Frontiers in Water and Environment

Research Article **Volume**
1(1), 2023, 35-46
ISSN: 2785-9029

Potential co-culture of fungi in degradation of 1,4-Dioxane

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SUBMITTED: 28 April 2021; REVISED: 18 May 2021; ACCEPTED: 21 May 2021;

ABSTRACT: Biodegradation using fungi become a concern for a decade to remove pollutants, it is due to cost-effectiveness, inherent eco-friendly properties, and potential for complete decomposition of harmful compounds. A single strain of fungi was studied to degrade various pollutants has been commonly investigated. However, the degradation of 1,4-Dioxane by co-culture white-rot fungi has not been investigated. Dioxane contamination is known as public health due to its adverse health effect, including carcinogens and persistence in the natural water system. Therefore, this study has aimed to investigate the degradation of 1,4-Dioxane and compared it with the addition of inducer. Co-culture of fungi (*Trametes versicolor* F200 and *Pestalotiopsis* sp. NG007) can improve degradation of 1,4-Dioxane after the addition of inducers. It degraded dioxane 71% at 50 ppm and 47% at 500 ppm for 60 days. $MnSO_4$ as an inducer was capable to enhance the activity of enzymes and degradation rate.

KEYWORDS: 1,4-Dioxane; biodegradation; *Trametes versicolor* U97; *Pestalotiopsis* sp. NG007, co-culture

1. Introduction

1,4-Dioxane is a synthetic chemical with colorless, chemically stable, highly soluble, and flammable compound. It is widely used as a solvent and stabilizer of chlorinated solvents, especially 1,1,1-trichloroethane (TCA) [1]. Although TCA was eventually banned in the U.S in the 1990s, 1,4-Dioxane has continued to be used as a solvent for paints, lacquers, pesticides, oils, textile processing, polishing compositions, as a wetting and dispersing agent in paper, coating, plasticizers and can be found in personal care products and cosmetics [2] [3]. The extensive use, improper storage, the disposal and limited regulation on this xenobiotic, results in a considerable release of 1,4-Dioxane into the environment so it can be detected in industrial sewage, landfills, rivers, and groundwater [4] [5].

The U.S. Environmental Protection Agency certify dioxane contamination is a public health due to its adverse health effect, including carcinogen and persistence in natural water system [4]. Its solubility in water generate the high heat capacity and high polarity in water-dioxane or other solvent-dioxan mixtures, but also creates challenges for separation via dehydration [3]. In 1970, the first study of 1,4-Dioxane detection in water has been considered as an emerging contaminant in drinking water because of its potential carcinogenicity, lack of

wastewater management, and absence of regulations in many countries [6]. Based on this reason, it is desirable to remove 1,4-Dioxane from surface water and groundwater. Conventional physical and chemical processes such as coagulation and activated carbon adsorption have low ability to remove dioxane because of its high solubility [7].

Biodegradation is an interesting method to degrade pollutants because of its cost-effectiveness, inherent eco-friendly properties, and potential for complete decomposition of harmful compounds [5]. Fungi can be potential as biological agent for degradation processes. Fungi are extraordinary microorganisms found in every habitat and being tolerant to many extreme environmental conditions [8]. Furthermore, fungi have a high content of functional groups in the cell wall such as amino, amide and phosphate groups encourage the absorption of pollutant. Fungi can form mycelial networks to undergo degradation, detoxification and maintain stress resistance [9]. The degradation performed by the activities of ligninolytic enzyme including Manganese Peroxidase (MnP), Lignin Peroxidase (LiP), laccase and 1,2-dioxygenase and 2,3-dioxygenase [10] [11].

Recently, the application of fungal co-culture become a new tendency for biotechnological processes such as, ligninolytic production, biodegradation and bioremediation. Fungal with different phenotype and genotype interact and form connection (symbiotic, competitive or antagonistic relationships). These interactions have posibility for degradation and nutrient cycling. The synergistic relationship enhance the degradation abilities over the monoculture [12]. Moreover, generally single microbial strain are not very effective to degrade pollutant with high complex molecule. However, fungal co-culture need to be used. Fungal co-culture renowned effectively degrade polycyclic aromatic hydrocarbons and volatile organic compounds [13].

