GENERAL INTRODUCTION

1.0 BACKGROUND OF STUDY

An ever-expanding population and high rates of economic development in Malaysia have resulted in the generation of a vast amount of waste. The population of Malaysia has shown a sharp increase from 23.49 million in 2000 to 26.75 million in 2005, an increase of 13.8 % in the ten years period (Tarmudi *et al*., 2012). Meanwhile, it was estimated that by July 2012, the populations of Malaysia have increased to 29,179,952 million, with a population gowth rate at 1.542% (CIA World Factbook, 2013). In addition, for the last two decades, Malaysia has experienced a rapid economic growth and it grew almost three times higher than the world's average annual economic growth rate at 2.5% per annum (Juzhar, 2002 as cited in Tarmudi *et al*., 2012). These contributed to the increased of annual quantity of solid wastes generated in Malaysia cities from 2.5 million tonnes in year 1991 to 5.9 million tonnes in 2005, with an annual growth rate of 2.0% (Tarmudi *et al*., 2012). Furthermore, in year 2008, 23,000 tonnes of waste is produced each day in Malaysia, and this amount was increased to 28,565.32 tonnes in 2012 (Jabatan Pengurusan Sisa Pepejal Negara, [JPSPN], 2013) and expected to rise to 30,000 tonnes by the year 2020 (Global Environment Centre, [GEC], 2009). In Selangor alone, waste generated in 1997 was over 3000 t/day, increased to 4435.30 t/day in 2012 (JPSPN, 2013) and the amount of waste is expected to rise up to 5700 t/day in the year 2017 (GEC, 2009). Based on the amount of waste generated, (Local Government Department, [LGD], 2003) reported that about 76% of waste was collected, and from that amount only less than 5% of the waste is being recycled, while the remainder is taken to the disposal site (landfill). Over 40% of 175 disposal site are operating as dumpsite (LGD, 2003). Also, according to the report by Local Government Department, (2003), currently only 5% of the waste is being recovered and it is targeted

by the year 2020 that more than 20% of waste will be recycled and about 15% are at the stage of intermediate processing such as shredded and crushing (Figures 1.1 & 1.2).

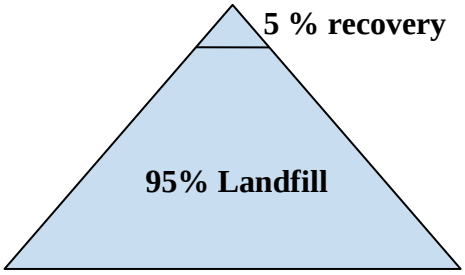


Figure 1.1: Waste Hierarchy (Current Status)

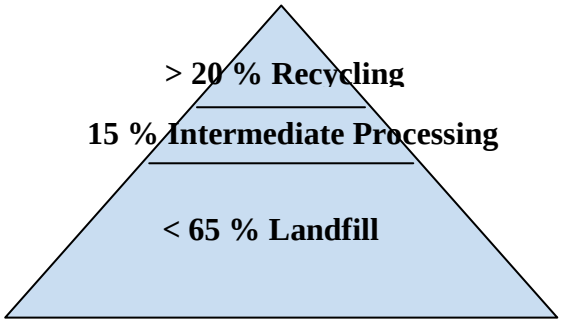


Figure 1.2: Waste Hierarchy (Targeted 2020)

Source: Report by Local Government Department, Ministry of Housing & Local Government of Malaysia [MHLG] (2003).

Landfilling still remains one of the main methods for disposing of municipal and industrial solid waste. The degradation of the organic fraction of the waste in the landfill in combination with the percolation of rainwater generates a polluted liquid called leachate (Maranon *et al*., 2006). Leachate (or liquid pollutants) is generated when water passes through the refuse and becomes a great threat to the surrounding soil, groundwater and even surface water. In Malaysia, landfilling is still the most popular way for the treatment of municipal solid waste (MSW). Landfilling takes up a lot of land besides leads to serious pollution in its surroundings.

