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SHORT COMMUNICATION

Molecular identification and biodegradation of 3-chloropropionic acid (3CP) by filamentous fungi-*Mucor* and *Trichoderma* species isolated from UTM agricultural land

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ABSTRACT

Aims: This study was carried out to further characterize fungal species that could degrade 3-chloropropionic acid (3CP) as sole source of carbon and energy.
Methodology and Results: Both fungi were able to grow on 3CP after 10 days on solid minimal media. Based on sequencing of its segment of 18S rRNA these isolates were identified as *Mucor* sp. SP1 and *Trichoderma sp*. SP2. The isolated strains were not able to grow on media plates containing 10 mM of 2,2-dichloropropionate (2,2DCP) as sole source of carbon. 3CP degradation was observed in liquid minimal medium containing 10 mM 3CP after 18 days culture period. The chloride ion released was detected in both growth medium containing *Mucor sp.* SP1 and *Trichoderma sp*. SP2. At least 80% of 10 mM 3CP was utilized in the growth medium.
Conclusion, significance and impact of study: Dehalogenase enzyme that can degrade α-chloro-substituted haloalkanoic acids for example 2,2DCP is well studied up to protein crystallization. Very few reports on the degradation of β-chloro-substituted haloalkanoic acids such as 3CP and none from fungi. This study is considered important because it can be compared to that of well-documented α-chloro-substituted haloalkanoic acids degradation. This is the first study to indicate fungal growth on 3CP as sole carbon and energy sources.

Keywords: Haloalkanoic acid, 3-chloropropionate, biodegradation, pollutant degradation, *Mucor* sp. SP1, *Trichoderma* sp. SP2.

INTRODUCTION

Xenobiotics are unnatural environmental contaminants that are widely distributed as environmental pollutants (Rieger *et al*., 2002). Halogenated organic compounds are common xenobiotics produced as industrial products and have numerous applications such as in pharmaceuticals, herbicides, fungicides, insecticides, solvents and plasticizers. Despite wide applications they are considered as an important class of hazardous environmental pollutants due to their toxicity, carcinogenicity and persistency in the environment. These pollutants can be degraded into harmless substances by using both non-biological and biological pathways, of which the latter are considered to be more economical, safer and environmental friendly. Dehalogenation is one of the critical steps during microbial degradation of halogenated compounds. During this reaction, halogen substituent, which often contributes to xenobioticity and toxicity properties of these compounds, is usually substituted by hydrogen or a hydroxyl group. Halogen removal reduces the recalcitrance of the compound, and also the risk of producing toxic intermediate compounds during subsequent metabolic steps (Janssen *et al.*, 2001). There have been very few reports available on degradation of halogenated aliphatic compounds especially 3-chloropropionate (3CP). Moreover, many studies have focused on biodegradation of 2-haloacid or α-chloro substituted alkanoates, such as 2,2-dichloropropionic acid (2,2DCP) and D,L2-chloropropionic acid (D,L2CP) (Motosugi *et al*., 1982; Barth *et al*., 1992; Thasif *et al*., 2009; Wong and Huyop, 2011), whereas the degradation of β–chloro substituited alkanoates (3CP) has not been well documented and there are very few reports to date on

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degradation of this compound by bacteria and no report so far from fungal (Mesri *et al*., 2009; Yusn and Huyop, 2009). Therefore, current study reports on the identification of fungal species and degradation of 3CP by two fungal isolates. The fungal species were carefully isolated from the agricultural soil.

MATERIALS AND METHODS

Growth media preparation

Cells were grown in a chloride-free minimal medium contained basal salts $(NH_4)_2SO_4$ (0.05%), K_2HPO_4 (0.05%), KH_2PO_4, $MgSO_4{\cdot}7H_2O$ (0.02%), $MgCl_2{\cdot}2H_2O$ (0.03%), yeast extract (0.05%) plus trace elements solution, $FeSO_4{\cdot}5H_2O$ (0.05%), and $CaSO_4$ (0.02%) adjusted to a pH of 6.8. The minimal media was autoclaved at 121 °C for 15 min at 15 psi. Carbon sources (1 M, 3CP and/or 1 M 2,2DCP) were sterilised separately and added aseptically to the media to the desired final concentration. In order to prepare solid medium, agar No.1-(Oxoid) (1.5% w/v) was added prior to sterilisation.

