Are Fungi the Future for the Bioremediation of Contaminated Soils?

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Abstract

White rot basidiomycetes are known to degrade recalcitrant pollutants such as polychlorinated biphenyls (PCBs) that pose serious risks to the environment and health. We compared the biodegradability of Aroclor 1248 (3,3',5,5'-tetrachlorobiphenyl) by four different white-rot fungi and examined the effects of phenol oxidase and peroxidase produced by the fungi. The four white-rot fungi that were studied were *Phanerochaete chrysosporium, Agaricus bisporus* (portobello) *Lentinula edodes* (shiitake) and *Grifola frondosa* (maitake). The biodegradation of Aroclor 1248 was determined via gas chromatography, and enzyme activity was measured via an enzyme assay. This study has shown *P. chrysosporium* and shiitakes to be effective bioremediators of Aroclor 1248 in contaminated substrate.
Keywords: PCBs; mycoremediation; bioremediation; white rot fungi; basidiomycetes; P*hanerochaete chrysosporium; Agaricus bisporus* (portobello); *Lentinula edodes* (shiitake); *Grifola frondosa* (maitake); soil contamination; Phenol Oxidase; Peroxidase; Enzyme Assay; Microwave Assisted Extraction; Gas Chromatography, UV-Vis Spectrophotometry

Introduction

Polychlorinated biphenyls (PCBs) are persistent organic pollutants (POPs) that were used extensively in various industrial applications such as hydraulic and dielectric fluids until banned by the EPA in 1979 (EIP Associates, 1997). However, their persistence and widespread use during the 20th century has made them ubiquitous in the environment (Pointing, 2001). They have been found in remote locations such as the eggshells of Arctic seabirds (Braune and Mallory, 2017), as well as in penguins and Antarctic birds of prey (Wolschke et al, 2015). Because of their degree of stability, PCBs have a propensity to both bioaccumulate and biomagnify in the food chain (Fensterheim, 1993). Their accumulation has been linked to a

variety of health effects, ranging from endocrine disruption to birth defects to possibly cancer (Cogliano, 2016). Therefore, their removal from contaminated sites is of the utmost importance (Ross, 2004; Svobodová et al, 2009). Unfortunately, conventional methods for PCB mitigation rely heavily on chemical landfills, which do little more than to contain the problem, and incineration, which is extremely energy-intensive (Woodyard, 1990). Both methods are exorbitantly expensive as well; from 1978 to 1990 alone, PCB contamination management cost the U.S. government over $1 billion (Woodyard, 1990). The need for a cheaper solution to mitigating such a ubiquitous pollutant could not be clearer, and is a major factor in the recent studies into bioremediation as a viable alternative to PCB pollution mitigation (e.g. Anastasi et al, 2008; Chekol et al, 2004; Passatore et al, 2014).

Some of this research has focused on phytoremediation, or uptake of pollutants by plants, as a means of extracting PCBs from contaminated substrate (Chekol et al, 2004). However, these methods have more recently been argued to have limited efficacy; the lipophilicity of PCBs, while enabling them to easily accumulate in animal tissue, does not make them easily taken up by plant roots (Passatore et al, 2014). Additionally, the PCBs that are taken up can be detected as intact molecules once in the plant's tissue (Chekol et al, 2004). This demonstrates that, much like with chemical landfills, the pollutant is only being contained, albeit through a much less invasive procedure than conventional methods (Woodyard, 1990).

Mycoremediation by white-rot fungi presents another alternative method of PCB mitigation, due to their production of lignolytic enzymes. The ecological purpose of these enzymes is to degrade lignin, an extremely stable organic molecule (Baldrian 2008), although they have also been demonstrated to degrade a variety of other recalcitrant organic materials (e.g. Arun and Eyini, 2011; Čvančarová et al, 2012; Tsutsumi et al, 2001). At the smallest scales,

