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Effect of Culture Technique of *Ganoderma Australe* Mycelia on Percentage Removal of Leachate Organics

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ABSTRACT

Leachate (liquid pollutant), which is highly contaminated with organic matter and toxic substances is a major problem that arised from landfill. Biological methods have proven to be effective to remove organic matters that are abundant in leachate. This study is intended to compare the used of free mycelia and immobilized mycelia of the white-rot fungi, Ganoderma australe for the removal of landfill leachate organics. The organics fraction of landfill leachate was measured by biological oxygen demand (BOD_5), and chemical oxygen demand (COD). The experiment revealed that free mycelia of G. australe showed capability in removing leachate BOD_5 but not COD. However, the use of immobilized G. australe displayed the best result in the removal of BOD_5 and COD leachate after 4 weeks of treatment in flasks with 93.09% and 17.84% percentage removal of BOD_5 and COD, respectively. Therefore, G. australe can be considered potentially useful in the treatment of landfill leachate as they can help in removing BOD and COD due to their biodegradative abilities.

Keywords: *landfill leachate, biological methods, biological oxygen demand (BOD), and chemical oxygen demand (COD)*

INTRODUCTION

An ever-expanding population and high rates of economic development in Malaysia have resulted in the generation of a vast amount of waste. Based on the amount of waste generated, only less than 5% of the waste is being recycled, while the remainder is taken to the disposal site (landfill) (Local Government Department, [LGD], 2003). Leachate (or liquid pollutants) generated at landfill becomes a great threat to the surrounding soil, groundwater and even surface water. Landfill leachate is highly contaminated and has high concentration of organic matter and toxic substances such as metals (Ding *et al.*, 2001). Therefore, the leachate must be treated appropriately before being discarded in the environment.

White-rot fungi are of current interest to be used for the bioremediation of a broad spectrum of persistent xenobiotics (Wan Razarinah *et al.*, 2015). The fungi have been implicated in the transformation of a large amount of organopollutants structurally related to lignin for example *P. sanguineus*, *Coriolus pubescens* and *Trametes* sp. in degradation of lignosulphonates (Eugenio *et al.*, 2008). Therefore, white rot fungi are of current interest to be used for the bioremediation of a broad spectrum of persistent xenobiotics, thus also can be used to treat wastewater, including landfill leachate. Saetang and Babel (2010) stated that the advantages of using immobilized microorganisms for pollutant degradation is due to their economically cheaper, easier to handle and the immobilized fungus is reusable for several batches. Therefore, the aim of this study is to investigate fungi capability in removal of leachate organic materials using free mycelia and immobilized mycelia of white-rot fungi, *G. australe*.

MATERIALS AND METHODS

Stock Culture Maintenance

Fungal cultures were maintained on malt extract (MEA) (Oxoid) agar slants, and inoculum was prepared by sub-culturing onto MEA grown for 7 days at 28±2 °C. The culture was obtained from the Mycology Laboratory, Institute of Biological Sciences, University of Malaya, Malaysia (Wan Razarinah *et al.*, 2014).

Preparation of Mycelial Suspension

Four plugs (6-mm^2 diameter) of a 7-day old fungal colony growing on MEA media in Petri plates were transferred into 250-ml Erlenmeyer culture flasks containing 100 ml of Glucose-yeast-malt-peptone (GYMP) growth medium under sterile conditions. The GYMP growth medium contained the following: $MgSO_4.7H_2O$ (1.00 g/L); KH_2PO_4 (1.00 g/L); K_2HPO_4 (1.00 g/L); NH_4Cl (1.00 g/L); glucose (15.00 g/L); peptone (8.00 g/L); yeast extract (8.00 g/L); and malt extract (8.00 g/L). The pH of the media was adjusted to 6.00 before autoclaving using 1.0 M HCl at room temperature. Inoculated flasks were then agitated on an orbital shaker for 48 h at 28±2 °C at 150 rpm.

Leachate Samples

The Leachate samples used in this experiment was collected from the pond of untreated leachate at the sanitary landfill in Selangor. The leachate was filtered to remove suspended solids before measurement and was analyzed for pH, COD, BOD_5, and NH_3-N according to the Standard Method for the Examination of Water and Wastewater (APHA, 1998) using a Hach DR 2800 spectrophotometer and appropriate chemical kits.