Sample preparation

Soil sample was collected from an agricultural land in the compound of UTM that was previously exposed to the herbicide and pesticide. For soil sample preparation, 0.5 g of soil was mixed in 20 mL of distilled water. The mixture was stirred and left until soil particles settled down, and then, 0.1 mL of aliquot was spread onto solid minimal media contained 10 mM 3CP as a carbon source. Plates were incubated at 30 °C. Then, the fungi growth was monitored at an appropriate time interval (from 5 to 10 days). The isolated mycelium pieces were transferred into a fresh solid minimal media containing the same concentration of 3CP and the plates were incubated at 30 °C until a pure culture was seen.

Stock cultures were maintained at 4 °C on Sabouraud agar medium (0.5% yeast extract, 1% peptone, 2% glucose, and 2% agar) slants added 10 mM 3CP. To standardize the inoculum, inoculum plate was prepared by transferring mycelium pieces from Sabouraud slants to Sabouraud plates containing 10 mM 3CP, followed by incubation at 30 °C.

The ITS analysis for fungal identification

The genomic DNA of the fungi was prepared using Promega Wizard® Genomic DNA Kit. The Internal Transcribed Space (ITS) regions of rRNA genes were amplified by PCR using a pair of universal primers ITS1 – TCCGTAGGTGAACCTGCGG and ITS4 - TCCTCCGCTTATTGATATGC as suggested by White *et al.*, 1990. Each PCR reaction mixture (total volume 50 µL) contained 0.25 unit/mL of *Taq* polymerase (Promega), 4 µM of $MgCl_2$, 1 µM of each dNTPs, 0.2 µmol of each primer, nuclease free water, and 100 ng of DNA template. The PCR protocol consisted of a denaturing process at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 55.5 °C for 2 min and 72 °C for 2 min, incubation at 72 °C for 10 min, using GeneAmp® PCR system 9700. Amplicons were purified using QIAquick PCR purification kit (QIAGEN). The PCR product was sequenced at 1st BASE® Laboratory Sdn. Bhd. Serdang, Selangor.

Phylogenetic analysis based on ITS gene sequence

Gene sequences were compared with GenBank database using BLASTn search tool (Altschul *et al.*, 1997). Multiple sequence alignment was constructed using profile alignment command of CLUSTAL-W from MEGA software version 4 (Tamura *et al.*, 2007). Using the same software, phylogenetic trees with bootstrap test (1000 replications) were constructed using Neighbour–joining method (Saitou and Nei, 1987).

Measuring chloride ion released in the growth medium

Chloride ion released was detected in the growth medium by measuring the chloride ion at the appropriate time intervals. Degradation of 3CP was measured by determining the release of chloride indicated by a colorimetric method employing mercuric thiocyanate as previously reported by Bergman and Sanik (1957). Samples (1.0 mL) were removed and assayed for halide ions. Each assay was carried out in triplicates. The absorbance of the mixture was measured at $A_{460\ nm}$ and was proportional to the chloride ion concentration.

RESULTS

Morphological analysis and toxicity of the substrate

After 10 days of incubation, both fungi showed visible growth on 10 mM 3CP minimal media plate. Morphological observation suggested that they belong to two different fungi [Figure 1(a) and 1(b)]. Therefore, they were designated as strains SP1 and SP2. The control plates without 3CP as a carbon source indicated no visible growth. These fungi were subcultured into different concentrations of 3CP liquid media containing 1 mM, 5 mM, 10 mM, 20 mM and 30 mM. The results showed that SP1 and SP2 grew well on 10 mM 3CP concentration. The growth was decreased at 20 mM 3CP, whereas at 30 mM 3CP the growth was inhibited. Growth was tested on 5 mM and 10 mM 2,2DCP. But no growth was observed in the minimal medium containing 2,2DCP as a carbon source.