To date, the study of biodegradation of 1,4-Dioxane has a lot concern with the use of bacteria. Several bacteria reported can utilize 1,4-Dioxane as the sole carbon and energy sources such as *Pseudonocardia benzenivorans* B5, *Pseudonocardia benzenivorans* D17, and *Afipia brromeae* D1 [1] [14]. A few fungi are explored to degrade 1,4-Dioxane [4]. Although degradation of 1,4-Dioxane by fungi has been many explored, the degradation and the oxidation mechanisms of 1,4-Dioxane by co-culture white-rot fungi have not been investigated.

In this study, the ability of free cell and crude enzyme of fungi, *Trametes versicolor* U97 and *Pestalotiopsis* sp. NG007, were to degrade 1,4-Dioxane in liquid medium was examined. To enhance degradation, several mediators and consortium between two fungi were evaluated. This study also investigated the enzymatic activities involving the degradation rate and mechanisms of dioxane degradation.

2. Materials and Methods

2.1. Degradation of 1,4-Dioxane by free cell of T. versicolor U97

A malt extract liquid medium (malt extract 20 g/l, glucose 15 g/l, and polypeptone 1 g/l) was used for degradation experiments. Three 5 mm plugs of strain were added into the Erlenmeyer flask containing 20 ml of medium and then pre-incubated for 7 days to obtain a similar radial growth and minimize fungus growth variation. After pre-incubation, each inoculated flasks was supplemented with dioxane (50 and 500 ppm). The harvest was conducted on days 7, 15, 21, and 30.

2.2. Degradation of 1,4-Dioxane by crude enzyme of T. versicolor U97

T. versicolor U97 grown on wood meal was extracted and dissolved in 50 mM malonate buffer (pH 4.5) as described in our previous study [15]. Further, 1.5 g of crude enzyme (equal with 0.4 U/ml MnP) was added into 20 ml distilled water in 100 ml Erlenmeyer flask together with dioxane (50 and 500 ppm). The cultures were incubated for 7, 15, 21, and 30 d.

2.3. Degradation of 1,4-Dioxane by co-cultures T. versicolor U97 and Pestalotiopsis sp. NG 007

Strains *T. versicolor* U97 and a filamentous fungus, *Pestalotiopsis* sp. NG007 were pre-cultured togeteher in liquid medium. Co-cultures between *T. versicolor* U97 and *Pestalotiopsis* sp. NG 007 was used to degrade 1,4-Dioxane with the compositions of 1:1 (w/w).

2.4. Addition of mediators for enhancing degradation

To enhance the degradation, several mediators e.g.: 1 mM Mn^{2+}-1 mM H_2O_2; 1 mM Cu^{2+}-1 mM H_2O_2; 1 mM veratryl alcohol - 1 mM H_2O_2, in co-cultures *T. versicolor* U97 and *Pestalotiopsis* sp. NG007 were used to degrade 1,4-Dioxane.

2.5. Residual dioxane analysis

Depletion of 1,4-Dioxane was determined by injection of 1 µl samples to gas chromatography coupled with mass spectrometry (GC-MS Shimadzu QP-2010), equipped with a TC-1 column (30 m, id: 0.25 mm). The carrier gas was helium delivered at a constant flow rate of 1.5 ml/min with a column pressure of 100 kPa and interface temperature of 250°C. The temperature program was set at 40 °C for 2 min, raised at 5 °C/min to 90 °C, then 10 °C/min to 250 °C, and maintained at 250 °C for 5 min.

3. Results and Discussion

3.1. Degradation of 1,4-Dioxane by free cell of T. versicolor U97

Biodegradation is an interesting process to obliterate diverse pollutants by using biological activity. In general definition, this process utilized the ability of organisms such as fungi to remove the environmental contaminants [3]. The biodegradability is associated with the concentration and composition of hydrocarbons. The high level of hydrocarbon strongly inhibits fungal growth, generates unfavorable biodegradation and may cause death of fungal [16]. The effect of concentration was studied at 50 and 500 ppm of 1,4-Dioxane concentrations. In the preliminary study, *T. versicolor* U97 has able to degrade 1,4-Dioxane 50 and 500 ppm for 65 and 40% on agar medium at 7 d (data was not shown). The higher concentration of 1,4-Dioxane effect in slower degradation because of difficulties the fungi for oxidizing the substrate. In line with result on agar medium, *T. versicolor* U97 can degrade several concentrations of 1,4-Dioxane in liquid medium with different degradation capacities. *T. versicolor* U97 able to degrade 1,4-Dioxane up to 60% at 50 ppm and 28% at 500 ppm for 30 days (Fig. 1.).