At present, the management of landfills was not properly carried out and this has led to pollution by leachate from dumpsites. This is supported by Vesilind *et al*. (2002),

which stated that groundwater contamination of leachate cannot be avoided totally since no landfill is sufficiently fixed. Kang *et al*. (2002) also stated that contamination of groundwater by leachate from unsanitary or even inappropriately designed or constructed sanitary landfill presented serious problems to water resource conservation because of the number and size of landfills. As example, in United Kingdom, Cornish Guardian (2013) reported that leachate from Connonbridge landfill site in East Taphouse has leaked from the site and polluted the Widdowpath stream due to overflowed from the lined area into an unlined area. This problem becomes worsen as some of the traditional remediation technologies (e.g., excavation, pump and treat, and perhaps capping) were less cost effective (Christensen *et al*., 1994 and Kang, & Park, 2000).

'The landfill leachate newsblog', (2008) commented that the management of 260 landfills in Malaysia were not properly carried out, thus this has led to the pollution of the river by leachate from dumpsites. This problem has arisen in Malaysia over the past few years and leachate is getting into rivers and then into water supplies and imparting a bad taste into drinking water in thousands of homes. Leachate is becoming a great threat to the surrounding soil, groundwater and even surface water. In many cases, landfill leachate is highly contaminated and has high concentration of organic matter and toxic substances such as metals (Ding *et al*., 2001). Ammonia has been identified as the most significant long-term component of leachate (Bilgili *et al*., 2007) since there was no significant change in ammonia concentrations over a 30-year period in conventional landfill leachate.

Therefore, leachate must be treated appropriately before being discharged in the environment. The treatment of landfill leachates is very complicated, expensive and requires various process applications due to their high concentration of chemical oxygen demand (COD) and nitrogen (Ellouze *et al*., 2009). Several options have been

applied for leachate treatment – biological, chemical, physical (membrane separation and thermal treatment) processes. Maranon *et al*. (2006) reported various processes that have been employed, such as anaerobic and aerobic biological degradation, chemical oxidation, coagulation–precipitation, activated carbon adsorption, and membrane processes. However, chemical and physical treatments are costly and ineffective. Other report by Andrés *et al*. (2004) stated there are three categories of techniques to treat leachate that are considered such as leachate recycle, physicochemical treatment, and biological treatment. However, in general, physicochemical processes were found inefficient to remove organic matter totally. Meanwhile in article titled "Landfill Leachate Treatment" (2011) has described the application of non-biological treatments of leachate in many countries are inherently more expensive to run than biological processes, and depending on the destination of the concentrate produced which may be very much less sustainable than biological treatment.

On the contrary, biological treatment is totally accepted as the most possible treatment for landfill leachate. It converts the contaminants into other less polluted chemicals, and hence will remove those contaminants from the waste. Malaysian solid wastes contain very high organic waste and consequently high moisture content (Consumers' Association of Penang [CAP], 2001). In an article titled ("Amount & component," 2012) reported that in the Ninth Malaysia Plan approximately 49.3% of waste consists of food waste (organic waste), followed by 17.1% paper, plastic (9.7%) and iron (1.6%) while 22.3% are other wastes For this reason, it is suggested that aerobic biological treatment is the best option to encounter the leachate waste problem in Malaysia. According to Zouboulis *et al*. (2001), biological processes based upon suspended-growth biomass, such as conventional activated sludge process, was proven to be effective for the removal of organic carbon and nutrients. Nevertheless, conventional biological process encounters the problems of inadequate sludge settling

and the need for longer aeration times. Therefore, bioaugmentation (addition of microorganisms and/or enzymes) that can help improve the performance of wastewater treatment system should be investigated. Bioaugmentation which is an ex situ bioremediation technology is the addition of bacterial cultural products to wastewaters. This bacterial cultural products containing different strains of microorganisms and/or enzymes, with the purpose of providing a sufficient quantity and diversity of microorganisms or constituents, which can help in improving the performance of wastewater treatment system.

