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A novel putative non-ligninolytic dehalogenase activity for 3-chloropropionic acid (3CP) utilization by *Trichoderma asperellum* strain SD1

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ABSTRACT

Aims: *Trichoderma asperellum* strain SD1 grows on 3-chloropropionic acid (3CP), a β-haloalkanoic acid, and produces a putative extracellular dehalogenase that can degrade this acid. Here we further characterized the fungal enzyme system responsible for biodegradation of 3CP.
Methodology and results: The primary qualification of the ligninolytic potential in *T. asperellum* strain SD1 was performed using guaiacol oxidation. When strain SD1 was grown in liquid minimal medium with the presence of 3CP as the sole carbon source, no lignin peroxidase, manganese peroxidase, or laccase activity was detected. The ligninolytic condition was achieved only in the presence of glucose or when guaiacol was present as an inducer. Under non-ligninolytic conditions, 3CP was utilized by strain SD1. Therefore, 3CP was utilized under ligninolytic conditions as well as under non-ligninolytic conditions, suggesting that extracellular peroxidases and laccase are not involved in the degradation of 3CP by *T. asperellum* strain SD1.
Conclusion, significance, and Impact of study: Very few studies have explained the degradation of β-chloro–substituted haloalkanoic acids such as 3CP by dehalogenases. This is the first report to identify a novel putative β-haloacid dehalogenase that degrades 3CP under ligninolytic and non-ligninolytic conditions. *T. asperellum* strain SD1, thus has the potential in the development of dehalogenating enzymes for industrial biocatalytic processes, in future.

Keywords: Halogenated aliphatic acid, degradation, *Trichoderma asperellum*, ligninolytic enzyme, reductive dehalogenase

INTRODUCTION

Halogenated aliphatic acids (HAAs) are a threat to the environment and are persistent in the biosphere (Leisinger, 1996). The sources of HAAs are both naturally occurring (Ellis *et al.*, 2001) and anthropogenic (Wilson *et al*., 1986). These compounds tend to be deposited in fatty tissues of animals and circulate in the food chain. Long-term exposure to these compounds is linked to many health problems such as cancer and neurological disorders (Mishra and Sharma, 2011). These halogenated xenobiotics can be eliminated by biological methods that transform them into harmless substances (Fetzner, 2010; Marco-Urrea and Reddy, 2012).

Recently, microbial enzymes that act on halogenated organic compounds have attracted a great deal of attention. Microbial enzyme systems break carbon-halogen bonds and allow these compounds to be utilized either as a carbon source or as alternative electron acceptors (Bagherbaigi *et al*., 2013). The catalytic mechanisms of these microbial systems are very broadly classified as hydrolytic, oxidative, or reductive (Fetzner, 1998).

Microbial degradation of β-chlorinated haloalkanoic acids such as 3-chloropropionic acid (3CP) is limited because microorganisms that degrade α-chloroalkanoatesare unable to dechlorinate β-substituted haloalkanoic acids, which differ only in terms of their chlorine substitution (Bollag and Alexander, 1971; Kohler-Staub and Kohler, 1989; Fetzner, 2010). Filamentous fungi have been proposed as an alternative organism that can degrade these compounds because of their broad enzymatic capacities (Sharma, 2011). The low specificity of fungal enzyme system has been hypothesized able to breakdown the β-carbon and chlorine bond in 3CP (Hatakka and Hammel, 2011). We previously described the isolation process and characteristics of *Trichoderma* strains found in pesticide-contaminated agricultural soil that are able to degrade 3CP. Of the seven isolates, *T. asperellum* strainSD1was determined to have efficient and

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rapid degradation capabilities. We observed that a reductive dehalogenation reaction is involved as a co-metabolic mechanism in the degradation of 3CP (Shanmugaprakasham, 2014).