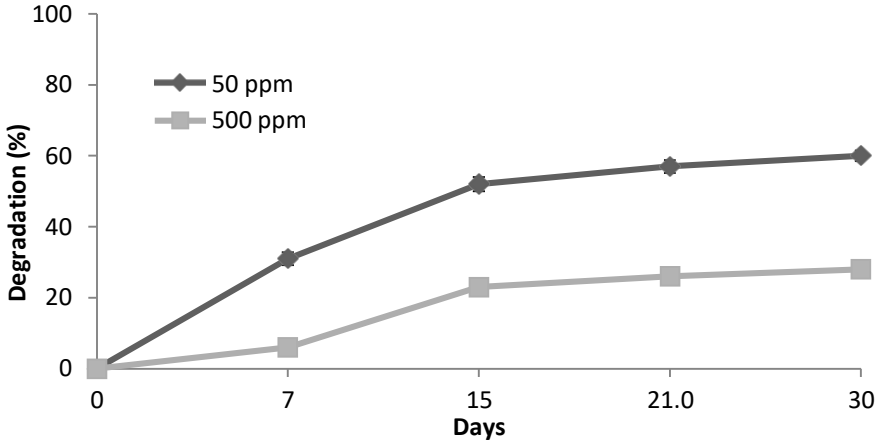

Figure 1. Degradation of 1,4-Dioxane by free cell of *T. versicolor* U97 in various concentrations.

Currently, the research of 1,4-Dioxane has been focus in recent years. Several microorganisms have been reported for their capacity to degrade 1,4-Dioxane. *Cordyceps sinensis* is known to metabolize 1,4-Dioxane under aerobic conditions with an initial 1,4-Dioxane concentration of 0.034 M at 30 ^{o}C incubation [4]. A gram negative propanotroph *Azoarcus* sp. DD4 able to degrade 1,4-Dioxane initially from 10.4 mg/L to <0.4 μg/L within 14 days [17]. Two bacteria, *Mycobacterium vaccae* JOB5 and *Rhodococcus jostii* RHA1 have able to degrade 20 ppm 1,4-Dioxane 87-95% for 72 h with addition of inducers, 1-butanol and propane [18]. *Rhodococcus aetherivorans* JCM 14343 able to completely degrade 20 mg/L 1,4 dioxane within 30 h and indicates the utilization of 1,4-Dioxane, 1,4-butanediol, 1-butanol, phenol, glucose and acetic acid for growth [19]. Filamentous fungi *Pseudallescheria boydii* ZM01 able to utilize 20 mM THF and 1,4-Dioxane as a substrate within 5 days [9].

In accordance with the research by Tusher et al. (2019), the high concentration of pollutant has a difficult to be degraded [20]. Therefore, enriched bacteria consortium 112 was used to degrade 1,4-Dioxane. This consortium completely degraded 50 and 100 mg/L of 1,4-Dioxane within 10 and 12 days. However, 1,4-Dioxane at concentration 500 dan 1000 mg/L exhibited the uncomplete degradation even after 25 days. The bacteria consortium 112 degraded only 68.2% and 28.6% of the 1,4-Dioxane at initial concentration of 500 and 1000 mg/L, respectively [20]. The study by Lee et al. (2020), enriched culture in various concentration of 1,4-Dioxane, ranging from 5 mg/L, 50 mg/L, 200 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L and 2000 mg/L showed differs degradation rate. The high concentrations (1500 mg/L and 2000 mg/L) was took more time to be degraded among other concentrations, approximately 200 h [21].

Fungal activity is recognized as a green technology to remove contaminants. The mechanism of biodegradation were characterized into two groups: metabolism and co-metabolism. Dioxane biodegradation via metabolism and co-metabolism initiated by the secretion of enzymes. Metabolism pathway is defined as a process of the use of organic contaminants as carbon and energy sources to support fungal growth. Metabolism degradation is challenging because of the high concentration of 1,4-Dioxane affects the microbial growth rate. In contrast, co-metabolism is established as a process of contaminant degradation with the addition of other carbon sources to sustain the microbial growth and enhance pollutant degradation [3] [5] [22].