Bioremediation using various microbial organisms is one way to remove pollutants from the environment. Most research within the field of bioremediation has focused on bacteria, with fungal bioremediation (mycoremediation) attracting interest just within the past two decades. The toxicity of many of the pollutants limits natural attenuation by bacteria; nevertheless fungi (white rot fungi) can withstand toxic levels of most organopollutants (Aust *et al*., 2004). Microorganisms are responsible for the production of several enzymes. Enzymes by microorganisms assure a potential and unlimited supply. Besides that, it also makes it possible for the genesis of new enzymatic systems that cannot be obtained from plant or animal sources. Fungi are the most important source for enzymes and they excrete a wide variety of enzymes than bacteria. Four main genera of white rot fungi have shown potential for bioremediation: *Phanerochaete*, *Trametes*, *Bjerkandera*, and *Pleurotus* (Hestbjerg *et al*., 2003). The main mechanism of biodegradation employed by this group of fungi, however, is lignin degradation system of enzymes. These extracellular lignin-modifying enzymes (LMEs) have very low substrate specificity so they are able to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin (Cajthaml *et al*., 2002: Mansur *et al*., 2003; Pointing, 2001, Veignie, *et al*., 2004). Furthermore, fungi that are GRAS (Generally Regarded as Safe) strains are good candidate and they

produce extracellular enzymes, which are easier to be recovered from fermentation broth.

1.1 PROBLEM STATEMENTS

The growth in population and economic developments has resulted in the generation of vast amounts of waste. Most of the wastes were dumped at the landfill sites which then generate leachate. Previous literatures had reported on the variation of leachate characteristics that are due to several factors such as the age of the landfill, the nature of the waste (solid or liquid), the source of the waste (municipal, industrial, commercial, mining) and the amount of precipitation. Leachate that was generated in the landfill site contains hazardous compounds such as organic matters, ammonia and heavy metals. Ramirez-Sosa *et al*. (2013) reported that leachate has high concentration of dissolved biodegradable (i.e. BOD) and non-biodegradable (i.e. COD) compounds, which mainly of humic and fulvic nature, as well as heavy metals, ammonia nitrogen and organochlorine compound. Thus, this leads to severe contamination of the surrounding environments including groundwater, soil and nearby river especially when there is no adequate treatment and disposal of these liquids (Ramirez-Sosa *et al*., 2013). Hasan *et al*. (2011) reported that contamination of ammonia (NH_3-N) in Malaysian rivers exceeding standard limits hence became a major problem to drinking water treatment plants. Therefore, leachate must be treated appropriately before being discarded in the environment. Leachates have been treated by chemical/physical methods, and also by biological methods. In Malaysia, leachate treatment using chemicals/physical methods has been studied by several researchers. Bashir *et al.* (2012) used the ion exchange technique for the treatment of stabilized landfill leachate, which is particularly for the removal of color and nonbiodegradable substances (measured as chemical oxygen demand [COD]). Meanwhile, Aziz *et al*., (2012) who

has treated raw leachate using powdered activated carbon (PAC) supplemented sequencing batch reactors (SBR) technology found that PAC-SBR able to enhance the removal efficiencies of phenols, total iron, zinc, ammonia, nitrite, nitrate, color, suspended solids, chemical oxygen demand, biochemical oxygen demand, and total dissolved salts. In other leachate treatment, carbon–minerals composite adsorbent was used by Halim *et al*. (2012) for removing contaminants such as ammonia, chemical oxygen demand (COD), and color from semi-aerobic landfill leachate.

On the other hand, Abu Amr and Aziz (2012) reported that the combination method of Ozonation and Fenton process (i.e., $O_3/H_2O_2/Fe^{2+}$) achieved higher removal efficiencies for COD, color, and NH_3–N compared with other studied applications. Bashir *et al*., (2009) investigated the electrochemical oxidation of stabilized leachate from Pulau Burung semi-aerobic sanitary landfill by conducting laboratory experiments with sodium sulfate Na_2SO_4 (as electrolyte) and graphite carbon electrodes. This resulted in 70% BOD removal, 68% COD removal, 84% color removal, 0.04 BOD/COD ratio and 9.1 pH. This depicted that electrochemical treatment using graphite carbon electrode was found to be effective in BOD, COD and color removal but was not effective in increasing the BOD/COD ratio or enhancing biodegradability of the leachate (Bashir *et al*., 2009). Aforementioned leachate treatments revealed that chemical/physical methods, which is best suited to remove specific pollutant (such as heavy metals) were found to be expensive, ineffective and needs high maintenance. While, biological methods have proven to be effective to remove organic matters and ammonia (Renou *et al*., 2008) that are abundant in leachate. Organic pollution that acts as substrates for microorganisms may cause oxygen depletion and having severe consequences for the stream biota. Organic content in leachate is measured as biological oxygen demand, BOD (i.e. biodegradable substances) and chemical oxygen demand, COD (i.e. nonbiodegradable substances). Ammoniacal nitrogen is measured for the