Co-metabolic reductive dehalogenation was not observed in any previously reported bacterial dehalogenation pathways of 3CP (Lin *et al*., 2011; Bagherbaigi *et al*., 2013; Hamid *et al*., 2013). Because of their saprophytic nature, fungi secrete ligninolytic enzymes that also have similar reductive dechlorinating capabilities (Khindaria *et al*., 1995). However, ligninolytic fungi also have a cell-bound reductive dehalogenase system (Reddy *et al*., 1998). To determine the mechanism of this presumed reductive dechlorination reaction, we identified the enzyme(s) involved. Here we report the potential of a ligninolytic and presumptive reductive dehalogenase in *Trichoderma* isolates.

MATERIALS AND METHODS

Chemicals

Most chemicals used in this study, including 3CP, propionic acid, and 2-methoxyphenol (guaiacol), were purchased from Sigma-Aldrich (USA) at 98% purity. Chromatography-grade acetonitrile was obtained from Thermo Scientific (Malaysia). Other chemicals were analytical pure-grade and are commercially available. Stock solutions were prepared with double deionized water, sterilized with membrane filtration (0.2 µm), and stored in dark bottles at 4 °C until use.

Fungi

Trichoderma asperellum strain SD1 was previously isolated from contaminated area as described in Shanmugaprakasham (2014). Strain SD1was maintained on potato dextrose agar (Difco Laboratories, Detroit, MI, USA). It was kept at room temperature until used. Subcultures were routinely made every 30 to 60 days.

Qualitative assay of guaiacol oxidation

All isolates from previous study (Shanmugaprakasham, 2014) were initially screened for ligninolytic enzyme production, which was performed by inoculating fragments of mycelium on potato dextrose agar containing 4 mM guaiacol (Atalla *et al*., 2010). Production of ligninolytic enzymes such as peroxidases and laccases by the isolates was observed as the presence of an intense brown-red color under and around the fungal colony resulting from guaiacol oxidation.

Culture conditions and inoculum preparation

The fungal isolate was inoculated into a chloride-free minimal medium (Parvizpour *et al*., 2013). This medium contained 0.50 g $(NH_4)_2SO_4$, 0.60 g KH_2PO_4, 0.40 g K_2HPO_4, 0.5 g $MgSO_4$, 0.05 g $MnSO_4$, 0.01 g $ZnSO_4$, and 0.5 mg $FeSO_4$ per 1 L of medium. The pH of medium was adjusted to 5.5 using 1 M HCl or 1 M NaOH, prior to autoclave. Five disks (5 mm × 5 mm) were obtained from 7-day-old mycelia from strains grown on potato dextrose agar. All five disks were aseptically inoculated into a 100 mL sterile minimal medium in 500-mL Erlenmeyer flasks. The fungal cultures were incubated statically at 30 °C for 15 days; aeration was by diffusion through a cotton-wool stopper.

Growth medium

To assess the production of ligninolytic enzymes during catabolic degradation of 3CP, the fungus was grown in 100 mL culture at 4 different conditions:

(a) In minimal medium supplemented with sterilized 3CPas the sole carbon source with final 3CP concentration of 10 mM. Incubation was continued for 15 days at 30 °C, statically.
(b) In glucose-minimal medium supplemented with filter-sterilized 3% glucose as the sole carbon source. Fungus was incubated statically for 15 days at 30 °C.
(c) In 3CP+glucose minimal medium. The amount of 3CP and glucose were described as in a) and b), respectively.
(d) In minimal medium supplemented with sterilized 3CP as the carbon source + guaiacol at final concentration of 1 mM. Guaiacol was added to the liquid minimal medium to induce ligninolytic enzymes.

At appropriate time intervals, samples from growth medium were collected and centrifuged at 10,000 ×*g* with 4 °C for 10 min. Chromatography analysis was performed on supernatant (see below) to analyze enzyme activities, dechlorination, and disappearance of 3CP. All assays were carried out in duplicates.