In this research, all of the study was performed via co-metabolism pathway. A malt extract liquid growth medium was used as an additional substrate. Co-metabolism biodegradation has several advantages that can be obtained. Co-metabolism pathway could effectively remove phenolic compounds. The additional substrate that used for co-metabolism degradation could be used as energy and carbon source for microbial growth, notably when the phenolic compound does not provide sufficient carbon. Moreover, the additional substrate can also improve the microbial resistance to high-strength phenolic compounds [23], for example the study by Barajas-Rodriguez et al. (2018), the result showed that propane as the additional substrate can support microbial growth. The growth rates of co-metabolism consumption of 1,4-Dioxane by mixed culture of *Rhodococcus ruber* ENV425 and ENV487 were higher compared with metabolic consumption by *Pseudonocardia dioxanivorans* CB1190. Thus, the propanotophic mixed culture decreased 1,4-Dioxane from 1,000 to 1 µg/L in less time than *Pseudonocardia dioxanivorans* CB1190 [24].

In co-metabolism system, the degradation of 1,4-Dioxane is expressed by the catalytic enzymes. Therefore, this study analyzed the enzymatic activities including, 1,2-dioxygenase, 2,3-dioxygenase, MnP, LiP and laccase (Table 1).

Table 1. Enzymatic activities by *T. versicolor* U97 at 15 d without and with 1,4-Dioxane

	Laccase	1,2-dioxygenase	2,3-dioxygenase	MnP	LiP
Without dioxane	1,6	45,9	1,8	9	52
With dioxane	0	57,5	0	13	49

Based on the result, the free cell of *T. versicolor* U97 shows the activities of 1,2-dioxygenase, MnP and LiP during the degradation of 1,4-Dioxane. The degradation of 1,4-Dioxane is often present in monooxygenase expression, however, rarely studied among dioxygenase and ligninolytic enzymes [19] [25] [26].

3.2. Degradation of 1,4-Dioxane by crude enzyme of T. versicolor U97

Besides the degradation of 1,4-Dioxane by free cell of *T.versicolor* U97, this research was also carried out the comparing of biodegradation mechanism by crude enzymes from *T. versicolor* U97. The extraction of crude enzyme using malonate buffer resulted only MnP retained in this crude enzyme. In 30 days, this crude enzyme degraded 1,4-Dioxane 53 and 18% at 50 and 500 ppm, respectively (Fig. 2). This result was in accordance with the study by Lourenco et al. (2017), *T. versicolor* showed the important role of MnP in degrading pollutants. MnP activity was the highest among other ligninolytic enzymes. During the degradation of two-phase olive mill waste (TPOMW), MnP activity reached 15-139 U/L [27].

MnP is a heme belongs to class II peroxidase catalase. MnP has high redox potentials and remarkably wide ranges of phenolic and non phenolic compound. MnP able to degrade macromolecule into small molecule through the reducing of carbon atoms and molecular weight into carbon dioxide and water [28]. Their catalytic pathway comprise the oxidation of Mn^{2+} to Mn^{3+} in the present of phenolic compound stabilized by reacting with carboxilic acid such as tartrate as ion chelator, the oxidation lead to resulting unstable radicals. In addition, the redox mediators such as thiyl or lipid radicals required for the oxidization nonphenolic compound [29].