Identification of fungi based on molecular and phylogenetic analysis

Primers ITS 1 and ITS 4 were used to amplify the rDNA-ITS regions for both fungi. The amplified fragment was analyzed using agarose gel electrophoresis as shown in Figure 2. The sizes of the amplified fragments for SP1 and SP2 were approximately 600 bp as expected. The full length of the sequences was subjected to BLASTn search

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in order to compare with the nucleotide sequences from the Genebank database (NCBI). The results of BLASTn illustrated that the isolate SP1 and SP2 matched to the *Mucor* sp. and *Trichoderma* sp., respectively as shown in Table 1.

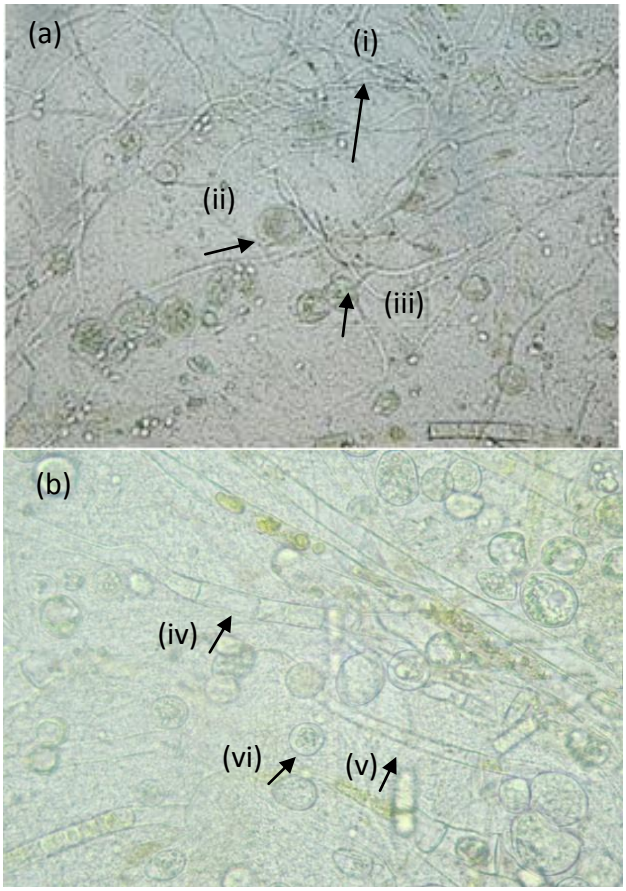

Figure 1: Morphological study for both strains under a light microscope (1000x magnification). Both strains were grown in 10 mM 3-chloropropionic acid incubated at 30 °C observed after 10 days in culture: (a) SP1; (b) SP2. Arrows indicate: (i) hyphae of the mycelium; (ii) sporangiospore; (iii) columella; (iv) conidiophores; (v) hyphae; (vi) conidia (spore).

Table 1: A proposed genus and species for SP1 and SP2 based on BLASTn analysis.

Strain	Accession	Description	% identity
SP1	FN650641.1	*Mucor indicus*	98%
SP2	JN004180.1	*Trichoderma asperellum*	99%

Further analysis was carried out using Neighbor-joining phylogenetic tree with bootstrap test. The phylogenetic trees of SP1 and SP2 were presented in Figure 3 and 4, respectively. Therefore, SP1 and SP2 were designated as *Mucor* sp. strain SP1 and *Trichoderma* sp. strain SP2, respectively.

