Effect of glucose on enzyme activity and color removal by *Trametes versicolor* for high strength landfill leachate

J. Saetang and S. Babel

**ABSTRACT**

This research was carried out to study the treatment of landfill leachate by immobilized *Trametes versicolor* BCC 8725. Leachate was collected from Nonthaburi disposal site of Thailand from a pipe as discharged from landfill to the stabilization pond. Batch experiments were conducted to determine the effects of carbon source (glucose) on the biomass growth of fungi and the treatment of leachate in terms of color, Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) removal. Enzymes produced by *Trametes versicolor* BCC 8725 were also analyzed. Experimental results indicated a higher biomass growth when glucose was added, indicating that the growth of fungi is dependent on the co-substrate. The percentage of color removal is approximately 58% and 12%, respectively, with and without glucose. BOD and COD removals were 37% and 40% with glucose addition within 12 days at optimum conditions. Enzyme analysis indicated that laccase was the main enzyme produced. In addition, Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) were also detected. The fungi were able to produce the enzymes. The peak concentrations of LiP, MnP and laccase activity were found to be 384, 1,241, 2,534 unit/litre (U/L) with glucose, indicating that the color removal rates were proportional to the enzyme activity.

**Key words** | biomass growth, co-substrate, decolorization, laccase, landfill leachate, *Trametes versicolor*

**INTRODUCTION**

In Thailand, more than 14.3 million tons/year or 39,200 tons/day of municipal solid waste is produced in urban areas all over the country with an annual increasing rate of about three percent (Pollution Control Department 2002). Nonthaburi landfill site (location used in this study) has a total area of about 0.194 km² and receives more than 900 tons/day of municipal solid wastes from Nonthaburi province, Thailand (as of 2007). The site has been in operation since 1982, and at present, the total accumulated dumped waste is more than one million cubic meters. Each cell is about 0.0384 km² and is normally filled up in one year.

Even though some physical and chemical methods are effective for the removal of high strength organic and inorganic materials, the high cost per unit volume is the major drawback (Emrah et al. 2007). When a leachate containing high strength of organic matter, suspended solids as well as heavy metals is discharged without treatment, it can stimulate algal growth through nutrient enrichment and deplete dissolved oxygen. Color is one of the major problems in leachate which is hard to remove and causes stratification problems in water bodies. Biological methods are more economical, ecological, and effective, and can be used for leachate treatment.

White rot fungi are widely used in bioremediation processes since these organisms have the ability to degrade a wide range of environmental pollutants (Fu & Viraraghavan 2001). White rot fungi produce various isoforms of extracellular enzymes including laccase, manganese
peroxidase and lignin peroxidase that can degrade pollutants (Wesenberg et al. 2003) and, thus, can be used to treat wastewater, including landfill leachate. Several factors make white rot fungi particularly suitable for bioremediation applications. They tolerate high concentrations of pollutants, can degrade a wide variety of recalcitrant compounds, are environmental-friendly, and are more economical (Srikanlayanukul et al. 2006). The non-specificity of the ligninolytic enzymes allows for the degradation of a range of pollutants, without extensive acclimation (Pointing 2001). Decolorization can be achieved either by adsorption or oxidative degradation by the enzymes (Fu & Viraraghavan 2001). They do not require preconditioning to particular pollutants. They can decolorize as well as biodegrade toxic chemicals. Previous studies have shown a need for a carbon source for fungi (Swamy & Ramsay 1999). The fungi can be grown on a number of inexpensive agricultural wastes such as cassava, bagasse and sawdust. Many studies have been carried out on biodecolorization, for example, decolorization of textile dyes and their effluents using white rot fungi (Sathiya Moorthi et al. 2007), biological treatment of a pulp and paper industry effluent by Fomes lividus and Trametes versicolor (Selvam et al. 2002), but no attention has been paid to the treatment of leachate by white rot fungi. Trametes versicolor is used in this study because of its previously shown potential for decolorization and organic treatment (Raghukumar & Rivonkar 2001).

This study investigates treatment of leachate as discharged from landfill to the stabilization pond from Nonthaburi disposal site of Thailand by using immobilized white rot fungi, namely Trametes versicolor BCC 8725 on polyurethane foam (PUF), with and without use of glucose as carbon source in batch experiments. Enzymes produced by immobilized Trametes versicolor BCC 8725 were also analyzed. Removal efficiency was observed in terms of color, BOD, and COD removal.