Treatment of Leachate with Free Fungal Mycelium in Batch Culture

In this experiment, mycelial broth (10 mls) was transferred into 250-ml Erlenmeyer flasks containing 100 mls leachate prepared as follows: i) 50% leachate: 50 ml leachate and 50 ml distilled water and ii) 100% leachate: 100 ml leachate only. Both leachate media were autoclaved (SLM-50 and SLM-100) and not autoclaved (LM-50 and LM-100) before inoculating with mycelium pellets. All treatments were incubated at 28±2 °C and 150 rpm for 4 weeks. Three important leachate components: BOD_5, COD and ammoniacal nitrogen (NH_3N) together with pH were measured before and after 4 weeks of incubation. Three flasks were replicated for each treatment. The results were subjected to the t-test to compare the value of percentage removal using SPSS software (SPSS Version 14.0, Chicago, IL).

Preparation of Immobilized Fungal Mycelium

About 50 pieces of Ecomat (2 cm x 2 cm) were put into 500 ml beaker. The beaker was covered with aluminium foil and then sterilized using an autoclave for one hour prior to use.

Four pieces of sterilized Ecomat and 5 ml of mycelial suspension were added to 250-ml Erlenmeyer culture flasks containing 50 ml of GYMP growth medium. The flasks were agitated at 100 rpm on an orbital shaker. The Ecomat covered with fungal mycelium within 4 days were used for the study.

Treatment of Leachate using Immobilized Mycelium in Batch Culture

Treatment of leachate using immobilized mycelia were carried-out in 250 ml Erlenmeyer culture flasks containing ecomat covered with fungal mycelium of *G. australe*. The excess GYMP medium was removed and 125 ml of 50% and 100% leachate (as mentioned in Section 2.4) were added into each 250 ml Erlenmeyer flask. Dilution was done using distilled water. The flasks were then agitated on an orbital shaker for 4 weeks at 28±2 °C at 150 rpm. Every week (for four weeks), samples were collected and measured for pH, BOD_5, COD and ammoniacal nitrogen. All processes were done under sterile conditions at ambient temperature. Three flasks were replicated for each treatment.

RESULTS

Treatment of leachate was carried out using free and immobilized fungal mycelium of *G. australe* on Ecomat. Ecomat is a high tech organic fibre that is made from 100% oil palm residues or empty fruit bunches (EFB). It is a highly refined eco-friendly product and fully biodegradable. The raw leachate used in this experiment was collected from the pond of active and untreated leachate. Comparison of the leachate characteristics with industrial effluent standards in Malaysia is as shown in Table 1.

Results for initial analysis showed that most of the parameters well exceed the standards (Wan Razarinah *et al.*, 2015). The BOD_5 and COD values were very high with 11360 mg/L for BOD_5 and 16000 mg/L for COD however, the ammoniacal nitrogen concentration was quite low with 21.3 mg/L which, indicating the leachate was very fresh. The heavy metal concentrations were below the standard levels (Table 1). Leachate used in this study was filtered since Saetang and Babel (2009) reported that BOD and COD were reduced to some extent because of a high amount of suspended solids. The content of BOD_5 and COD before and after treatment was measured according to the APHA (1998).

Effect of Culture Technique and Leachate Concentration on Percentage Removal of Leachate Organics

Ganoderma australe was selected in this study since they show a good growth in leachate (Wan Razarinah, 2014). The effect of culture technique-free cell mycelia and immobilized mycelia of *G. australe* on percentage removal of BOD_5 and COD were compared. Immobilization of cells is the attachment of cells in distinct solid phase that permits exchange of substrates, products, and also inhibitors. Meanwhile, free mycelia are web or mat of hairlike fibers that grow in a medium culture (Kuo, 2006). Figure 1 depicted the result of leachate degradation by free cell mycelia and immobilized mycelia of *G. australe* incubated in flask containing liquid growth medium (GYMP) incorporated with 100% (raw) leachate for 4 weeks. The result showed that the percentage removal of BOD_5 by immobilized mycelia of *G. australe* was higher compared to free mycelia after 4 weeks of incubation. However, at the end of 4 weeks of incubation, the percentage removal of COD only occurred when 100% leachate was treated with immobilized mycelia of *G. australe* (17.84%). Hence, the results show that immobilized mycelia of *G. australe* were better than free cell mycelia in reducing the BOD_5 and COD of 100% leachate.