3CP degradation study using 3CP as the sole source of carbon

3CP degradation was followed over a period of 18 days and samples were analyzed for chloride ion released (Figure 5). 3CP concentration of 1 mM consumed by the

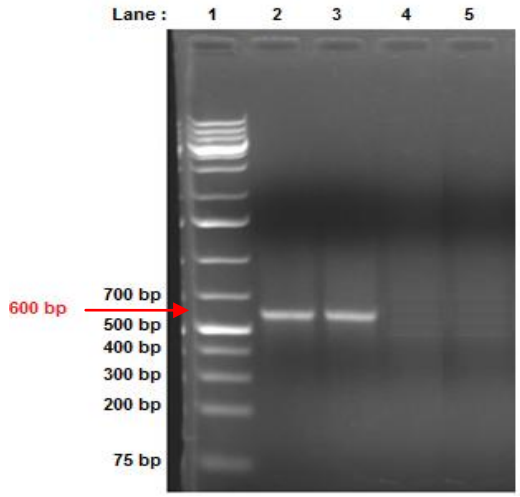

Figure 2: Agarose gel electrophoresis of PCR amplified fragment from fungal SP1 and SP2. Lane 1: 1 kb DNA ladder; Lane 2: SP1; Lane 3: SP2; Lane 4: Control 1 (SP1 amplification without ITS1 primer); and Lane 5: Control 2 (SP2 amplification without ITS1 primer).

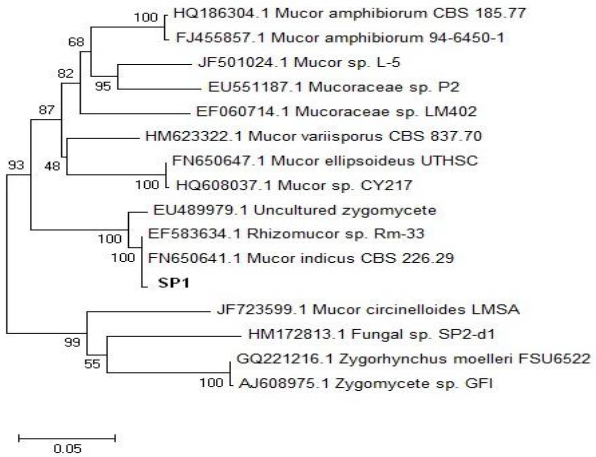

Figure 3: Phylogenetic tree of strain SP1 and the related fungal species.

fungus is equivalent to 1 µmolCl^-/mL. If all 10 mM of substrate utilized by the fungi would be 10 µmolCl^-/mL of chloride ion released will be detected in the medium. Current investigation showed that at least approximately 80% of 10 mM 3CP was utilized as shown by the released of chloride ion in the growth medium. A control experiment without substrate used, showed no growth.

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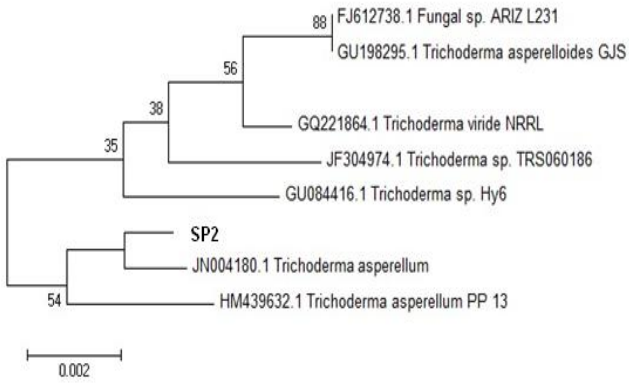

Figure 4: Phylogenetic tree of strain SP2 and the related fungal species.