Dechlorination study

Chloride ions released by the cleavage of carbon-halogen bonds were detected with colorimetric methods (Bergmann and Sanik, 1957). A 1 mL sample (supernatant) was added to 100 µL of 0.25 M ammonium ferric sulfate in 9 M nitric acid and mixed thoroughly. Mercuric thiocyanate-saturated ethanol (100 µL) was added to the mixture, and the solution was mixed by vortexing. The color was allowed to develop for 10 min at room temperature, and A_{460nm} was measured with a Jenway 6300 Series spectrophotometer. The halide concentration was determined by comparing the absorbance of the test sample to a standard curve of known concentrations of halide.

Enzyme assay

Crude culture filtrates were used for the estimation of extracellular ligninolytic activities. Lignin peroxidase (LiP) activity was determined with spectrophotometric analysis at A_{310nm} of H_2O_2-dependent veratraldehyde formation from veratryl alcohol (Tien and Kirk, 1984). Manganese peroxidase (MnP) activity was determined using 1mM guaiacol (0.25 mL) as the substrate in the reaction

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mixture, which was composed of 0.2 mL of 0.5 M sodium tartrate buffer (pH 5.0), 0.1 mL of 1 mM $MnSO_4$, 0.1 mL of 1 mM H_2O_2, and 0.3 mL of crude enzymes. The oxidation of substrate at 30 °C was followed spectrophotometrically at A_{465nm} (Singh *et al*., 2013). Laccase activity was determined by incubating the reaction mixture which contained 1 mL of 1 mM guaiacol in 0.1 M sodium phosphate buffer (pH6.0) and 1 mL of crude enzyme solution at 30 °C for 10 min. Oxidation was followed by an increase in absorbance at A_{495nm}. One unit of activity was defined as the amount of enzyme that led to the oxidation of 1 mol guaiacol per min (Sivakami *et al*., 2012).

Chromatography analysis

The concentration of 3CP in the growth medium was determined with liquid chromatography analysis. 3CP was extracted using 5 M NaOH precipitation techniques (Shanmugaprakasham, 2014). The extracts were filtered with a 0.2 µm nitrocellulose filter before high-performance liquid chromatography (HPLC) analysis. The extract was analyzed on an Agilent 1100 HPLC system (Agilent, USA) fused with an Agilent ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 µM) under 210 nm. In addition, isocratic elution with a mobile phase consisting of 20 mM potassium sulfate/acetonitrile (60:40) was used. Standards were also run and used to determine the 3CP concentrations.

RESULTS

Qualitative assay of guaiacol oxidizing potential

Trichoderma asperellum strain SD1 was screened for guaiacol oxidation, which indicates the activity of ligninolytic enzymes in this strain. Production of ligninolytic enzymes were indicated by an intense brown-red color under and around the mycelium (Figure 1).This qualitative assay suggested that the fungus *T. asperellum* strain SD1 was the most promising for ligninolytic enzyme production, and thus this strain was further analyzed.

Effect of 3CP as the sole carbon source

Figure 2 shows biodegradation of 3CP by *T. asperellum* strain SD1 cultures. No ligninolytic enzyme activity was detected in the cultivation medium throughout the 15-day incubation. The ligninolytic system can be suppressed by the presence of the substrate 3CP as the sole carbon and energy source (Dhawale *et al*., 1992).

Dechlorination is important evidence of enzymatic cleavage and release of halogens from 3CP (Jing and Huyop, 2007). Chloride release was accelerated after the sixth day of incubation. Within 15 days, ~90% of supplemented 3CP in the liquid cultures was successfully reduced. This indicates that *T. asperellum* strain SD1 induced a non-ligninolytic system to degrade 3CP and may indicate the production of a novel putative haloalkanoic dehalogenase in this fungal strain. Growth of strain SD1 was not observed in minimal medium without 3CP because of the absence of a source of energy (data not shown).