Table 1: Landfill leachate characteristic

Parameter	Value	*Industrial effluent standard
- Biochemicals Oxygen Demand (BOD_5) @ 20°C, 5 Days (mg/L)	11360 ± 703.42	**20**
-Chemical Oxygen Demand (COD) (mg/L)	**16000 ± 1130**	**400**
-BOD_5/COD	0.71 ± 0.08	**NA**
-Total Suspended Solids (TSS) (mg/L)	130 ± 13.45	**50**
-Ammoniacal Nitrogen (NH_3N) (mg/L)	21.3 ± 3.17	**5**
-Total Carbon (TOC) (mg/L)	4700 ± 145.26	**NA**
-Total Nitrogen (TKN) (mg/L)	98 ± 13.45	**NA**
-pH @ 25°C	8.05 ± 0.05	**6.0 – 9.0**
HEAVY METAL		
Leads as Pb (mg/L)	0.06 ± 0.02	**0.1**
Cadmium as Cd (mg/L)	ND < 0.002	**0.01**
Copper as Cu (mg/L)	0.02 ± 0.01	**0.2**
Iron as Fe (mg/L)	4.44 ± 0.11	**5**
Zinc as Zn (mg/L)	0.18 ± 0.03	**2**
Magnesium as Mg (mg/L)	23.01 ± 0.90	**NA**

ND: Not detected; NA: Not available
*** Source: Environmental Quality Act, (EQA). [Regulation 13 (2009) Schedule 2], (2009)**

Leachate concentration is one of the factors influencing the percentage removal of leachate organics. Percentage removal of leachate BOD_5 and COD after 4 weeks treatment with free cell mycelia and immobilized mycelia of *G. australe* in different concentration of leachate are shown in Figure 1. Result showed that treatment of leachate with free mycelia of *G.*

australe exhibited that 50% leachate demonstrated slightly higher BOD_5 removal than 100% leachate where for 50% leachate, 89.33% of BOD_5 was removed compared to 85.28% for 100% leachate. In contrast, slightly higher BOD_5 removal was demonstrated by 100% leachate (93.09%) than 50% leachate (81.57%) when the leachate was treated with immobilized mycelia of *G. australe*. However, statistical analysis revealed that BOD_5 removal of the two concentration of leachate showed no significant difference ($p>0.05$) for both treatments. Meanwhile, COD removal of leachate by free cell mycelia of *G. australe* only obtained in 50% leachate with 24.72%. Whilst, treatment of leachate with immobilized mycelia of *G. australe* shown that percentage removal of COD was higher (44.66%) for diluted leachate than concentrated leachate (17.84%). Thus, this study indicated that the treatment of leachate BOD_5 and COD with immobilized mycelia of *G. australe* was more efficient at lower organic concentration of leachate; though the fungal was also able to treat concentrated leachate.

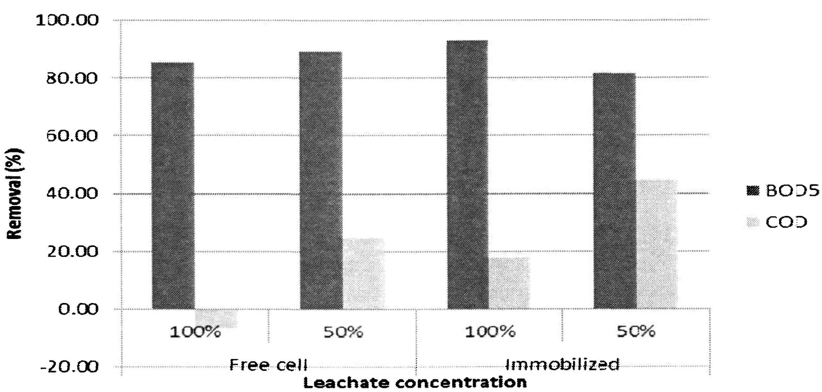

Figure 1: Percentage removal of 100% and 50% leachate BOD_5, and COD by free and immobilized mycelia of *G. australe* on Ecomat after 4 weeks incubation in submerged cultures incubated at room temperature, shaking at 150 rpm

Meanwhile, Figure 2 depicted the result of leachate degradation by immobilized *G. australe* incubation in flask containing liquid growth medium (GYMP) incorporated with 100% leachate for 4 weeks. The result indicated that the percentage removal of BOD_5 increased every week. After 7 days of incubation, 86.56% of BOD_5 removal was obtained, and it's followed by 90.78% after 14 days, 91.19% after 21 days and after 4 weeks of incubation the percentage removal of BOD_5 was 93.09%. Figure 2 also reveals that the percentage removal of COD was slight decreased after two weeks of incubation. The results show that 22.60% of COD removal was attained after 7 days of incubation, and increased to 23.48% after 14 days of incubation. However, after 21 days of incubation the percentage removal of COD was decreased to 16.41% and at the end of 4 weeks of incubation, the percentage of COD removal was slightly increased to 17.84%. The percentage removal of BOD_5 and COD that showed in Figure 2 was determined every week as to confirm the stage of removal percentage.