amount of ammonia, a toxic pollutant that often found in landfill leachate and waste products. Nevertheless, conventional biological process encounters the problems of inadequate sludge settling and the need for longer aeration times. Application of biological methods such as bioremediation process involved microorganisms that primarily mediated by bacteria and fungi. Previously, majority of bioremediation process used bacteria.

However, currently much attention have been focused to the use of fungi in bioremediation due to their ability to adjust rapidly to a variety of environments, they can grow in medium with wastewater and most importantly, they can release enzymes that are able to breakdown organic materials. Furtheremore, study of using local fungi (including white-rot fungi) for leachate bioremediation is still lacking. In addition, from the literature, there's a gap of knowledge that can still be noticed particularly in the removal of leachate pollutant such as ammonia by potential local fungal strain. Therefore, bioaugmentation by fungi that can help improve the performance of wastewater (especially leachate) treatment system was investigated.

By doing this research we can permanently eliminate contaminants through biochemical transformation since enzymes encounters its substrate (the target pollutants) and splits the substrates into component parts of the molecule – non-toxic molecules; and also the study of different operation strategies on the efficiency of biological treatment process can be used to optimize performance, especially for the removal of nitrogen compounds and biodegradable organic compound in leachate.

1.2 RESEARCH OBJECTIVES

The major aims of this study are to investigate the efficiency of bioaugmentation technology by using fungi to remediate leachate. The specific objectives of the present study are:

(i) to characterize and compare leachate from various landfills,

(ii) to screen fungi species capable of growing in leachate as potential candidate for bioremediation,

(iii) to remediate leachate using selected fungi through free-cell mycelia, immobilized mycelia and using extracellular enzymes.

1.3 SCOPE OF STUDY

The study examined the treatment of leachate by fungi. Characterization of leachate was monitored on the leachates obtained from landfills in Selangor. The differences of landfill status revealed the variation in leachate characteristics. Thus, treatment of leachate from one landfill source may differ from other landfills. In order to determine the effect of leachate treatment by fungi, only four important leachate parameters viz biological oxygen demand (BOD), COD, ammoniacal nitrogen (NH_3-N) and pH are being accounted. BOD and COD were chosen since they represent the measurement of organic content which is high in leachate. Meanwhile, since high concentration of NH_3-N in water makes it more toxic, so the measuremnet of NH_3-N in leachate treatment is also important to be considered. Due to the potential of using fungi and fungi enzymes in leachate treatment, the scope of work that have been proposed for this study include the study of leachate characteristics collected from different landfills in order to determine leachate characterization, screening of potential fungi to be used in leachate treatment and finally, in the last part is a study of different methods to treat several important leachate components (Figure 1.3), which include application of free-cell mycelia of selected fungi, immobilization of selected fungi and bioaugmentation of crude enzyme produced by selected fungi.