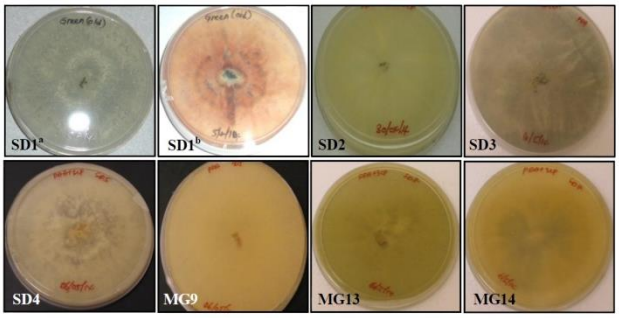

Figure 1: Guaiacol oxidation experiment. All seven six strains were analyzed as control to SD1, *T. asperellum* strain SD2, *T. harzianum* strain SD3, *T. viridae* strain SD4, *T. asperellum* strain MG9, *T. harzianum* strain MG13, and *T. reesei* strain MG14. SD1[a], Control plate (without guaiacol addition) of strain SD1. SD1[b], A brown-red halo formed under and around the mycelia colony of *T. asperellum* strain SD1. The remaining strains without brown coloration were negative for guaiacol oxidation.

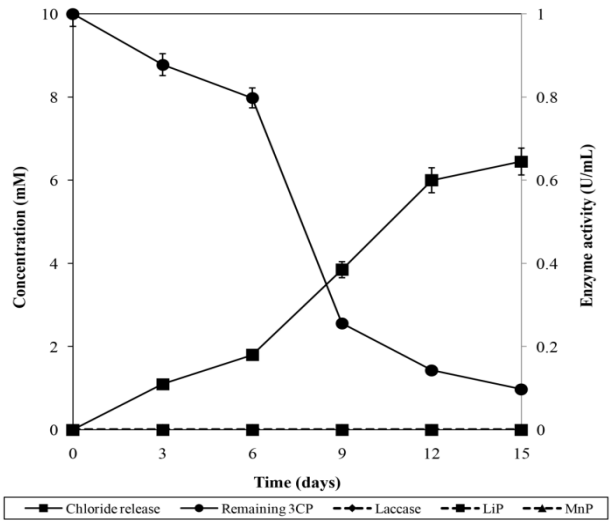

Figure 2: Biodegradation of 3CP by *T. asperellum* strain SD1. Strain SD1 was grown in liquid minimal medium supplemented with 10 mM 3CP as sole carbon source for 15 days. On appropriate time intervals, samples of the growth medium were taken and assessed for ligninolytic enzyme activities (shown as units/mL). Enzyme activity for laccase, MnP, and LiP was not detected at any incubation days (data points for laccase MnP and LiP cannot be seen on the graph because they overlap as values are similar). Samples of the culture medium were also assayed for chloride release and remaining amounts of 3CP (shown as millimolar amounts). The data points and error bars are the mean ± standard error.

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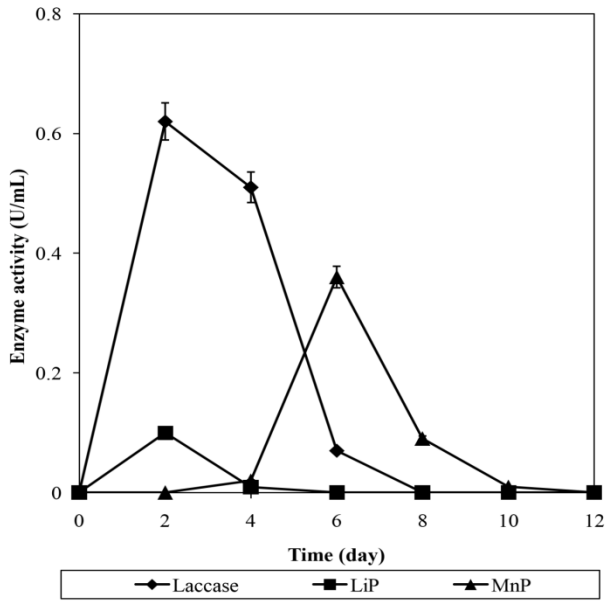

Figure 3: Ligninolytic enzyme activity of *T. asperellum* strain SD1 cultures in glucose minimal medium, without addition of 3CP. Fungus was inoculated and incubation was done statically for 15 days at 30 °C. Samples of growth medium were collected at indicated days for analysis of ligninolytic enzymes that expressed in units/mL. Analysis was performed in duplicates and the data points were expressed in the mean ± standard errors.

