Research article

Edbeib, M.F., Biodegradation of 3-chloropropionic acid and production of propionic acid by novel isolated fungus Trichoderma sp. MF1. International Journal of Life Sciences and Biotechnology, 2020. 3(1): p. 41-50. DOI: 10.38001/ijlsb.677005

Biodegradation of 3-chloropropionic acid and production of propionic acid by novel isolated fungus *Trichoderma* sp. MF1

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ABSTRACT

Biologically synthesized chemicals have drawn a growing interest recently. The main objectives of the study are to isolate 3-chloropropionic acid (3CP) degrading fungus and to examine its ability to produce Propionic Acid (PA) as a by-product. 3CP is widely used in many pesticide and herbicide synthesis due to biocidal properties but it is toxic and recalcitrant to be removed from the biosphere. Bioremediation approaches through dehalogenation have promised removal of these xenobiotics. Fungi are selected due to its broad enzymatic capacities which is not limited like bacteria. In this research, several fungal isolates have been purified, among the isolates, strain designated as MF1 has shown greater potential in 3CP degradation. Using morphological and molecular approaches, MF1 was identified as *Trichoderma* sp. Amplification of ITS genome region (Accession No. MT126695) revealed that the MF1 isolate had 99% identity to *Trichoderma asperellum* strain AF14. Strain MF1 growth rate is 1.42cm/day on solid medium and it was able to produce biomass up to 0.855g/L in liquid minimal medium supplemented with 10mM 3CP. Whereas the growth in control medium containing 1% glucose has resulted in biomass of 1.814g/L. 90.32% of 10mM 3CP were successfully de-chlorinated within 20 days. Confirmed by HPLC, PA was the major product of dehalogenation with highest concentration of 2.72mM at day 10. Presented data can be used for the designing of by-product extraction. Dehalogenation of 3CP by *Trichoderma asperellum* MF1 have not only been successfully removed xenobiotic pollutant but also have open for new strategy on synthesis of industrial required chemicals.

ARTICLE HISTORY
Received
19 January 2020
Accepted
4 March 2020

KEY WORDS
Pollutant degradation, dehalogenation, *Trichoderma* sp., 3-chloropropionic acid, propionic acid synthesis.

Introduction

Propionic acid (PA) is widely used as an additive in animal feed and an intermediate in biodegradable polymers which can potentially replace the petroleum-based polymers. Commercial production of PA is chemical synthesis from petroleum feedstock [1]. Growing demand of this acid draws attention towards a more economical energy efficient technology. 3-chloropropionic acid (3CP) has revealed synthesis of significant by-products of PA.

Various xenobiotic have been used extensively in agriculture for the sustained production. These compounds are highly toxic, recalcitrant and raised public concern as

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they are harmful threat to environment. Microorganisms have been identified with ability of utilizing these halogenated compounds by dehalogenation mechanism [2, 3]. However, biodegradation β-halogenated compounds like 3CP which are widely used in intermediates for pharmaceutical and pesticide productions was limited in bacterial remediation [4-6]. 3CP has been classified as chlorinated aliphatic acids and also known as β-chlorine substituted haloalkanoates. This compound has been identified as a chemical intermediate in many herbicide and chemical fertilizers [7].

Therefore, fungal remediation was proposed in this research as a new approach in bioremediation of 3CP. Our main objective was to isolate 3CP degrading fungi from pesticide exposed soil samples. Microbial dehalogenation usually produces an intermediate which subsequently converted to another form or directly utilized as carbon source. The intermediates serves as carbon source to the organism, if it is not toxic. In present study, we have proven that fungal biodegradation of 3CP have resulted in synthesis of industrial useful co-product PA.

PA is widely used in many industries as additive in animal feed and intermediate in biodegradable polymers. PA is also used in the manufacture of herbicides, chemical intermediates, artificial fruit flavours, pharmaceuticals, cellulose acetate propionate, and preservatives for food, animal feed, and grain. This important industrial substrate is expensively synthesized by chemical reactions. However, our newly isolate *Trichoderma asperellum* MF1 has been proven to synthesize PA as co-metabolic product in dehalogenation of 3CP.