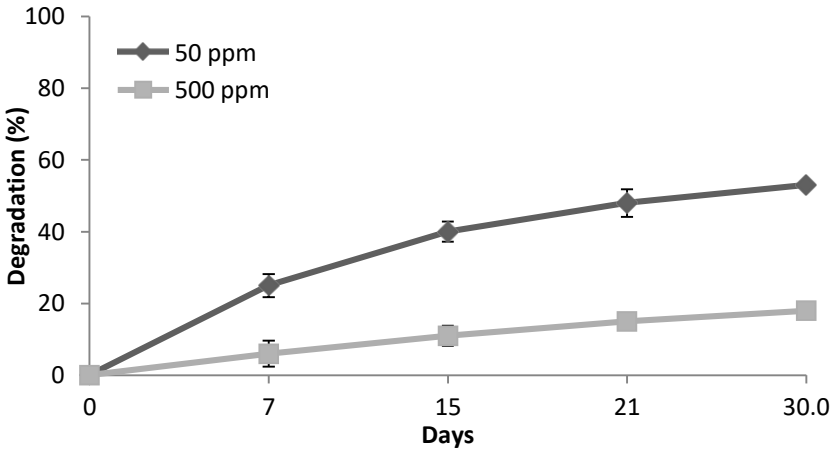

Figure 2. Degradation of 1,4-Dioxane by crude enzyme of *T. versicolor* U97 in various concentrations

Another study, the utilization of crude enzyme also dealing with other environmental problems exhibit the extraordinary degradation. The purified laccase from *Trametes hirsuta* was completely degrade 100 ppm of 1,4-Dioxane within 2 hours [30]. Monooxygenase from *Xanthobacter flavus* DT8 was able to completely mineralize 1,4-Dioxane with concentration from 10 mg/L to 1200 mg/L [5]. *Psudonocardia* sp. ENV478 can metabolize 1,4-Dioxane supplemented with THF via enzyme dioxane monooxygenase (DXMO) [22].

3.3. Degradation of 1,4-Dioxane by free cell of T. versicolor U97 and Pestalotiopsis sp. NG007.

The previous studies, 1,4-biodegradation was mostly examined by pure culture, a single strain were isolated and designated for 1,4-Dioxane biodegradation. However, only few studies examined 1,4-Dioxane biodegradation on mixed cultures [21] [31]. In the present study, *Pestalotiopsis* sp. NG007 was used as co-culture of *T. versicolor* U97. These fungal co-culture were studied to degrade 1,4-Dioxane in co-metabolism pathway with malt extract liquid medium as an additional substrate. Since *Pestalotiopsis* sp. NG007 is fungus producing 1,2-dioxygenase and *T. versicolor* U97 is fungus producing ligninolytic enzyme, consortium of these fungi could improve degradation of dioxane (Fig. 3.).

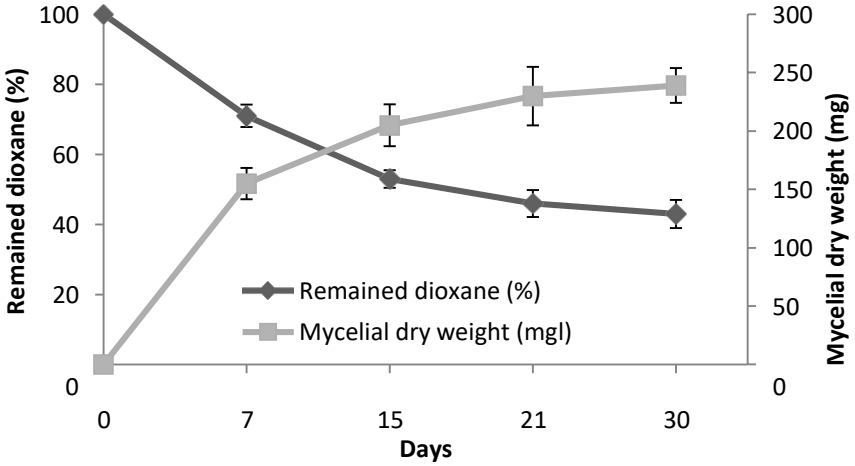

Figure 3. Performance of co-culture *T. versicolor* U97 and *Pestalotiopsis* sp. NG007 to degrade 1,4-Dioxane (50 ppm)

Pestalotiopsis sp. NG007 is a marine-derived fungus that commonly known toproduce secondary compounds and metabolites. This fungus is spreadly everywhere in the world. *Pestalotiopsis* sp. NG007 are known to have a rich set of enzymes and several studies were already demonstrated to degrade organic matter [32] [33] [34]. *Pestalotiopsis* sp. NG007 reported has a high 1,2-dioxane activity during the biodegradation of petroleum hydrocarbons. NG007 showed their capability to utilize 21-39% petroleum hydrocarbons for metabolism and growth. About 51-67% of aliphatic can be assimilated by the strain [35].