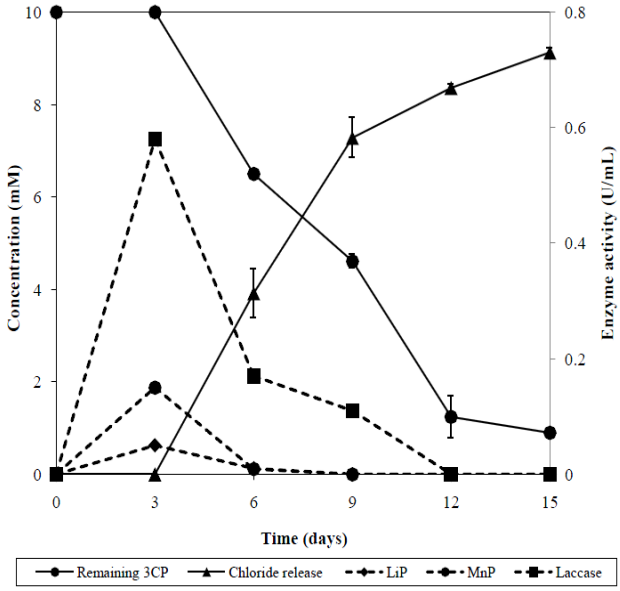

Figure 4: Ligninolytic degradation of 3CP by *T. asperellum* strain SD1 cultures. Strain SD1 was grown in liquid glucose minimal medium and 10 mM 3CP was added to the grown cultures after 3 days of incubation. Incubation continued for 15 days and samples of medium collected on appropriate time intervals, for chloride detection (shown as millimolar amounts) and ligninolytic enzyme assays (shown as units/mL). All data plotted in mean ± standard error bars.

Ligninolytic enzyme production in glucose medium

To evaluate the ligninolytic potential of *T. asperellum* strain SD1 cultures, glucose-supplemented minimal medium was used. Figure 3 shows the ligninolytic enzyme activity of *T. asperellum* strain SD1 grown in control cultures of glucose minimal medium, with 3% glucose as a carbon source. LiP activity was extremely low, with a maximum level on the second day of incubation (0.1 U/mL).Of the remaining two ligninolytic enzymes, laccase activity was strongly detected, with a maximum level of 0.62 U/mL on the second day of incubation. MnP activity was delayed and was detected on the fourth day of incubation and later. The maximum MnP activity was 0.36 U/mL on the sixth day of incubation.

Figure 4 shows ligninolytic degradation of 3CP by *T. asperellum* strain SD1 grown in glucose-minimal medium, supplemented with 3CP after three-days of incubation. LiP, MnP, and laccase enzymes were induced in the presence of glucose as the only available carbon source in minimal medium. Addition of 3CP did not completely suppress ligninolytic enzyme production. Similar to what is shown in Figure 3, higher laccase activity was detected earlier during the incubation and reached a maximum of 0.58 U/mL on the third day of incubation. However, MnP activity was lower compared in Figure 3. These results suggested that the presence of 3CP inhibited production of ligninolytic enzymes if 3CP was the only available carbon source in *T. asperellum* strain SD1 cultures. Under ligninolytic conditions induced by the glucose-minimal medium, 3CP was efficiently degraded and chloride was released (Figure 4). Dechlorination and the disappearance of 3CP were accelerated, but only after production of ligninolytic enzymes. Within 15 days of incubation, 90% of supplemented 3CP was successfully dechlorinated by the enzymatic system.