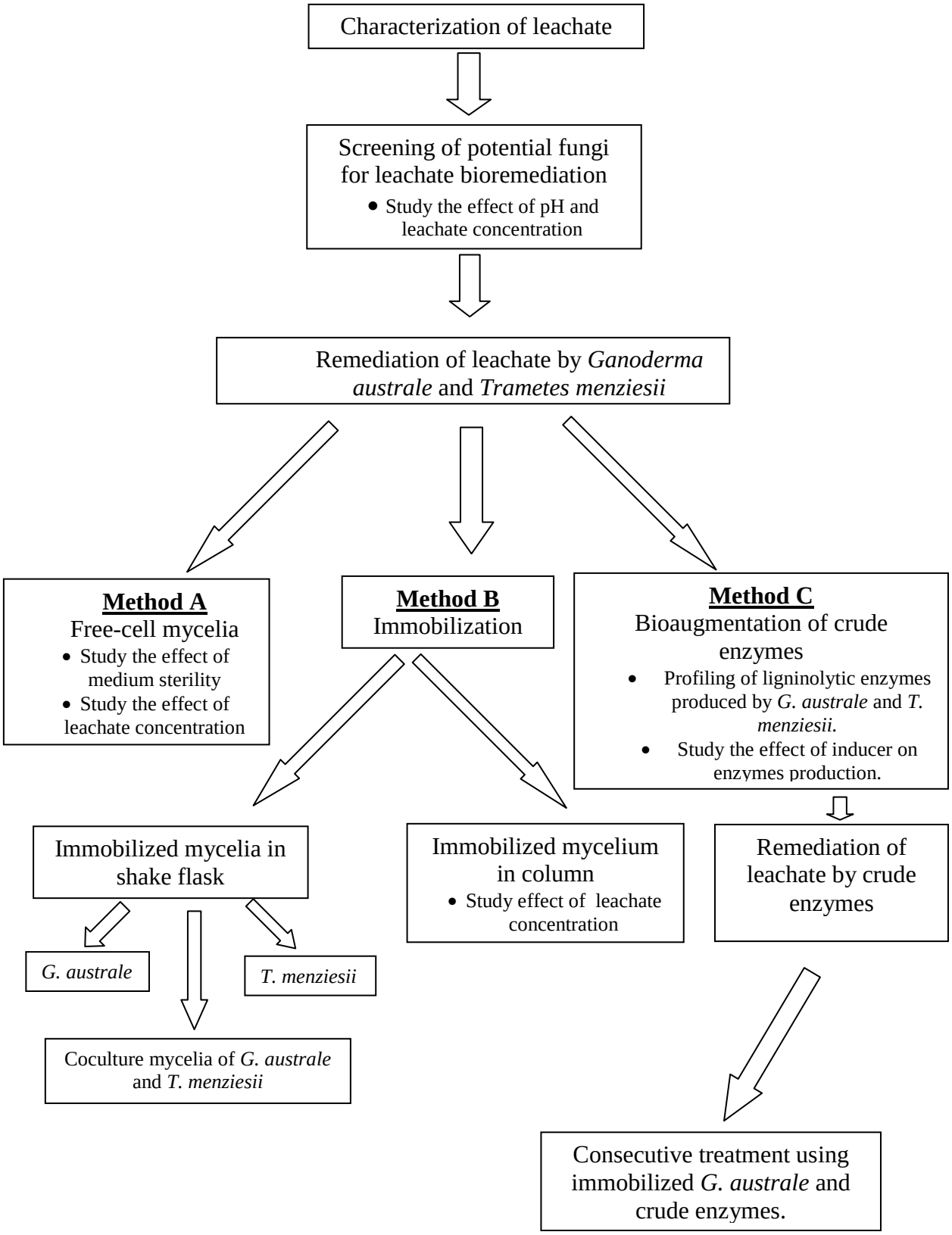


Figure 1.3 Scope of Work

LITERATURE REVIEW

2.1 Solid Waste Management

Increasingly lifestyles, continuing industrial and commercial growth in the past decade have been accompanied by rapid increased in both municipal and industrial solid waste production.

Growth population in Malaysia has increased drastically. The average annual population growth rate in Malaysia for year 2010 is 1.6%, and estimated to continue to grow at a rate of 2.4% per annum (Department of Statistics, Malaysia, [DS], 2012). Same author also revealed that the total population of Malaysia stands at over 28 million in 2012, compared with 23.3 million in 2000. This scenario created the increasing in waste amount since Malaysians are generating waste products at a rather alarming rate, much faster than the natural degradation process. According to Doble, and Kumar (2005) solid is defined as waste other than water that is collected and transported. The solid waste can be classified into different types depending on the source: household waste (also called municipal waste), industrial waste, hospital or biomedical waste. Solid waste is one of the three major environmental problems in Malaysia. It plays a significant role in the ability of nature to sustain life within its capacity.