The white-rot fungus *Trametes versicolor* is recognized as a producer of ligninolytic enzymes [36]. Laccase from *T. versicolor* was extensively studied because of their potential to degrade numerous organic pollutans. This enzyme can decolorize mono azo including Orange 2 and Acid Orange 6. The decolorization percentage afrer 120 hours was 72.8% and 45.3% for Orange 2 and Acid Orange 6, respectively [37]. LiP from *T. versicolor* F200 was degrade lignin in black liquor. During degradation process, the result shows that LiP (571.21 U/l) was higher than MnP (84.83 U/I) and laccase (120.86 U/I) [38].

Dioxygenase and ligninolytic enzymes have an important role in the biodegradation of 1,4-Dioxane. The study by Zhou et al. (2016) showed that dioxygenase was open the aromatic ring of 1,4-Dioxane via ortho-cleavage pathway [39]. In addition, Kumar & Chandra (2020) reported the extracelullar ligninolytic enzymes could attack on several types of bonds such as β-O-4 ether bond, biphenyl bond, O-demethylation enzyme systems, and aromatic organic pollutants and carried out one-electron oxidation to produce free cation radicals. These cation radicals may undergo chemical reaction including hydroxylation or C-C bond cleavage generate hydrophilic products [40]. Nakamiya et al. (2005), reported that generally the degradation pathway of 1,4-Dioxane can be considered as the sequential production of ethylene glycol, glycolaldehyde, glycolic acid, and oxalic acid, followed by incorporation of the glycolic acid and/or oxalic acid into the tricarboxylic acid (TCA) cycle. Afterwards, carbon dioxide (CO_2), oxalic acid and / or components of the cell generated as a by products (Fig. 4) [4].

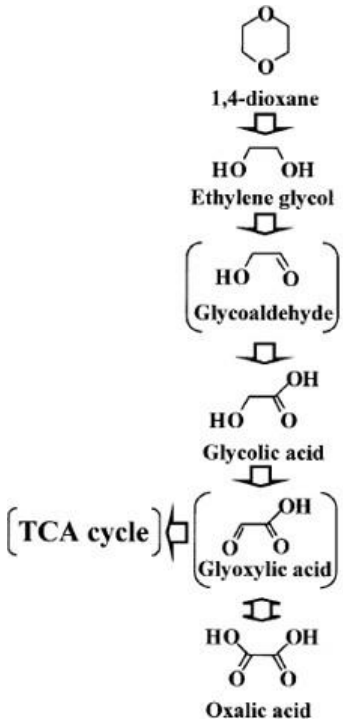

Figure 4. Degradation pathway of 1,4-Dioxane by *Cordyceps sinensis* [4].

Based on the result, pure culture and co-culture are potential to degrade 1,4-Dioxane. Unfortunately, this consortium only slightly improved degradation of dioxane may due to antagonism effect on nutrients competition (Fig. 2 and Fig. 3). This competition effect can be seen in reduction of fungal biomass during degradation [41]. In addition, several factors such as temperature, pH, and oxygen level also influence the successful of biodegradation [42]. It can affect the enzyme activity, may depletion of biodegradation rate and inhibit the utilization of carbon within the pollutant [43]. The research by Polasko et al. (2019), the co-culture consists of mixed consortium KB-1 (Dehalococcoides) and aerobically bacteria strain *Pseudonocardia dioxanivorans* CB1190 were capable to degrade TCE in anaerobic/aerobic conditions. However, each of microbes took different time for degradation depend on oxygen level. During the anaerobic incubation period, KB-1 took the time for degradation of TCE ro cDCE. After the transition to aerobic condition, CB1190 showed the biodegrade of its transformation product, cDCE. It may cause by the oxidative stress on the enzymes that generate the alternative respiratory chain in anaerobes, as well as the production of reactive substances, such as superoxide radicals and hydrogen peroxide [31].

3.3. Degradation of dioxane by free cell of T. versicolor U97 and pestalotiopsis sp. NG007.

To accelerate the dioxane degradation, veratyl alcohol, $MnSO_4$ and $CuSO_4$ were used as mediators.