Induction by guaiacol

Strain SD1 was grown in minimal medium with 10 mM 3CP and in the presence of 2 mM guaiacol to induce ligninolytic enzymes. The production of ligninolytic enzymes in the liquid cultures resulted in guaiacol oxidation (Table 1). In contrast, control medium (with 10 mM 3CP but without guaiacol) did not show ligninolytic activity. The presence of guaiacol induced the production of ligninolytic enzymes, even in the presence of 3CP as sole carbon source. Table 1 shows that LiP and MnP activities were similar, with 1.024 and 1.216 U/mL, respectively, after 10 days of growth in the presence of guaiacol in the medium. In contrast, laccase activity was higher. Without guaiacol in the minimal medium, no

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Table 1: Ligninolytic activity in the presence and absence of 2 mM guaiacol that was added to the minimal medium supplemented with 10 mM 3CP as sole carbon source.

Time in culture (days)	LiP activity (U/mL)		MnP activity (U/mL)		Laccase activity (U/mL)		3CP degradation (%)	
	+Guaiacol	–Guaiacol	+Guaiacol	–Guaiacol	+Guaiacol	–Guaiacol	+Guaiacol	–Guaiacol
0	0	0	0	0	0	0	0	0
5	0.889±0.032	0	0.952±0.003	0	1.468±0.040	0	72.35	21.40
10	1.024±0.017	0	1.216±0.058	0	2.508±0.106	0	100.0	76.51

ND, not detected
% of degradation was determined based on the total chloride released
Values are the mean (n = 3) ± standard error.

ligninolytic enzymes were detected in *T. asperellum* strain SD1. This demonstrates the inhibition of the production of these enzymes by 3CP. The percentage of 3Cp degradation was calculated based on the chloride release from the cultures. In the presence of guaiacol, 3CP was successfully and completely (100%) degraded within 10 days (Table 1). During growth for a similar length of time without guaiacol, ~76.51% of 3CP was degraded.

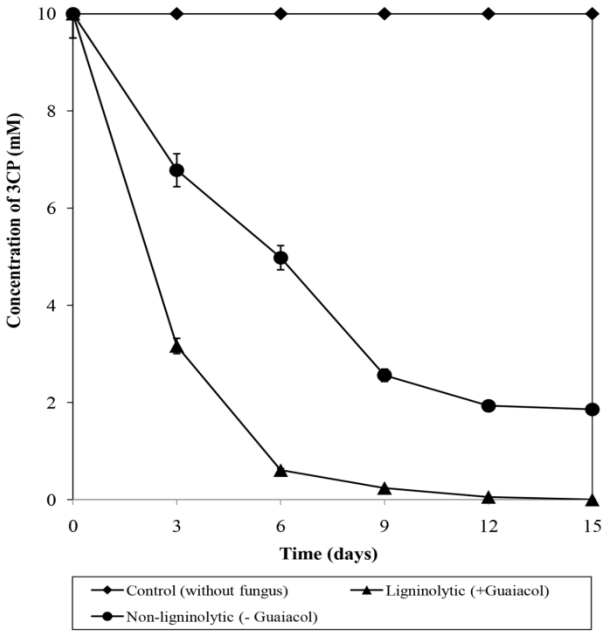

Figure 5: Ligninolytic and non-ligninolytic degradation of 3CP. Ligninolytic condition was achieved by adding guaiacol (2 mM) to liquid minimal medium supplemented with 10 mM 3CP as sole carbon source. Fungus in non-ligninolytic conditions was inoculated to minimal medium supplemented with 3CP (10 mM) but guaiacol was not added. Remaining 3CP in the supernatant of growth medium collected was analyzed by HPLC techniques. The data points and error bars are the mean ± standard error.

Comparison of ligninolytic and non-ligninolytic conditions in the presence of 3CP

Utilization of 3CP by *T. asperellum* strain SD1 was evaluated under both ligninolytic and non-ligninolytic conditions. Ligninolytic conditions were created by adding guaiacol (2 mM), and both conditions were created by growth in liquid minimal medium supplied with 10 mM 3CP. Approximately ~95% of supplemented 3CP was successfully reduced in the treatment medium within 6 days of incubation under the ligninolytic condition induced by guaiacol (Figure 5). The ligninolytic enzyme system rapidly broke down 3CP and released chloride ions into the medium. Under the non-ligninolytic condition, 3CP was more slowly utilized over the 15 days of incubation. Without guaiacol, strain SD1 did not produce ligninolytic enzymes (Table 1). However, dehalogenation (~80%) still occurred, which may indicate the existence of another enzymatic system that catalyzes dehalogenation of 3CP and release of free chlorides into the medium. However, this observation was not tested further.