Consumers' Association of Penang, [CAP] (2001) reported in the year 2000 the per capita generation of solid waste in Malaysia varies from 0.45 to 1.44kg/day depending on the economic status of an area. In general, the per capita generation rate is about 1kg/day. Zakaria *et al*. (2005), stated in 1994, the amount of wastes generated and collected in Kuala Lumpur was around 3 million kg/day. In the year 2000, the amount of wastes generated has increased to about 7.9 million kg/day. Meanwhile, Global Environment Centre, [GEC] (2009) affirmed that in 2002, solid waste generated in

Malaysia was 19,100 tonnes per day of waste. However, generation of solid waste is expected to reach about 30,000 tonnes per day in the year 2020, which is approximately equivalent to 10.95 millions tonnes per year due to the increasing population and development (National Solid Waste Management Department of Malaysia, 2005). The most recent information on solid waste generation rate in Malaysia are summarised in Table 2.1.

Disposal of solid waste is done almost solely through landfill method since it is regarded as one of the most economical means of handling waste. According to CAP (2001) there are about 177 disposal sites in Peninsular Malaysia. In most cases, open dumping is being practised and takes place at about 50% of the total landfills. Chandravathani (2006) reported, Alam Flora Sdn Bhd Chief Executive Officer mentioned that statistics show only three to five per cent of our solid waste is being recycled while the rest ends up in drains, abandoned properties and landfills. In Malaysia scenario, many landfills remain in the hands of local authorities who cannot afford upgrading works therefore landfills (open dumps) have any pollution control. Related to that, more than 90% of the municipal solid waste is directly disposed of on land. For that reason, landfilling still remains one of the main methods for disposing of municipal and industrial solid waste.

Table 2.1 Waste Generation in Malaysia

Year	Total Amount of Solid Waste Generated (tonnes per day)
2002	19,000
2008	23,000
2012	28,565
2020 (expected)	30,000

Source: Report by Jabatan Pengurusan Sisa Pepejal Negara (JPSPN). (2013) & Global Environment Centre, [GEC] (2009).

Landfill is a site for the disposal of waste materials by burial. It is also the oldest form of waste treatment. Landfill has been the most common method of organized waste disposal and remains so in many places around the world. Renou *et al*. (2008) reported that the sanitary landfill method used for the ultimate disposal of solid waste material continues to be widely accepted due to its economic advantages. It has been shown to be the cheapest, in terms of exploitation and capital costs. In addition, Doble, and Kumar (2005) also stated that landfill is the main method used to dispose of municipal solid waste (MSW) where 85 to 90% of domestic waste and commercial waste is disposed of in this way. According to Ding *et al.* (2001) landfilling is still the most popular way for MSWs treatment in China. Similar as in Kuwait, landfilling is the main disposal method for domestic waste where about 90% of all the domestic wastes is disposed to landfill (Al-Muzaini, 2006). In United States and throughout the world, sanitary landfills have been suggested to be the most economical and environmental acceptable method for disposal of municipal solid waste (MSW) (O'leary, & Tchobanoglous, 2002; Deng, 2007). Bouazza, and Van Impe, (1998) reported in Australia, 96% of wastes are landfilled, in the United States, approximately 80% of wastes are landfilled, 10% incinerated and 10% recycled and in Europe, 66% of the municipal solid waste and 70% of the industrial hazardous wastes are deposited in landfills.