Material and Methods

Sample collection and isolation

Pesticide exposed soil were collected in sterile polythene bags from local oil palm plantation area. Samples were stored at 4°C in a refrigerator for further use. Samples were prepared as described by Parvizpour, Hamid [8].One gram of soil sample was serially diluted and spread on the potato dextrose agar plates supplemented with 10mM of 3CP. All plates were incubated at 30°C for 5 to 14 days. Colonies were purified by transferring the 1 cm^3 of mycelium to a new agar plates.

Growth medium

Minimal media (100 ml) containing 3CP as the only source of carbon and energy was used to incubate the fungul culture. Herein, chloride-free minimal media was prepared in distilled water as 10x concentration basal salts containing $NaH_2PO_4.2H_2O$ (10.0 g/L), $K_2HPO_4.3H_2O$ (42.5 g/L), $(NH_4)_2SO_4$ (25.0 g/L). Trace metals at 10x concentrations: $C_6H_9NO_6$ (1.0 g/L), $MgSO_4$ (2.0 g/L), $FeSO_4.7H_2O$ (120.0 mg/L), $MnSO_4.4H_2O$ (30.0 mg/L), $ZnSO_4.H_2O$ (30 mg/L) and $CoCl_2.6H_2O$ (10 mg/L) were also added [9]. Basal salts (10 ml) and trace metals (10 ml) were added to distilled water (80 ml) and sterilized (121°C for 15 min at 15/psi). 3CP was filter sterilized through a 0.2 μm nylon-membrane and added aseptically to the media. Delta 320 pH meter (AES, Combourg, France) was used to monitor pH of the media and was adjusted to pH 5.5 ±0.2 using sodium hydroxide and/or hydrochloric acid. Fungal isolates were incubated at 30°C in a rotary incubator with agitation at 200 rpm. 1% of Glucose replaced 3CP as carbon source in control sample.

Morphological and molecular characterization

Selected isolate was grown in minimal medium with 10mM of 3CP to characterize their morphology. Purified isolate was identified based on fruiting bodies and spores viewed under light microscope after staining with lactophenol blue. The genomic DNA of the fungal isolate was prepared by modified methods of [10]. Internal Transcribed Space (ITS) regions of rRNA genes were amplified using upstream primer of ITS1 – 5' TCCGTAGGTGAACCTGCGG 3' and downstream primer of ITS4 – g' TCCTCCGCTTATTGATATGC 3'. PCR conditions are detailed in (Table 1). A phylogenetic tree was constructed using MEGA 6.0 software through Neighbour-Joining bootstrap method. The ITS partial sequence of top blast results (Accession numbers are shown in the figure 3) from NCBI and other different species from same genus were selected to investigate the evolutionary relationship.

Table 1 ITS rRNA PCR amplification conditions of *Trichoderma asperellum* MF1.

Steps	Cycles	Temperature (ºC)	Time (minute)
Initial denaturation	1	95	5
Denaturation	34	95	1
Annealing		55.5	2
Extension		72	2
Final extension	1	72	10
Hold	1	4	∞

Biodegradation of 3CP

Isolate grown in 100 mL of minimal medium adjusted to pH 5.5; supplemented with 10mM 3CP, at 30°C and 200rpm. The depletion of 3CP was detected by chromatography analysis (HPLC) using C18 column (4.6 x 150mm, 5µM) under wavelength of 210 nm. Samples were separated using an isocratic elution with a mobile phase containing potassium sulphate (20 mM) to acetonitrile at ratio of 60:40, in deionised water. The retention time of 3CP was 1.254 min at flow rate of 1.0 ml/min flow rate and 30°C column temperature. PA also confirmed through similar chromatography method detected at 1.190 min.

Growth Analysis

The growth was measured by determination of biomass produced, recovered by filtration using Whatman No. 1 filter paper (No. 1). Dehalogenation was supported with detection of free halide release by modified method of Bergmann and Sanik [11].

Results

Morphological and molecular characterization

In this study, MF1 isolate have shown a high growth rate (1.42 cm/day) on solidified minimal medium with 3CP (Fig. 1 a and b). Morphological characteristics of the strain MF1 showed that the isolate are among the genus *Trichoderma.* Specie of *Trichoderma* are mainly distinguished from other isolates through their conidiophores (Fig. 1 c).