MATERIALS AND METHODS

Fungi and leachate collection

T. versicolor BCC 8725 was obtained from BIOTECH (Central Research Unit), Pathum Thani, Thailand. Lab grade Potato Dextrose Agar (PDA) was used as culture media. The fungi were grown in culture tubes containing PDA at 25°C for 1 week. Leachate was collected from a pipe as discharged from landfill to the stabilization pond in March 2008. The Landfill cell was about 8 months old. The leachate was stored at 4°C to avoid any decomposition.

Sub-culture of fungi

Potato Dextrose Agar (PDA) was used as a culture media. The boiled agar was poured into culture tubes (20 * 25 mm) with caps. The tubes were then kept in an autoclave at 121°C (at a pressure of 15 psi) for 15 min and cooled until solidified. The fungi were spread over the PDA in the culture tubes and kept in the incubator at 25°C for 1 week. Sub-culture was done once a week to obtain active fungi.

Leachate characterization before treatment

The leachate was filtered through Whatman No.1 (0.45 μm pore size) filter paper to remove suspended solids before measurement and was analyzed for pH, color, COD, BOD₅, NH₃-N, and Total Kjeldahl Nitrogen (TKN) according to the Standard Methods for the Examination of Water and Wastewater, 20th Edition (1998). The color of the leachate was measured by using ADMI Tristimulus Filter Method (Hewlett Packard Spectrophotometer Model 8452A Diode Array).

Mycelial suspension and immobilization of fungi on PUF

Mycelial suspension

Mycelial suspension was prepared by punching four pieces of fungi from the culture tube by using a sterile loop in 100 ml of sterile lab grade Potato Dextrose Broth (PDB). The 250-ml Erlenmeyer flasks were plugged with cotton and were agitated for 24 hours in a rotary shaker at 150 rpm.

Polyurethane foam (PUF) sterilization

The pieces of PUF were cut with a size of 1 * 1 * 1 cm and the foam pieces were autoclaved before use. Eighty pieces of PUF were first put into the 1,000 ml PDB in 2,000 ml flasks so that PUF pieces were not too crowded. The flasks were...
plugged with cotton and then sterilized in an autoclave for 15 minutes prior to use.

**Fungi immobilization on PUF**

To 1,000 ml of PDB containing 80 pieces of PUF, 100 ml of mycelial suspension was added in a 2,000 ml flask. The flasks were kept at ambient temperature (30–33°C). The PUF pieces were covered with fungal mycelium within 4 days and were used for the study. Ten pieces of PUF with immobilized fungi were used for each varying condition.

**Biomass growth of fungi**

To find the biomass growth, 10 pieces of polyurethane foam covered with fungal mycelium for four days in PDB (as above) were placed in 100 mL of leachate in 250-ml Erlenmeyer flasks. The growth of fungi was determined every day by weighing the flask before and after the growth for 12 days. This provided the wet weight biomass growth in both attached and suspended form. Glucose was also added to see the effect on growth of fungi. All processes were done under sterile conditions at ambient air temperature (30–33°C).

**Batch experiments**

Batch experiments were conducted to find out the effect of glucose and the effect of contact time on color, BOD, and COD removal at optimum conditions.

**Effect of pH on color removal**

Ten pieces of PUF with immobilized fungi were put in flasks containing 100 ml of leachate. To study the effect of pH, the pH in each flask was varied by using 1 N NaOH and 1 N HCl solution to pH 3, 4, 5 and one flask was used as a control. The flasks were plugged with cotton and covered with aluminum foil. All flasks were shaken at 150 rpm for 1 to 5 days and color removal was observed. All processes were done under sterile conditions at ambient temperature. From the previous experimental results (paper accepted for publication), pH 4 was found to be the optimum and was used in this study.

**Effect of co-substrate and contact time on color removal**

Leachate (100 ml) was adjusted to the optimum pH and 10 pieces of PUF with immobilized fungi were then added. The concentration of co-substrate (glucose) was controlled at 3 g/L. This was based on previous experiments (paper accepted for publication) conducted at different glucose concentrations (1, 2 and 3 g/L). It was found that 3 g/L gave maximum removal. In one flask, no co-substrate was added (control). All flasks were shaken at 150 rpm for 12 days to find the effect of contact time. The leachate was filtered and color removal was monitored. To check the reproducibility, experiments were carried out in triplicate for both with and without glucose. Coefficient of variance was calculated.