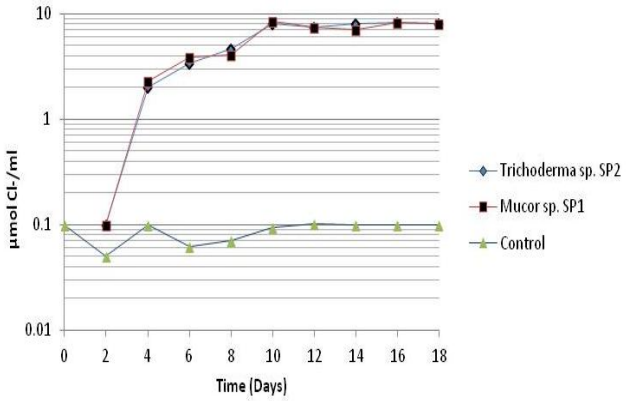

Figure 5: Growth curves measured using chloride ion released in the growth medium containing 10 mM 3CP. Control experiment with both bacterial cultures were mixed together without any carbon source. A 80% of 10 mM 3CP was utilized as shown by the released of chloride ion in the growth medium. A control experiment without substrate used, showed no growth.

DISCUSSION AND CONCLUSION

Both fungi were isolated from soil that was previously exposed to herbicides and pesticides. This is the first report on the ability of fungal isolates to degrade 3CP as sole source of carbon. This study describes the overall characterization of two fungal strains which are able to grow on 3CP as their sole source of carbon and energy. Based on 18S molecular analysis, the fungi belong to the following genera: *Mucor* sp. and *Trichoderma* sp.

Degradation of 3CP was demonstrated by the released of chloride ion in the growth media. Our findings highlight the importance of halogen substituent position in governing the susceptibility of halogenated aliphatic acids to degradation by microbial enzyme. Hence, this study is of considerable importance as it is comparable to that of other well-documented α-chloro-substituted haloalkanoic acids, such as 2,2-dichloropropionic acid and D,L-2-chloropropionic acid (Marchesi and Weightman, 2003). The isolation of these microorganisms that were able to degrade organohalogen particularly, 3CP would be useful in understanding the mechanism of enzyme action (Mesri *et al*., 2009). A phylogenetic analysis of the known haloacid dehalogenases group these proteins into two, namely Group I members, which act on both D and L-enantiomers of 2-halocarboxylic acid such as D,L-2-chloropropionic acid and Group II that strictly catalyses for only L-enantiomers. Both groups belong to HAD-superfamily (Koonin and Tatusov, 1994). This does not include of fluroacetate dehalogenase (Liu *et al.*, 1998).

Our preliminary study have found there is new group of dehalogenase that could act specifically on 3CP. Based on computational molecular analysis, 3CP could bind to the DehE (Group I dehalogenase) active site (Hamid *et al*., 2012). However, empirical investigations suggested otherwise (Huyop *et al*., 2004). In contrast, computational analysis showed that 3CP could not bind with DehD (Group I dehalogenase) active site (Sudi *et al*., 2012) and 3CP was not a substrate for DehD enzyme (Huyop *et al*., 2008). Both dehalogenases were previously isolated from *Rhizobium* sp. RC1 as reported earlier by Allison (1981) and Huyop *et al*., (2010).

Bacterial and fungus species have been reported to degrade both halogenated aliphatic and aromatic compounds (Chaudhry and Chapalamadugu, 1991). Both *Trichoderma* and *Mucor* were well known for their capacity to degrade halogenated compounds as reported earlier (Anderson *et al*., 1970; Bumpust and Austt, 1987; Katayama and Matsumura, 1993). *Mucor* is a microbial genus of about 3000 species of moulds commonly found in soil, digestive systems and plant surfaces. Most of *Mucor* species are unable to infect humans and endothermic animals due to their inability to grow in warm environments close to 37 °C. Thermotolerant species such as *Mucor indicus* sometimes cause opportunistic, and often rapidly spreading, necrotizing infections known as zygomycosis. *Trichoderma* genus commonly found in all soil, were the most prevalent culturable fungi. Many species in this genus can be characterized as opportunistic a virulent plant symbionts. Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants (Harman, 2006).

Current investigation is the first reported fungus species that were able to degrade 3CP. Isolation, gene identification and computational approach of the translated gene that can degrade 3CP may shed light on the enzymatic structure and function of the protein.

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