PCBs and lignin are not terribly chemically different; both rely heavily on aromaticity in their molecular structures (Stutz et al, 2017). It is therefore not surprising that these enzymes, phenol oxidase and peroxidase, have also been shown to degrade PCBs (Beaudette, 1998; Čvančarová et al, 2012; Köller, 2000). Although there have been a number of studies investigating the abilities of various white-rot basidiomycetes to enzymatically degrade PCBs, to the best of our knowledge, few have taken steps to grow the fungi in substrate that would be found in the natural environment. Some studies (e.g. Bruzzoniti et al 2012; Stella et al, 2017; Chekol et al, 2004) have looked at the efficacy of PCB breakdown in soil, but these did not go any further to recreate an accurate "natural" matrix; i.e. with wood and other organic matter. In order to truly understand which species would be most effective at a polluted site, it is important to minimize the inaccuracies inherent in studying fungi in a laboratory setting, especially with respect to substrate. To this end, we tested the effects of three white-rot fungi against Aroclor 1248, in a substrate comprised mainly of store-bought soil, and sterilized sawdust.

Methods

Species Selection:

Our species were chosen for several reasons, respectively. *Phanerochaete chrysosporium* is a well-established model white-rot fungus, and has been used in a variety of studies examining PCB bioremediation with lignolytic enzymes (e.g. Hiratsuka et al, 2005; Dietrich et al, 1995; Čvančarová et al, 2012). The amount of literature available on the behavior of phanerochaete in the presence of PCBs makes it a useful analog when considering less-studied species. One study (Beaudette et al, 1998) reported four white-rot fungi as more successful than phanerochaete at degrading PCBs; two of these were *Pleurotus ostreatus* (oysters) and *Trametes versicolor*

(turkey tails). Given the relative abundance of literature on these species as bioremediators (e.g. Pointing et al, 2001; Byss et al, 2008; Anastasi et al, 2008, Gao et al 2010), we have instead elected to study two species taxonomically close to oysters and turkey tails, respectively: *Agaricus bisporus* (portobellos), and *Grifola frondosa* (maitakes). *Lentinula edodes* (shiitakes) was chosen due to being widely known as an easy-to-grow white rot fungus, while having not been studied as much from a bioremediative perspective.

Fungal Culture Preparation:

Liquid cultures of *Agaricus bisporus* (portobello) *Lentinula edodes* (shiitake), and *Grifola frondosa* (maitake) were purchased from out-grow.com and agar-based cultures of *Phanerochaete chrysosporium* ATCC 34540 grown on a petri dish were obtained from Ecovative, in Troy, NY.

The liquid cultures of the first three species were cultivated in quarter-pint mason jars with 236 ml solution of malt and dextrose (1:1) using well water and were incubated at 23.5° C for 2 weeks. Each species had five jars of replicates.

10 ml of homogenized liquid cultures from each species (except *Phanerochaete chrysosporium*) were then added to Mason jars containing 225 ml of sterilized rye grain. The agar based culture of *Phanerochaete chrysosporium* which was grown on petri dishes was directly added onto the sterilized rye grains. Each species had eight jars of replicates. By this point, portobello cultures showed no biological activity, and were discontinued from the study.

After 2 weeks of incubation period at 23.5° C, the mycelium colonized grain spawns were added to sterilized hardwood sawdust substrate and were incubated at 23.5° C for 3 more

weeks in order for the mycelium to colonize the sawdust substrate. Each species had six jars of replicates.

Mini-Superfund Site Preparation:

The topsoil used in the study to simulate a PCB contaminated field site, was purchased from a commercial source. Aroclor 1248 was inoculated into the soil according to methods described in Lopez-Avila et al (1995). After sterilizing twelve quarter-pint Mason jars containing 100 ml of soil using an autoclave, each soil samples were inoculated with 10 ml of 100 ppm Aroclor 1248 in hexanes. The moisture content of the soil samples was adjusted to 70%.

The mycelium colonized sawdust substrate of each species was added to the contaminated soil samples in a 3:1 ratio by volume. Three replicates were used for each species. Three control replicates, containing 10 ml of 100 ppm Aroclor 1248 in the sterilized soil and sawdust matrix, were also analyzed. For the controls, the sterilized sawdust was not added to the matrix until less than 12 h before the 2.5 d analyses.

PCB Analysis via GC:

The concentration of PCBs in the soil samples were analyzed according to methods described in Lopez-Avila et al (1995). Soil samples, taken at 2.5 days and 5 days following PCB inoculation, were oven-dried and homogenized before mixing with 20 ml of hexane-acetone (1:1). The soil sample solutions were then microwave digested at 115° C for 30 minutes. The supernatant was then filtered using glass fibers. Nitrogen gas was used to evaporate the extra solvent present in the sample solutions. Any losses in volume incurred during the nitrogen blowdown were compensated with addition of hexane-acetone (1:1) solution.