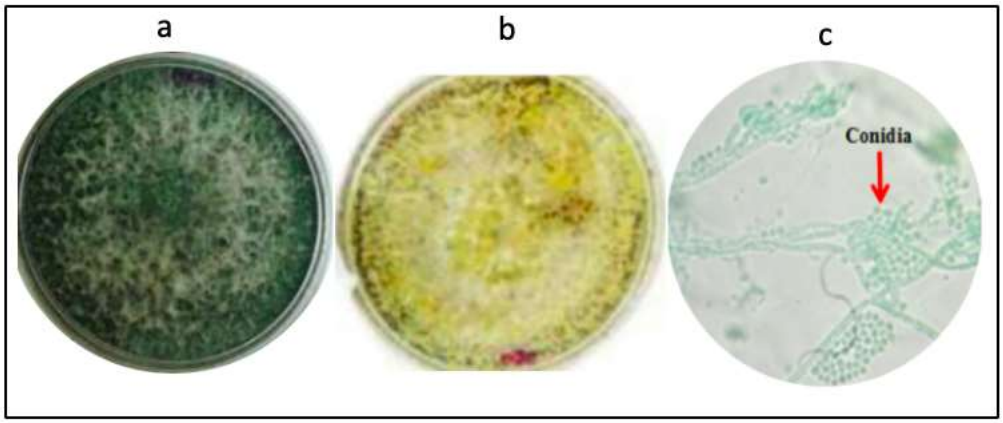

Fig 1 Morphological analysis of MF1. (a) Grown on PDA agar, (b) on solidified minimal medium supplemented with 10mM 3CP. All plates incubated at ambient temperature for 14 days, (c) shows the conidiophores of *Trichoderma* species MF1.

Based on staining result, MF1 classified as a *Trichoderma* species. Further confirmation was done through genetic identification by the amplification of the ITS gene (Fig. 2).

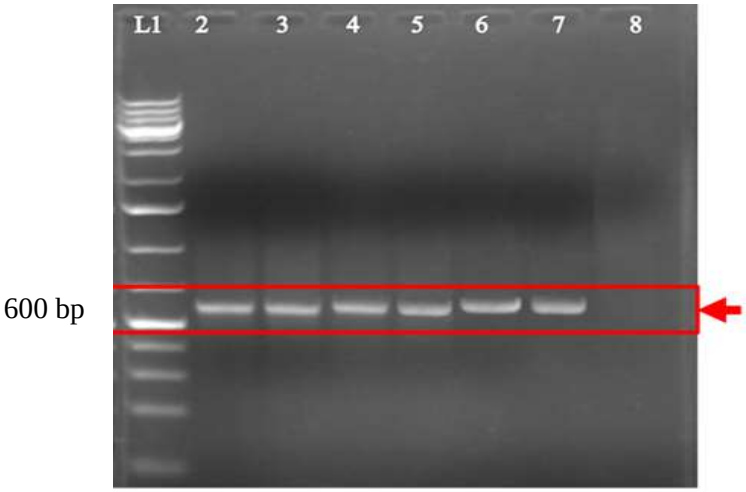

Fig 2 The amplified ITS region for *Trichoderma* strains. Lane 1: DNA ladder; Lane 2: strain MF1; Lane 3: strain MF2; Lane 4: strain MF3; Lane 5: strain MF4; Lane 6: strain MF5 and Lane 7: strain MF6. Lane 8: negative control.

Approximately 600 base pairs of the amplified ITS region was obtained by aligning the forward and reverse sequences. Phylogenetic relatives to the corresponding isolates were identified by using BLASTn online analysis tool. The isolate showed 99% similarity to *T. asperellum* strain AF14 (JX677934.1), the phylogenetic tree is shown in Fig. 3.