**Enzyme assays**

**Lignin peroxidase (LiP)**

The assay was done by monitoring the oxidation of dye Azure B in the presence of H₂O₂ (Arora & Gill 2001). The reaction mixture contained (final concentration) sodium tartarate buffer (50 mM, pH 3.0), Azure B (32 μM), H₂O₂ (100 μM) and 0.5 ml of enzyme extract. The reaction is initiated by adding 0.5 ml of H₂O₂. One unit of enzyme activity is equivalent to an absorbance decrease of 0.1 unit min⁻¹ ml⁻¹.

**Mn peroxidase (MnP)**

MnP assay is based on the oxidation of phenol red (Orth et al. 1993). Five ml of reaction mixture contained 1.0 ml sodium succinate buffer (50 mM, pH 4.5), 1.0 ml sodium lactate (50 mM, pH 5.0), 0.4 ml manganese sulphate (0.1 mM), 0.7 ml phenol red (0.1 mM), 0.4 ml H₂O₂ (50 μM), gelatin 1 mg ml⁻¹ and 0.5 ml of enzyme extract. The reaction was initiated by adding H₂O₂ and conducted at 30°C. One ml of reaction mixture was taken and 40 μl of 5 N NaOH was added to it. Absorbance was taken at 610 nm. After every minute the same steps were repeated with 1 ml of the reaction mixture up to 4 min. One unit of enzyme activity is equivalent to an absorbance increase of 0.1 units min⁻¹ ml⁻¹.
Laccase (Lac)

Laccase production was assessed by measurement of enzymic oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm ($\varepsilon = 5.6 \times 10^4$ cm$^{-1}$ M$^{-1}$) (Bourbannais & Paice 1988). The reaction mixture contained 300 $\mu$L of extracellular fluid, 300 $\mu$L of 1 mM ABTS and 0.1 M Na Acetate buffer (pH 4.5). Oxidation of ABTS was measured by determining the increase in absorbance of the mixture on a Shimadzu UV-160 spectrophotometer at 420 nm. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 $\mu$mol ABTS in 1 min.

To determine the error associated with these techniques used for three enzyme analyses, 3 samples were run 10 times each. A variance of less than 10% was found and in light of the high degree of confidence associated with this assay, all subsequent samples were done in duplicate.

RESULTS AND DISCUSSION

Leachate characteristics

The characteristics of landfill leachate compared with the Industrial effluent standard of Thailand are shown in Table 1.

The color of leachate is dark-black. The leachate has very high organic strength in terms of BOD and COD. The leachate is stabilized as can be seen from the ammonia content which is 1,568 mg/L, and causes the pH to be basic.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Industrial effluent standard, Thailand (PCD 2006)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pH</td>
<td>–</td>
<td>5.5–9.0</td>
<td>7.9</td>
</tr>
<tr>
<td>2. Color</td>
<td>ADMI</td>
<td>No standard</td>
<td>2,074</td>
</tr>
<tr>
<td>3. BOD$_5$</td>
<td>mg/L</td>
<td>&lt;20 (did not exceed 60)</td>
<td>38,100</td>
</tr>
<tr>
<td>4. COD</td>
<td>mg/L</td>
<td>&lt;120 (did not exceed 400)</td>
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</tr>
<tr>
<td>5. BOD/COD</td>
<td></td>
<td></td>
<td>0.547</td>
</tr>
<tr>
<td>6. Ammonia</td>
<td>mg/L</td>
<td></td>
<td>1,568</td>
</tr>
<tr>
<td>7. TKN</td>
<td>mg/L</td>
<td>TKN &lt; 100</td>
<td>3,432</td>
</tr>
</tbody>
</table>

Biomass growth of fungi and effect of co-substrate on color removal

The fungi need to be supplied with an external carbon source (Cerniglia 1997). Figure 1 shows Biomass growth vs. Color removal with time of *Trametes versicolor* BCC 8725 in leachate at pH 4 with 3 g/L of glucose and without glucose addition.

Fungi followed a typical pattern of growth in response to the carbon source in the environment. Typically, a single unit of fungus would grow rapidly at the beginning. The exponential phase is followed by the stationary phase. Similar growth patterns were observed in the study done by other researchers (Carlile & Watkinson 1994). As the organic matter in the form of nutrients such as glucose became depleted, growth of fungi did not increase much. The fungi consume and grow readily on available carbon sources at the initial stages of growth and then produce secondary metabolites and extracellular enzymes for biodegradation in the presence of low concentrations of nitrogen (Gramss & Ziegenhagen 1999).