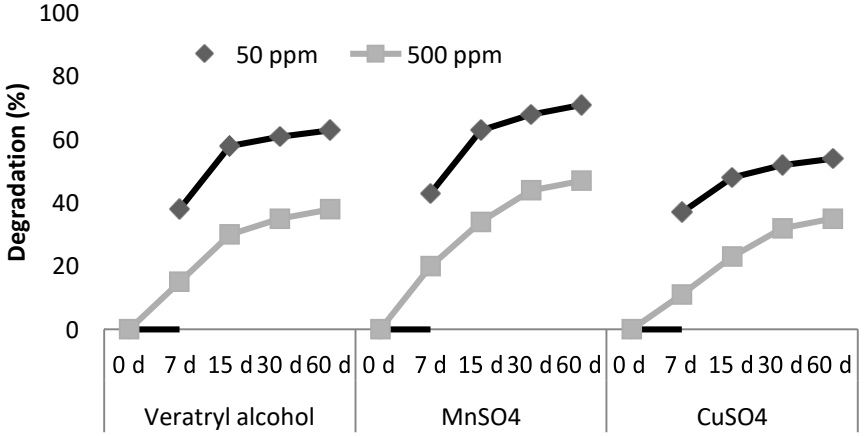

Figure 5. Effects of inducers on degradation of 1,4-Dioxane by co-culture *T.versicolor* U97-*Pestalotiopsis* sp. NG007

The degradation of 50 and 500 ppm of 1,4-Dioxane by co-culture of fungi (*T.versicolor* U97-*Pestalotiopsis* sp. NG007) was significantly enhance by $MnSO_4$ but was significantly inhibit by $CuSO_4$. The degradation of 1,4-Dioxane was up to 71% at 50 ppm and 47% at 50 ppm for 60 days (Fig. 5). According to Golveia et al. (2020), mediators are low molecular weight substrates that have a role as an "electron carrier" between the catalytic enzyme and the target pollutant. Because of their size, they can not directly enter the enzyme active site. Therefore, after becoming oxidized by the enzyme, the mediator remain the active site and could oxidize any substrate. Eventually, this redox mediator is capable to enhance the activity of enzyme [44]. Another study by Iqbal & Asgher (2013), only by addition of $MnSO_4$, the degradation of 1,4-Dioxane was significantly improved. Mn^{2+} stimulated MnP activity of immobilized *Trametes versicolor* IBL-04. The immobilized MnP has able to decolorize textile industry effluent in a Packed Bed Reactor System (PBRS) by 98.8% within 5 h [45].

In the previous result, *Pestalotiopsis sp.* NG007 was examined to degrade asphalt. The ligninolytic enzymes (MnP and laccase) of this strain were not sufficient to degrade asphalt. By using addition of Mn^{2+} and H_2O_2, the degradation of asphalts could be improved. Simultaneous addition of Mn^{2+} and H_2O_2 had a very good effect on the biodegradation of asphalt bacause it increased MnP and laccase activity [11]. Perez and Jeffries (1992) reported that a low concentration of Mn^{2+} was sufficient to increase MnP activity in batch cultures during degradation of pollutant. 4-hydroxy-3-methylbutan-2-one was detected as intermediate product in 1,4-Dioxane degradation [46]. Chen et al (2016) reported that 1,4-Dioxane was identified as a metabolic product in 1,4-Dioxane degradation by monooxygenase activity from *Xanthobacter flavus* DT8 [5].

4. Conclusions

This is the first known study to evaluate the ability of co-colture white-rot fungus, *Trametes versicolor* U97 and filamentous fungus, *Pestalotiopsis* sp. NG007 to degrade 1,4-Dioxane, and compare the efficiency of inducers on 1,4-Dioxane removal. We demonstrated that *T. versicolor* U97 degraded dioxane at 30 days. Co-culture of fungi can improve degradation of 1,4-Dioxane after addition of inducers. It degraded dioxane 71% at 50 ppm and 47% at 500 ppm for 60 days. Dioxygenase and ligninolytic enzymes have an important role in the biodegradation of 1,4-Dioxane. Moreover, the additional substrate, $MnSO_4$ as an inducer, can improve the degradation rate. This inducer can act as an redox mediator which capable to enhance the activity of enzymes.

Acknowledgments

I sincerely express my deep sense of gratitude to Dr. Sanro Tachibana (Professor) for his extraordinary cooperation, and valuable guidance and supervision.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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