPA-degrading bacteria was carried out by selecting the red colour colonies. Colonies which produced relatively little or no acid fail to mobilize the dyes and remain colorless. Loos (1975) stated that many pesticide molecules contain chlorine which may be released as chloride ions by the metabolic action of microorganisms. Chlorinated pesticide breakdown is shown by the reaction of indicator dyes. However, the indicator action depends on mobilization of the dyes at low pH and their movement into acid-producing colonies.

All isolates were capable of growth at the expense of MCPA (80 mg/l) as evidenced by the decrease of UV-absorbing compounds (200 to 310 nm) and by an increase in biomass produced during passages through Zobell 2216E containing only MCPA as a carbon source. The 107 isolates were sorted into two groups based on the biomass product and the % MCPA degradation. Strain KM221 was selected which have the highest capability of degrading MCPA compounds and biomass production.

Degradation of MCPA

The degradation of MCPA by strain KM221 was carried out in batch operation. As can be seen in **Figure 1**, the concentration of MCPA decreased gradually with microbial growth. It is likely that MCPA was effectively utilized by the bacterium and biomass concentration was not significantly decreased even after the stationary phase.

The specific growth rate obtained was plotted against MCPA concentration and the maximum growth rate (μ_{max}) and the saturated concentration on MCPA (C_s) were estimated to be 0.8024 h^{-1} and 5.10 mg/l MCPA, respectively (Figure 2).

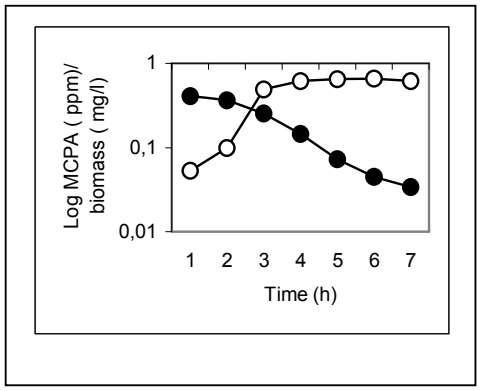

Fig. 1. Growth behaviour of strain KM221 and MCPA depletion in batch culture (o: biomass formation ; ● : MCPA concent-ration)

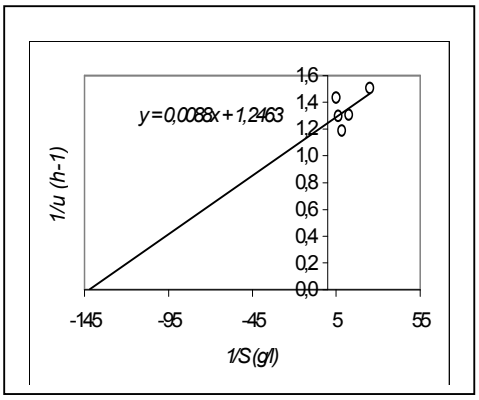

Fig. 2. Relationship between specific growth rate and MCPA concentrations

The metabolism of xenobiotic compounds have been shown to be carried on the plasmids (Don and Pemberton, 1985; Bhat *et al.,* 1995; Sabdono *et al*., 2000; Chaudry and Huang, 1988), the ability of the host organisms to degrade target xenobiotic compounds may depend upon the stability of the degradative plasmids or their genes. In case of MCPA-degrading bacterium, its degradative traits and plasmids may not be stable. Isolate that was originally capable of utilizing MCPA as a sole source of carbon did not grow on MCPA, if it was used as the only carbon source, in transfers subsequent to the initial isolation. This suggestion is supported by the observation that this strain is unable to degrade MCPA completely. Slow to degrade MCPA was shown when MCPA concentration obtained 5.1 ppm. It is possible that the expression of the MCPA degradation genes may require inducer molecules that may be produced by other bacteria of the mixed culture.

ISSN: 1410-5217
Accredited: 69/Dikti/Kep/2000

Phenotypic properties of strain KM221

Microscopy bacterium revealed that strain KM221 is rod, motile and gram-positive (**Figure 3**).

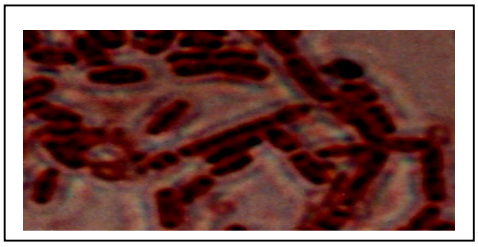

Fig. 3. Photomicrographs of strains KM221

On solid MCPA-Zobell 2216E agar the strain forms hair-like outgrowths, filamentous, colorless and opaque colonies. Liquification of gelatine and degradation of starch could be detected positively. Catalase reaction was positive. Nitrate was reduced under aerobic and anaerobic conditions. Good growth was observed on the following carbon sources : glucose, maltose and fructose. On the other hand, the following features were negative on lactose, sucrose, mannitol, inositol, xylose and galactose (**Table 1**).

Table 1. Characteristics of Strain KM221

Characteristic	Strain KM221
Morphology	Rod
Spores	+
Motility	+
Gram stain	+
Flagella	None
Catalase	+
Pigment	Colorless
Growth by hydrolysis of:	
Starch	+
Lipid	-
Casein	+
Gelatine	+
Growth by fermentation of:	
Lactose	-
Succrose	-
Glucose	+
Maltose	+
Mannitol	-
Inositol	-
Xylose	-
Galactose	-
Fructose	+
Growth on :	
Metl Red	+
Voges-Proskauer	-
Indole	-
Phosphate	+
Simon's Citrate	-
H2S	-

Urease	-
Catalase	+
Nitrate reduction	+
Amonification	-

Previously most research has focused on the selection and isolation of microbial communities which grow on halogenated compounds under aerobic conditions. Some Gram-negative bacteria, belonging to the *Protobacteria*, have been described in the publications that utilize chlorinated phenoxyalkanoic acids and their chlorophenol derivatives, i.e. *Alcaligenes eutrophus* JMP 134 (Don and Pemberton, 1985), *Pseudomonas* sp. (Bhat *et al.,* 1995), *Vibrio natriegens* PP202 (Sabdono *et al*., 2000), *Flavobacterium* sp. (Chaudry and Huang, 1988) and *Ochrobactrum antropi* strain LMG3331 (Lechner *et al*., 1995). However, unlike other bacterial degraders mentioned above, strain KM221 is gram positive. In addition, this strain is facultative anaerobic. These observations suggest that a wide variety of bacterial species have the potential to survive under chlorinated conditions.

The comparison of 16S rDNA with known 16S rDNA sequences from BLAST Database showed that the closest sequence similarity (98%) of strain KM221 was found to *Bacillus iodinum*. It is interesting to note that *B. iodinum* is the new species of MCPA-degrading coral bacterium. The 16S rDNA sequence obtained from MCPA-degrading coral bacterium strain KM221 have been deposited in GenBank under accession no. X76567.1.

CONCLUSION

The result of this investigation showed that *B. iodinum* KM221 is a new species of MCPA-degrading bacterium. This result also indicated that the use of coral bacteria to deplete MCPA in contaminated marine sites has potential. However, before the use of these bacteria in marine remediation efforts can be considered a viable alternative, the nature, stability, and toxicity of the coral-bound transformation products, under a variety conditions, must be elucidated.

ACKNOWLEDGEMENT

Sincere thanks is addresed to Esti Harpeni for her technical assistance, Simon Meinhard, Oldenburg University, Germany, for his assistance on molecular identification. This research was supported by Riset Unggulan Terpadu VIII No. 011.27/SK/RUT/2001, 26 Januari 2001.

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