DISCUSSION

We previously described the isolation of *Mucor* sp. SP1 and *Trichoderma* sp. SP2 as 3CP-degrading filamentous fungi from soil (Parvizpour *et al*., 2013). In our current study, we analyzed *T. asperellum* strain SD1, isolated from palm plantation soil that had been exposed to many industrial chemicals. *T. asperellum* strain SD1 efficiently degraded 3CP. In addition, chromatography analysis showed that degradation of 3CP by this strain also results in production of propionic acid as aco-metabolic reaction (Shanmugaprakasham, 2014). Thus, we have hypothesized there must be a cluster of enzymatic reaction(s) that favored the dehalogenation of 3CP by *T. asperellum* strain SD1.

The co-metabolic synthesis of propionic acid with the substitution of hydrogen after dechlorination is similar to previously described reductive mechanisms (Wiegel and Wu, 2000; Habash *et al*., 2004). Thus, our current investigation strongly suggests that previously reported synthesis of propionic acid in the breakdown of 3CP by *T. asperellum* strain SD1 may occur by a reductive dehalogenase or other extracellular enzyme(s) that catalyze reductive reactions. In fungi, the catalytic mechanisms of extracellular peroxidases and laccase of white rot fungi are similar (Novotný *et al*., 2004; Marco-Urrea and Reddy, 2012).

Work by Khindaria *et al*. (1995) has demonstrated that halocarbons such as CCl_4, $CHCl_3$, CH_2Cl_2 and trichloroethylene can be reductively dechlorinated by this mechanism. This reductive dehalogenation is a free

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radical–mediated process that is initiated by the oxidation of veratryl alcohol into a cation radical by lignin peroxidase enzymes. This veratryl alcohol cation radical in turn oxidizes organic acids such as ethylene diamine tetraacetic acid (EDTA) in growth medium or oxalate, which is secreted extracellularly by most fungal strains, into their respective radicals. This oxalate was then decarboxylated to release carboxylate anion radicals (CO_2•–),which finally dehalogenate aliphatic halogenated organic acids by reductive dehalogenation (Novotný *et al*., 2004; Marco-Urrea *et al*., 2008; Diwaniyan *et al*., 2010; Hatakka and Hammel, 2011).

Qualitative screening indicated that *T. asperellum* strain SD1 produces the ligninolytic enzymes LiP, MnP, and laccase. Guaiacol oxidation is one of the best qualitative methods for differentiating ligninolytic enzyme producers. Mtui and Nakamura (2008) demonstrated guaiacol oxidation by the marine fungal isolate *Laetiporus sulphureus* after 7 days of incubation. El Aty and Mostafa (2013) also used this method to screen for potential laccase-producing fungi. In the saprophytic environment, most wood-degrading fungi possess a unique wood-degrading system that is catalyzed by the ligninolytic enzymes LiP, MnP, and laccase (Novotný *et al*., 2004). Other report has demonstrated the potential for ligninolytic enzymes to degrade many recalcitrant and complex pollutants (Marco-Urrea and Reddy, 2012).

Ligninolytic enzyme production may not predominantly occur in the presence of 3CP, as the fungus utilizes glucose as its major carbon source. However, we demonstrated that our strain could degrade environmental pollutants in both the absence and presence of another carbon source such as glucose. This is important because most pollutant-degrading microbial isolates fail to break down pollutants in resource-rich nature because they do not utilize pollutants unless they are the sole carbon source.