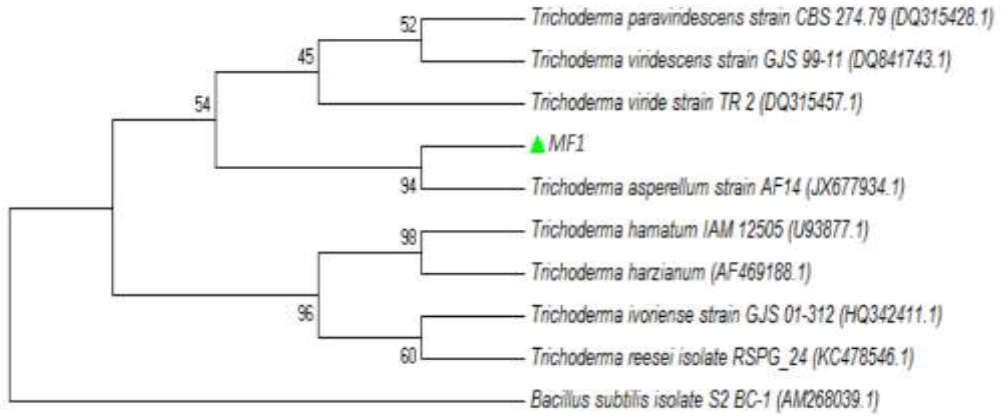

Fig 3 Phylogenetic tree showing the evolutionary relationship of isolate MF1 (Accession No. MT126695) with representative members from the genus of *Trichoderma*. The sequences were obtained from GenBank database. The tree was constructed by the neighbour-joining method using MEGA 6.0 software

Biodegradation of 3CP

Under optimized conditions, the harvested *Trichoderma asperellum* MF1 spores were subjected to growth in 10mM 3CP liquid minimal medium. MF1 produced biomass up to 0.855g/L. Whereas, in control cultures supplemented with 1% glucose as carbon source, MF1 produced biomass of about 1.814g/L (Fig. 4). This indicates the limited growth under treatment with 3CP. Increasing free chloride ions detected together with biomass have proven that there was dehalogenation of 3CP (Fig. 5).

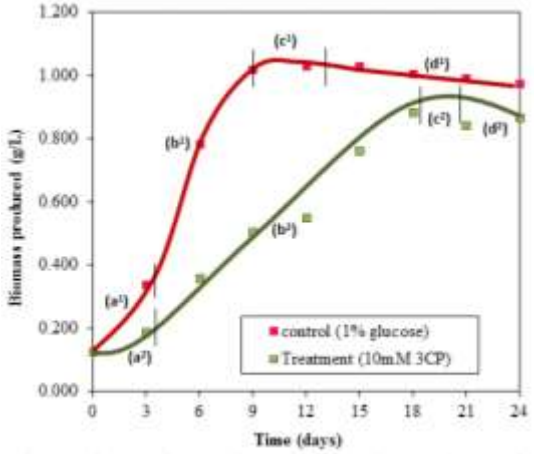

Fig 4 Growth of *Trichoderma asperellum* MF1 in presence and absence of 3CP. **$a^{1\&2}$:** lag phase; **$b^{1\&2}$:** log (exponential) phase; **$c^{1\&2}$:** stationary phase; **$d^{1\&2}$:** death phase. Growth determined through dry cell weight of biomass collected on the corresponding time.

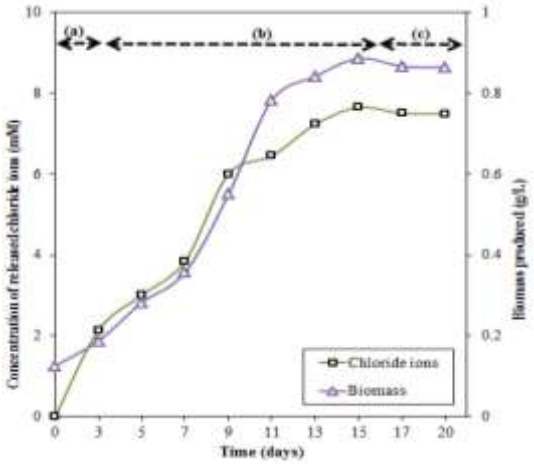

Fig 5 Dechlorination performance by *Trichoderma* strain MF1. Triplicate experiments conducted with liquid medium supplemented with 3CP at final concentration of 10mM.

Production of PA

Samples were further analysed by HPLC methods. The liberation of chloride ion almost corresponds with the disappearance of 3CP from the cultivation medium. Maximum chloride has been released through dehalogenation was 3.41mM/mL in 20 days of

incubation. About 90.32% of 3CP was disappeared from the cultivation medium. It is expected to be successfully oxidised by fungal enzymes in 20 days of treatment period. HPLC analysis also detects synthesis PA. Concentration of PA detected by HPLC reached 2.72mM at day 10 and increased to 3.77mM at day 20 of incubation (Fig. 6). Increasing concentration indicates this product was accumulated in the medium and not utilized by the fungal cell.