The analysis of PCB concentration in the sample solutions was performed using the Gas Chromatograph-Mass Spectrometer (Agilent 7890A Gas Chromatograph with 5975C Mass Selective Detector) in the Skidmore SAIL facility.

Enzyme Assays:

The enzyme assay to gauge the activity of enzymes produced by the mycelium was conducted according to the procedures outlined in Weintraub et al (2007). The phenol oxidase assay was performed with 200 µL of each soil sample in a well-plate, with 50 µl of 5 mM L-DOPA as an indicator. For control replicates, we used 50 mM acetate solution (pH 5), in place of either soil or L-DOPA, respectively. For the peroxidase assay, in addition to what was used for the phenol oxidase assay, we also used 10 µl of 0.3% hydrogen peroxide. We allowed the plates to incubate in the dark for up to 12 h, and measured absorbance at 460 nm using a Synergy HTX Multi-Mode Microplate Reader. Values are reported in nmol h^{-1} g^{-1}.

Results

Enzyme Assays:

Peroxidase activity was seen in shiitakes, phanerochaete and the control replicates at 2.5 d. Phanerochaete showed a statistically higher level of peroxidase activity than the control group, although this cannot be said for shiitake. We attribute the peroxidase activity in the control group at 2.5 d to the presence of peroxidase in the soil upon purchase. Maitake cultures showed an

extremely low level of peroxidase activity at 2.5 d, and was the only species to show an increase in peroxidase levels from 2.5 to 5 d (Fig. 1). All other treatments, including the control replicates, show a statistically significant decrease in peroxidase activity from 2.5 to 5 d. The only cultures with noteworthy phenol oxidase levels were the shiitakes, and then only at 2.5 d (Fig. 2). Slight increases of phenol oxidase at 5 d were seen in the other two species.

PCB Analysis:

At 2.5 d, shiitakes showed the highest degree of PCB degradation, with 18.791 ± 2.847 ppm Aroclor 1248 remaining in the substrate. An additional 2.5 days did not make a statistically significant difference for PCB breakdown by shiitakes, although it did put phanerochaete PCB concentrations at level comparable to those found in shiitakes. Although PCB concentrations in the phanerochaete treatment were at 31.157 ± 4.889 after 2.5 d, they fell to 22.611 ± 3.345 ppm after 5 d (Table 1). From this it appears that, although shiitake had an advantage over phanerochaete in the early days following exposure to PCBs, the two species performed comparably thereafter. Maitake showed little PCB degradation, and no statistical difference between 2.5 and 5 d (Fig. 3). Controls actually fared better than every species after 5 d, with PCB concentrations at 16.187 ± 0.686 ppm.

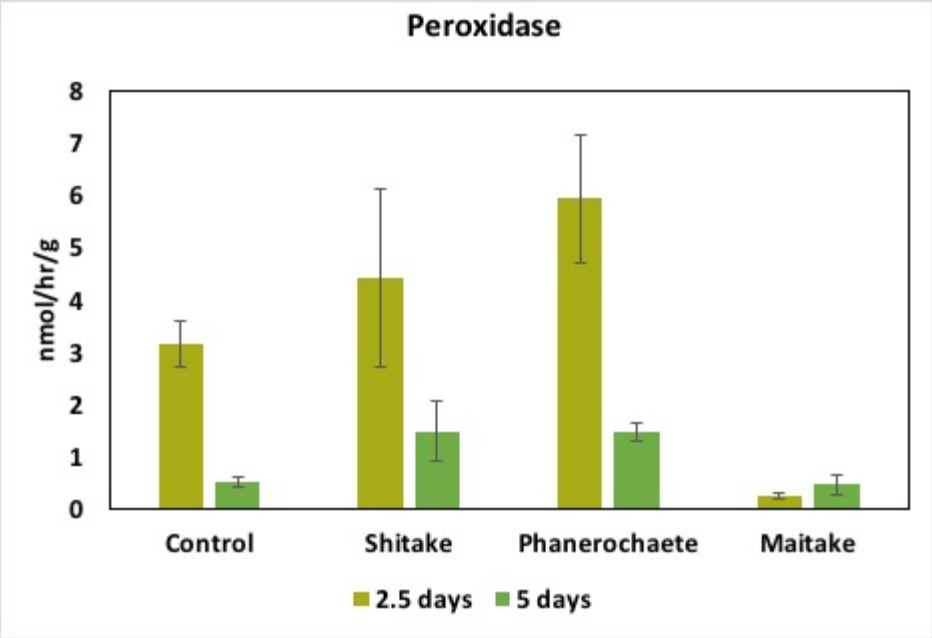

Figure 1. Peroxidase activity in fungi in soil 2.5 and 5 d following PCB exposure.