From Figure 1, it was found that the biomass growth is higher when glucose is added. The growth increases sharply until day 9 and tends to be constant for leachate with glucose. The maximum biomass of fungi is approximately 209.0 and 47.3 mg in 100 ml leachate with and without glucose, respectively, indicating that the growth of fungi is dependent on the co-substrate.

Özer Yeşilada *et al.* (1999) investigated the treatment of olive oil mill with an initial COD of 28,200 mg/L by *P. sajorcaju* within 15 days in an agitated incubator. The most relevant growth was observed in the early phase (3 days) and then gradually declined. They also found that after 3 days of growth, 60% of COD and 60% of color removal were achieved.

The color removal was proportional to the biomass growth. The maximum color removal of 58% and 12%, with and without glucose, respectively, was obtained. Thus, co-substrate is necessary for fungi growth and color removal. Experimental results in triplicate for both with and without glucose gave consistent results as reflected by the coefficient of variation which was less than 10% among the experiments conducted for each of the two categories. Amaral *et al.* (2004) found that when using real textile wastewater,
decolorization efficiencies reached to about 92% in a diluted system (approximately 50 mg dye/L) by *Trametes versicolor*. Ashish *et al.* (1995) found that *Trametes versicolor* can treat pulp mill wastewater with an influent of 18,500 color units. The maximum color removal was found to be 80% when glucose was used as co-substrate.

**Leachate characteristics after treatment**

The BOD and COD removal of leachate with and without glucose, before and after treatment, for the time course of 12 days is shown in Figure 2.

From Figure 2, fungi can not only remove color, but also BOD and COD. BOD and COD removal were 37% and 40% with glucose addition within 12 days at optimum conditions. Results indicated that fungi require co-substrate for growth and also gave higher removal efficiency for color, BOD and COD, as compared to when no co-substrate was added. The BOD and COD of glucose are about 2,200 mg/L and 39,744 mg/L, respectively. The initial BOD and COD of leachate used are 38,100 mg/L and 69,580 mg/L. So the total BOD and COD in the leachate with glucose are 40,300 mg/L and 109,324 mg/L. Based on the assumption that glucose was used for the growth of fungi and did not contribute to the BOD and COD of the leachate, mg removal of BOD and COD per mg of biomass was calculated. The mg BOD and COD removed per mg biomass for the leachate with glucose is 67.8 and 132.6. It is interesting to note that COD removal per unit of biomass is higher than BOD. This also indicates that the fungi require some source of readily degradable carbon otherwise the fungi growth is limited. When the growth is limited, the removal efficiency is low. However, the fungi can still treat the leachate because the fungi use the biodegradable organics in the leachate. In contrast, the biomass growth is higher in the presence of glucose, and the removal efficiency is also higher. Srikanlayanukul *et al.* (2006) found that by using real textile wastewater with an initial COD of 3,680 mg/L and 3 g/L glucose addition, immobilized *Coriolus versicolor* RC3 on PUF can remove 80% of color and 67% of COD in 48 hours.
The leachate used in this study is highly concentrated compared to other studies and the removal of color, BOD and COD is more than 50%, which is considered to be very good. This indicates that fungi can be used to treat high strength leachate. However, in order to comply with effluent standards, it should be coupled to other treatment methods.

**Enzyme analysis**

In order to find correlation between decolorization and enzymes, activities of enzymes released by *Trametes versicolor* BCC 8725 were analyzed without and with glucose, and the results are shown in Figure 3(a, b). Other researchers have also found the production of enzymes (especially LiP, MnP, laccase) in decolorization process that use white rot fungi, *Trametes versicolor* (Fulya et al. 2009).

The production of enzyme by *Trametes versicolor* BCC 8725 was determined as a function of time at the optimum conditions. From Figure 1, it can be seen that the biomass growth steadily increased and reached the highest on the 12th day when glucose is added, but the maximum enzyme released was on the 6th day. Therefore, it can be said that the enzyme activity is not growth-associated as compared to the biomass growth. Although in the absence of glucose, the enzymes were also detected, the presence of glucose was necessary for the color removal by *Trametes versicolor* BCC 8725. All enzymes produced by *Trametes versicolor* BCC 8725 had higher enzyme activity in leachate with glucose.