The reason for the non-ligninolytic condition or the absence of extracellular peroxidases and laccase is not well understood. In this study, no ligninolytic enzymes were detected in the presence of 3CP as the sole carbon source. Inhibition of ligninolytic enzymes is often linked with the presence of toxic pollutants in the growth medium (Dhawale *et al.*, 1992). For example, Ryu *et al*. (2000) showed that inhibition of ligninolytic enzymes during biodegradation of pentachlorophenol by *Phanerochaete chrysosporium*, a white rot fungus**,** is due to the pentachlorophenol in the growth medium. However, 3CP did not suppress the activity of LiP, MnP, or laccase enzymes in glucose supplemented minimal medium or in minimal medium added with guaiacol.

Despite these conflicting results, we have demonstrated a novel finding regarding the microbial dehalogenation of β-chlorinated haloalkanoic acids. Our findings focused on substitution of the halogen position after the dechlorination step in the dehalogenation mechanism. All reported bacterial β-haloacid dehalogenases belong to hydrolytic groups (Jing and Huyop, 2007; Mesri *et al*., 2009; Hamid *et al*., 2013). The carbon-halogen bond in 3CP is cleaved by a hydrolytic dehalogenase and subsequently forms 3-hydroxypropionic acid through addition of a hydroxyl (Lin *et al*., 2011; Hamid, 2014). In a previous study by Shanmugaprakasham (2014) only the synthesis of propionic acid was demonstrated as a metabolite of 3CP degradation mechanism by *T. asperellum* strain SD1. Synthesis of propionic acid was proven occurred in minimal medium supplemented with 3CP as sole carbon source. This study also has shown propionic acid was not detected under ligninolytic conditions (in glucose-minimal medium and in minimal medium added with guaiacol). Therefore, current findings in co-relation to previous findings have demonstrated that there is a reductive dehalogenase system in *T. asperellum* strain SD1 that responsible for dechlorination of 3CP under non-ligninolytic conditions. Further investigations on this novel aliphatic reductive dehalogenating enzyme which belongs to fungal group is important as these dehalogenases are widely applied in many commercially practiced industrial biocatalytic processes (Swanson, 1999).

Our findings are similar to the work of Reddy *et al*. (1998) on the discovery of a reductive dechlorination reaction that is involved in the biodegradation of 2,4,6-trichlorophenol by *P. chrysosporium*. This group described two components of the reductive dehalogenation enzyme system that are involved in the dehalogenation of chlorinated phenols. The initial dehalogenation of chlorine at the 4-position is catalyzed by LiP or MnP activity. However, the subsequent products suppress the ligninolytic system, and the removal of remaining chlorine is performed by reductive dehalogenase enzyme systems (Reddy and Gold, 2001). Biodegradation of pollutants under non-ligninolytic conditions is also possible (Dhawale *et al*., 1992).

An early report by Köhler *et al*. (1988) showed that dichlorodiphenyltrichloroethane degradation is not correlated with the production of ligninases. In addition, biodegradation of xenobiotics such as phenanthrene and pentachlorophenol by *P. chrysosporium* occurs under ligninolytic as well as non-ligninolytic conditions (Dhawale *et al*., 1992). Despite the many studies that have been performed, the ligninolytic role in the degradation of various pollutants remains unclear. Our assumption regarding the existence of a novel reductive dehalogenase is also supported by the report by Sutherland *et al*. (1991), which shows that enzymes other than ligninasesare involved in the degradation of halogenated hydrocarbons such as phenanthrene. Therefore, the results collectively indicate that ligninolytic enzymes are not essential for the degradation of 3CP by *T. asperellum* strain SD1.

CONCLUSION

Our findings strongly suggest that *T. asperellum* strain SD1could be used for degrading 3CP, which is a potentially hazardous pollutant. We have demonstrated 3CP degradation by this strain under both ligninolytic and non-ligninolytic conditions, and we found that ligninolytic enzymes were not the key enzymes involved in 3CP

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biodegradation activities. Purification and further characterization of this novel putative haloacid dehalogenase and the resulting valuable metabolite in this unique system are in progress. We are using a genetic approach to clarify the roles of ligninolytic enzymes and the reductive haloacid dehalogenase involved in 3CP degradation.

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