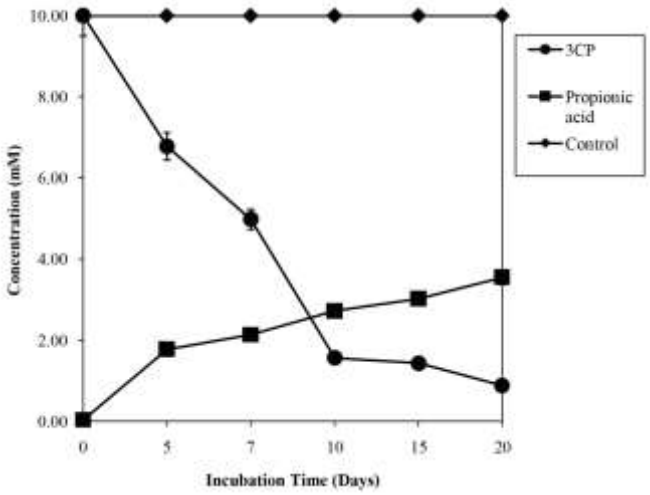

Fig 6 Disappearance of 3CP in culture medium and accumulation of PA in the culture medium.

Discussion

Bacterial remediation of β-chloro substituted organic acids such 3CP is restricted to certain groups only. This is due to the compound toxicity and recalcitrance, created by the position of halogen [7]. In current investigation, PCR amplification of ITS gene had proven that 3CP tolerant and degrading fungal isolates shared more than 99% to *Trichoderma* and *Aspergillus* species. These widely occurring organisms are of considerable environmental and biotechnological importance due to their broad metabolic diversity and array of unique enzymatic capabilities [12].

Trichoderma species are the most common fungi that were successfully isolated. Morphologically, spores formed through asexual reproduction are exposed and not enclosed within membrane such as in *Aspergillus* and *Penicillium*. Different from *Aspergillus*, the conidia of isolates form globular clusters and branched like in *Penicillium*. However, the absence of phialides, the branching tips was the key for

microscopic identification of *Trichoderma*. They also have branched conidiophore [13, 14].

Trichoderma species are the most ubiquitous saprophytic component, sustain for longer time even in increased pollution conditions [8, 15]. They are capable of synthesising various kind of enzymes which play a significant role in biotic and abiotic stress tolerance, hyphae growth, and plant growth enhancer as well as in pollutant degradation potential. Hamzah, Zarin [15] reported the degradation of up to 40% of total petroleum hydrocarbon (TPH), 100% of pristane and 74% of phytane compounds by *Trichoderma* species in 9 days of incubation at optimal physical and nutrient parameters.

There are four basic criteria which must be fulfilled in order for a given halogenated compound to be utilized by an organism as sole source of carbon and energy. Firstly, the organism must be adaptive to the concentration toxicity of supplemented halogenated compound. Secondly, the organism must either possess or synthesize dehalogenase in response to the halogenated compound which is capable of removing the substituent halogen(s) from the compound. Thirdly, the dehalogenation product should be non-toxic and easily converted to an intermediate of the organism's central metabolic pathway. Finally, the intermediate of halogenated compound should be able to enter cell either passively or by active transport in order to reach the site of central metabolic activity[16].

3CP degradation potential was previously described in *Trichoderma* sp. Parvizpour, Hamid [8] reported the dehalogenation of 3CP and the synthesis of 3- hydroxypropionic acid (3HP) as the by-product. 3HP is non-toxic carbon source which can be easily absorbed and utilized [4]. Fungal dehalogenation is different where most saprophytic fungi are equipped with lignin modifying enzymes which is non-substrate specific and able to breakdown various pollutants [17, 18].

In this study, we have proven that the accumulation of 3CP in fungal mycelium has resulted in synthesis of a reductive dehalogenase enzyme. The enzyme replaced the chloride ion with a hydrogen atom through reductive dehalogenation which is different from bacteria where it involving substitution of hydroxyl ion through hydrolytic dehalogenation [4].

Accumulation PA also proven with the biomass produced. One of the applications of PA in food industry is to delay the growth of mould and bacteria [19]. Thus, it has inhibitory

effect on *Trichoderma asperellum* MF1 and this explains the difference in maximum biomass produced in control and treatment cultures.

Conclusion

In conclusion, it is shown that biodegradation of environmental pollutants (dehalogenation of 3CP) by *Trichoderma asperellum* MF1 is producing an industrial important PA as the co-metabolites. Thus, breaking down of xenobiotic and producing non-toxic and industrially important intermediates.

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