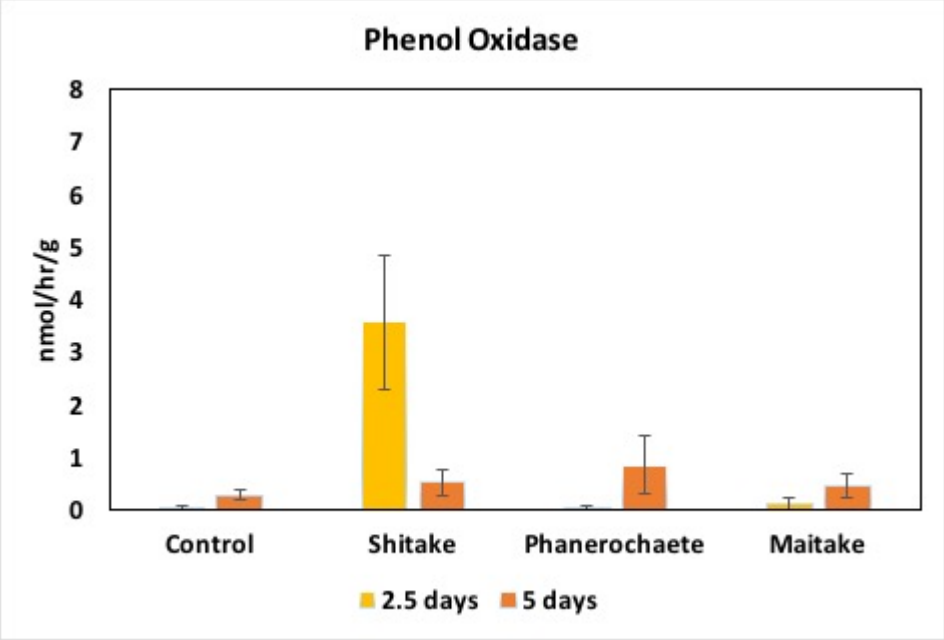

Figure 2. Phenol oxidase activity in fungi in soil 2.5 and 5 d following PCB exposure.