*Trametes versicolor* BCC 8725 was found to produce LiP, MnP and laccase that helped in degradation of organics. Lignin peroxidase (LiP) is a heme containing glycoprotein secreted during secondary metabolism in response to nitrogen limitation (Shrivastava et al. 2005). LiP is a strong oxidizer capable of catalyzing the oxidation of phenols and aromatic amines, chromatic ethers and polycyclic chromatic hydrocarbons (Collins et al. 1997). Manganese peroxidases (MnP) secreted by most white rot fungi are also glycosylated, heme containing enzymes that functionally require H₂O₂ (Jensen et al. 1996). Laccase is a blue multicopper system that may interact directly with phenolic components of lignin and in the presence of a mediator compound can react with a wide range of substrates (Wells et al. 2006).

It was found that laccase was the main enzyme which was produced at the highest level by *Trametes versicolor* BCC 8725. Pointing (2001) observed that purified laccases, LiPs and MnPs are able to decolorize dyes of different chemical structure. The fungi was able to decolorize 12% and 58% of the leachate without and with glucose, respectively. There were peak LiP, MnP and laccase activities of 193, 437 and 781 U/L without glucose (Figure 3(a)) and 384, 1,241, 2,534 U/L with glucose (Figure 3(b)). This indicated that enzyme formation increased when glucose was added. The co-substrate leads to higher decolorization as compared to without glucose. The co-substrate (glucose) has an effect on the amount of enzyme produced. Levin et al. (2005) found that MnP production was enhanced by higher glucose concentration.

Laccase was seen to increase gradually as the percentage of color removal increased. This suggested that laccase was the main enzyme contributing to the decolorization of the leachate. The three enzymes reached a peak on the 6th day in the leachate, both with and without glucose. The maximum color removal was found by the 9th day and then there was slow decolorization. LiP activity also slightly rose towards day 6 and dropped slightly towards the end of
the incubation period where most of the color was broken down and decolorized. Not much further decolorization occurred after the 9th day. For MnP in leachate without glucose, the enzyme gradually increased and had two peaks on the 6th and 12th day. Laccase activity during the peak period without glucose was just one-third of the laccase produced with the presence of glucose. The function of laccase is to detoxify highly reactive aromatic compounds by polymerizing, repolymerization, demethylation, or quinine formation (Thurston 1994). Laccase was found to be highest among three enzymes released and can help in degradation of complex organic compounds as seen from higher COD removal compared to BOD.

When observing biomass growth and color removal, the color removal tends to be constant after the 9th day while the biomass growth still gradually increased. In terms of enzyme activity and color removal, the enzyme activity reached a peak on the 6th day and dropped after that, whereas the color removal still increased slowly until the 12th day (Figure 1). This can indicate that when the enzymes were produced maximally, the decolorization was highest. This is in agreement with Mtui & Nakamura (2008). These results indicated that color removal rates were proportional to the enzyme activity. However, assistance in color removal of the leachate may be due to other enzymes present which were not assayed. The assayed enzyme activities still maintained high levels at the end of degradation.

CONCLUSIONS

It is possible to use T. versicolor BCC 8725 for leachate decolorization and removal of BOD and COD. Fungi require co-substrate for growth and also give higher removal efficiency for color, BOD and COD, as compared to when no co-substrate is added. Color removal of 58% and COD removal of 40% is observed when glucose is added as cosubstrate. Results obtained in triplicate indicate consistent results as reflected by the coefficient of variation which was less than 10%. The mg BOD and COD removed per mg biomass for the leachate with glucose is 67.8 and 132.6, indicating that high strength leachate can be treated. Compared to other studies, the removal of color, BOD and COD is good, even though the leachate is highly concentrated. Laccase is the main enzyme which contributed to the decolorization of leachate by Trametes versicolor BCC 8725. In addition, MnP and LiP could also be detected and helped in decolorization and degradation of organic compounds. In absence of glucose, the enzymes are also detected, but the presence of glucose is necessary for higher color removal by Trametes versicolor BCC 8725. All enzymes produced by Trametes versicolor BCC 8725 have higher enzyme activity in leachate with glucose. Color removal rates are proportional to the enzyme activity. Fungi used in this study cannot remove completely color, BOD and COD from leachate but can remove them to a large extent. Thus, this treatment should be coupled with other treatment systems. Finally, it can be concluded that this study demonstrates a novel process which provides an alternative way of managing leachate, since it is a natural biological process and can also be used for treatment of high strength leachate.

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