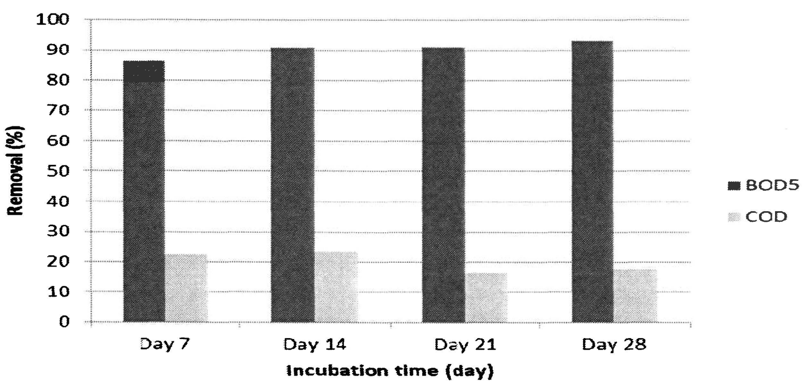

Figure 2: Percentage removal of 100% leachate BOD_5, and COD by *Ganoderma australe* immobilized on Ecomat at weekly intervals for 4 weeks incubated at room temperature, shaking at 150 rpm

DISCUSSION

White-rot fungi- *G. australe* was investigated for their ability to degrade BOD, and COD, from leachate. Different biological methods were studied that include treatment by free cell mycelia in batch culture and treatment using immobilized mycelium in culture flask. In this study, treatment of leachate with *G. australe* using free fungal mycelia in Erlenmeyer flask demonstrated promising percentage removal of leachate BOD_5. This study revealed that after 4 weeks, the percentage removal of BOD_5 in 100% leachate was increased from 85.28% by free mycelia of *G. australe* to 93.09% by immobilized *G. australe*. It was observed that the percentage removal of BOD_5 by these fungi was enhanced and accelerated in immobilized cell cultures. Meanwhile, COD removal showed variations among the treatments. This is consistent with previous studies done on pellets or immobilized cells of *T. versicolor* which showed that removal efficiency of BOD_5 and COD in leachate was found higher in the case of immobilized fungi compared to pellet form or mobilized fungi (Saetang & Babel, 2009). This is due to the reason that immobilization of fungal cells could stably maintain the production of various enzymes at levels higher than achieved with suspended or pellets forms.

In addition, this study also found that the concentration of leachate medium only showed a slight difference in terms of BOD_5 removal. This finding is quite similar with work done by Kissi *et al.* (2001) who found that COD reduction on different concentration of olive mill waste (OMW) obtained similar values although the initial values were very different. This may be due to the production of enzymes involved in the treatment. In addition, they also found no significant differences in enzyme production could be observed between *P. chrysosporium* incubation in 20% and 50% OMW. This indicated that *G. australe* can work better at lower organic concentration; though the fungal is also able to treat concentrated leachate.

This study also revealed that the leachate treatment by immobilized *G. australe* incubation in batch culture showed an increment in the percentage removal of BOD_5 in 100% leachate every week. Ehlers and Rose (2005) claimed that when fungal biomass is immobilized, the degradation capacity and tolerance to toxic pollutant concentrations can be increased. This is due to the fact that using an immobilized system provides greater degree of stability for the fungi and a high tolerance for elevated pollutant concentrations. According to Mtui and Nakamura (2002) immobilize mycelia can enhance enzyme production by facilitating mycelia-fluid contact, hence improving the mass and O_2 transfer rates. Previous result (Noorlidah *et al.*, 2013) shows that *G. australe* was able to produce ligninolytic enzymes such as LiP, MnP and Laccase. These enzymes were known for their ability to degrade a variety of environmental pollutants (Yateem *et al.*, 1998) that include leachate. According to Omar, *et al.* (1992) this could be attributed to the effect of culture techniques on fungal morphology and fungal metabolism (Bonnarme *et al.*, 1991). The results obtained showed the ability of white-rot fungi *G. australe* to remove BOD_5 and COD. This finding is coherent with

Kim *et al.* (2003) who reported the use of white-rot fungus *P. chrysosporium* for the biological removal of organics measured as COD. Coulibaly *et al.* (2003) noted that white-rot fungi have been attracting a growing interest for the biotreatment (removal or destruction) of waste water ingredients such as metals, inorganic nutrients and organic compound. This may be due to their capacities to adapt to severe environmental constraints.

CONCLUSION

Based on the result obtained, free cell cultures of *G. australe* used in this study showed capability in removing leachate BOD_5. However, using immobilized *G. australe* better result in the removal of BOD_5 and COD for concentrated leachate has been obtained. On the other hand, dilution of leachate (50% or 100%) did not significantly increase the removal efficiency. As a conclusion, white-rot fungal *G. australe* can be considered as a good candidate for leachate bioremediation.

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