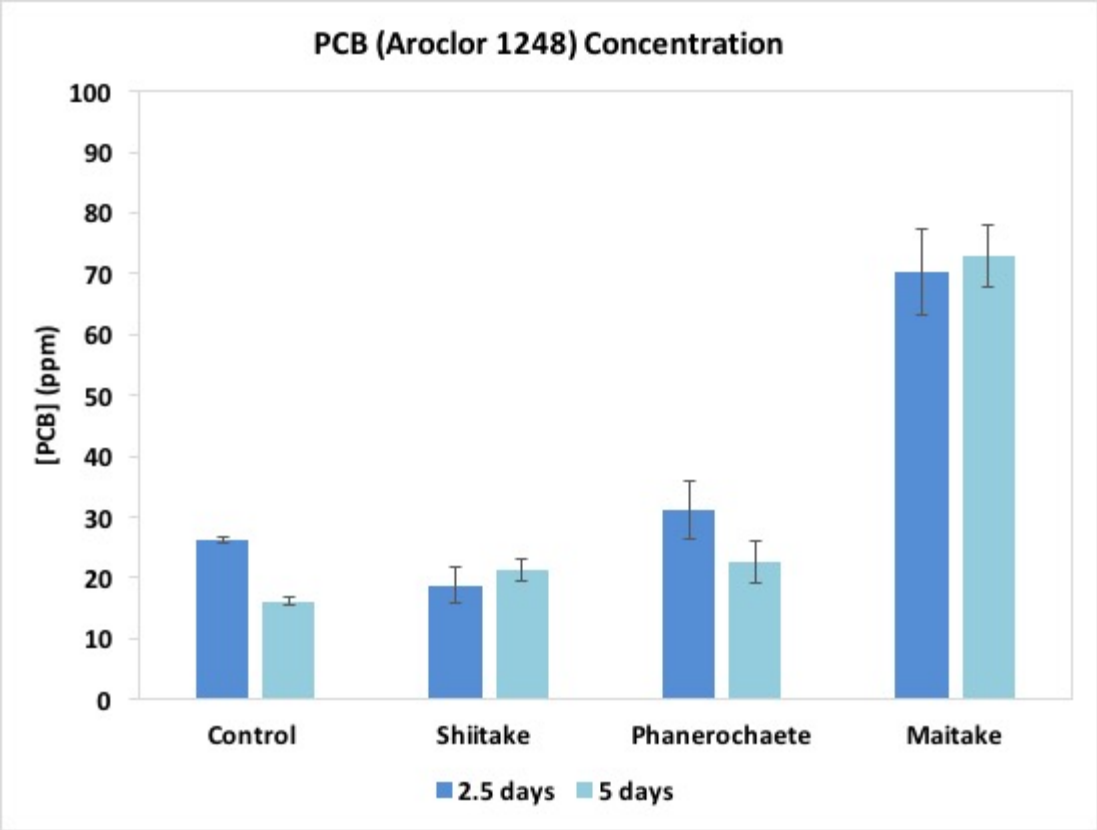

Figure 3. Aroclor 1248 concentrations at 2.5 and 5 d.

Species	2.5 Days	5 Days
Control	26.213 ± 0.486	16.187 ± 0.686
Shiitake	18.791 ± 2.847	21.254 ± 1.841
Phanerochaete	31.157 ± 4.889	22.611 ± 3.345
Maitake	70.255 ± 7.145	72.985 ± 5.401

Table 1. Mean PCB concentrations by species by day sampled in ppm, ± one standard error. All decimals are rounded to three significant figures.

Discussion

The PCB concentrations in the control replicates, as well as the difference between the 2.5 and 5 day measurements, may come as unexpected to some. Although initially taken aback by these figures, we attribute the low concentrations largely to breakdown from enzymes already in the soil upon purchase. This idea is corroborated by the peroxidase levels at 2.5 d. It is noteworthy that the controls and phanerochaete both showed peroxidase activity and comparable decreases in PCB concentration from 2.5 to 5 d. However, the lack of similar behavior from the shiitake cultures, despite having statistically comparable peroxidase activity to both the controls and phanerochaete and notably more phenol oxidase activity, is surprising. These results raise the question of whether other enzymes could be at play in any of the fungal cultures, or even the controls. For example, laccase, which has been demonstrated to have a degradative effect on naphthalene (Anastasi et al, 2008) has previously been shown to be produced by *Pleurotus ostreatus* (oyster), an established polycyclic aromatic hydrocarbon bioremediator (Arun and Eyini, 2011). Further study should include a wider array of enzymes, as well as analysis of enzyme activity over a longer term. The former will help to solidify our understanding of the connection between the production of certain lignolytic enzymes and PCB degradation, while the latter may help us to understand the temporal patterns seen in peroxidase production. At the same time, the reason for the controls having a lower PCB concentration than any of the fungi at 5 d may be elucidated.

Given that the peroxidase activity over time in shiitake and phanerochaete mirrors that seen in the control, it is possible that the fungi ceased peroxidase production several days following exposure to PCBs. This could then lead to the remaining peroxidase being used up through organic matter degradation, which would decrease the enzymes' concentrations. Despite

initial concerns of an acute toxic effect of the PCBs on the fungi, a post-study check on the fungi two weeks after PCB exposure showed no signs of this. Based on the small uptick in phenol oxidase production in the three fungi, it could be that the fungi had downregulated peroxidase production and were slowly beginning to upregulate phenol oxidase production. Some of the peroxidase seen in shiitake and phanerochaete may actually have been present in the soil, as was the case with the controls. However, the statistical difference between peroxidase activity in phanerochaete and the controls at 2.5 d, as well as those between phanerochaete, shiitake and the controls at 5 d, suggest that not all peroxidase activity can be attributed to being present in the soil from the beginning.

The greatest surprise in examining the data was the difference in PCB concentrations between maitake and every other species. Although it was expected that maitakes would show higher PCB concentrations than the other species based off of their lack of enzyme activity, the degree of difference, even from the controls, was unexpected. This raises the issue of whether the controls reflect the percent recovery of PCBs following digestion and filtration, as well as whether there was any sampling error or bias in the maitake replicates. We consider these scenarios unlikely, because if the PCB concentrations in the controls were an accurate reflection of our percent recovery, this would mean that either maitakes had produced PCBs during the incubation period, or that we had particularly egregious sampling error only for our six maitake replicates over two different days. Maitakes could not have produced PCBs, for obvious reasons, and the lack of any statistical difference in PCB concentrations between the 2.5 and 5 d replicates of maitake suggest that our sampling was consistent, and any sampling error would not have been serious enough to skew the results this badly. Instead, we propose that, while some PCBs may have been lost in the period from soil sampling to analysis, every treatment began

with PCB concentrations comparable to those seen in the maitake replicates, if not higher. The reason that the PCB concentrations in maitake replicates are so much greater than in the others would then be due to a much lower degree of PCB breakdown in the maitake replicates, because of significantly lower enzyme activity in the maitake replicates.

The paucity of enzyme activity in the maitake replicates does, however, give us pause. Considering that the soil used for the maitake replicates was no different than the soil used in the control replicates, and given that there was peroxidase activity in the controls at 2.5 d, it seems odd that there would be hardly any enzyme activity seen in the maitake replicates, even if the fungus itself did not produce any. In order to rule out any possibility of sampling error, future studies should consider homogenizing the entire jar's worth of substrate for sampling, although this will necessitate many more replicates. Despite this enigma, we maintain that none of the species were treated differently, either in sampling technique or in analysis, although we do concede that it would have been prudent to analyze samples for PCBs and enzyme activity immediately after PCB exposure; i.e. at 0 d, in order to establish a baseline for each species.

Despite the surprising nature of some of our results, we suggest that the juxtaposition of the control replicates against the maitakes speaks to the bioremediative potential of the enzymes produced by white-rot fungi (Čvančarová et al, 2012; Gao et al, 2010), by examining peroxidase in isolation. Despite having zero fungal activity, the controls showed a breakdown of PCBs over the short time they were watched, and also showed peroxidase activity at 2.5 d. Maitakes, on the other hand, showed extremely little enzyme activity, the highest PCB concentrations of any treatment, and no statistical difference between 2.5 and 5 d. Fungal biomass notwithstanding, we posit that our maitake replicates can, in a sense, be viewed as the true controls, since they grew most poorly out of our three species, and had even less enzyme activity than the controls. From

this perspective, our study affirms peroxidase as having bioremediative potential against PCBs. Additionally, we have demonstrated that shiitakes, which have not been studied as PCB bioremediators to the same extent as phanerochaete (e.g. Čvančarová et al, 2012; Beaudette et al, 2000; Stella et al, 2017), are at least as effective as phanerochaete in peroxidase production and in PCB degradation.

Future Studies

In this study, shiitakes have shown potential for biodegradation of PCBs. Therefore, future research should be focused on studying PCB breakdown capability of species taxonomically similar to shiitakes, in order to ascertain whether there are any that merit status as bioremediators.

The mechanism of PCB breakdown is still not understood properly and information on the degradation products generated from PCB breakdown by white rot fungi is rare (Köller et al, 2000). Therefore, future studies should study the mechanistic pathways of PCB breakdown as that would allow us to know the optimal conditions for bioremediation application by white rot fungi.

According to prior studies (e.g. Anastasi et al, 2008; Beaudette et al, 1998; Čvančarová et al, 2012), white rot fungi also secrete lignin peroxidase, manganese peroxidase and laccase. In our study, only the production of phenol oxidase and peroxidase by the fungi were assessed. These enzymes encompass a broader group and therefore, future research should incorporate studying more specific enzymes like lignin peroxidase, manganese peroxidase and laccase. It would also be worthwhile to conduct in situ studies in soils contaminated with PCBs, in order to account for ecological activity and competition from other organisms, assuming that a possible

goal of this line of research is to inoculate contaminated soils with the appropriate species to, we hope, eventually phase out the conventional remediation methods.

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