In Malaysia, landfilling and disposing of wastes in open dumpsites have been and is expected to remain the most common method for the disposal of municipal solid wastes. According to Kamaruddin *et al*. (2014), there are 261 landfills in Malaysia whereas more than 80 % of them are being controlled tipping or open dumping practice. Therefore, the potential of a landfill to pollute the environment cannot be avoided. The process of waste degradation occurred in a landfill may produce waste products in three phases: solid (which is basically degraded waste), liquid (called leachate, which is water

polluted with wastes), and gas (generally referred to as landfill gas) (Butt, & Oduyemi, 2003). The problems arises in a landfill has led to the introduction of new design standards for waste containment facilities and new regulations were put forward in several countries. “Landfill standards”, (2012) explained that “Regulation 232/98” was created to ensure that new or expanding landfilling sites must follow the specifications such as:

- designed for groundwater and surface water protection;
- minimize impacts to the environment from site operations; and
- to facilitate site closure and post-closure care.

Meanwhile, Bouazza, and Van Impe, (1998) elaborated that nowadays, new waste containment facilities must meet stringent government requirements that involved composite liner systems (claycgeomembrane), and the existing facilities must either be (cleaned up) and closed or retrofitted with pollution- reduction/prevention systems and monitored to ensure that current legal requirements for non pollution are met.

2.2 Leachate

The main pollutant to the environment in a landfill is leachate. Leachate (liquid pollutant) is formed by water passing through the refuse at landfill and thus becoming contaminated with various organic and inorganic pollutants. Doble, and Kumar (2005) stated leachate is water seepage from the landfill. The water causes leaching of soluble salts and partly biodegraded organic compound, responsible for foul-smelling, dark-colored leachate which also may contain fine particle of soil from the daily cover. This leachate contains organic, inorganic, and microbial contaminants extracted from solid waste. According to Maranon *et al*. (2006), the degradation of the organic fraction of the waste in the landfill in combination with the percolation of rainwater produces a polluted liquid called leachate. Leachate is one of the major problems of landfills since

it is composed of large amounts of both organic and inorganic compounds (Bodzek *et al*., 2006). Ding *et al*. (2001) stated that in many cases landfill leachates are highly contaminated and have much higher concentration of organic matter and toxic substances such as metals. This high strength organic wastewater of landfill leachate may cause corrosion of the pump station, difficulty in maintaining constant effluent chlorine residual, and sludge bulking and settling problems when discharged directly to a municipal wastewater treatment plant (Deng, 2007).

Since many of the landfills are sited close to streams and rivers, so the pollution persists and leachate has seeped into the surrounding area such land. The Department of Environment (DOE) (2008) found over half of the groundwater samples taken in 2004 from 27 wells near several landfills contained with arsenic, iron, manganese, sulphates and sewage pollution above acceptable values. This make landfills all over the country have, for years, been fouling our streams and will continue doing so. This situation has been and will be reducing our environmental capacity to sustain life.

Furthermore, most important problem that arises is the subsequent movement of the leachate into the surroundings environments such as soil, groundwater (Doble, & Kumar, 2005) or even surfacewater could lead to severe pollution problems. This is supported by an article in "Landfill Leachate Treatment" (2011) which stated landfills pose pollution threat to both ground and surface water resources. Meanwhile, Al-Muzaini (2006) also stated that contamination of groundwater by landfill is recognized a serious problem in many countries in the world. In an article entitled "Removal to sewer system" (2009) reported that the rivers impacted by leachate are often yellow in appearance and often support severe overgrowths of sewage fungus. The risks from waste leachate are due to its high organic contaminant concentrations and high ammoniacal nitrogen. Christensen *et al*. *(*1994, as cited in Kjeldsen, *et al*., 2002) stated the most common type of landfill that receives a mixture of municipal, commercial, and

mixed industrial waste, may be characterized as a water-based solution of four groups of pollutants (dissolved organic matter, inorganic macro components, heavy metals, and xenobiotic organic compounds). Furthermore, Bolton and Evans (1991) have shown that the composition of the landfill leachate from the same source as well as from different sources is extremely variable.

Several factors affect the composition of landfill leachate. These include the age of the landfill, the nature of the waste (solid or liquid), the source of the waste (municipal, industrial, commercial, mining) and the amount of precipitation. According to Lema *et al*. (1988), there are many factors affecting the quality of leachate such as age, precipitation, seasonal weather variation, waste type and composition (depending on the standard of living of the surrounding population, structure of the tip). A young leachate in the acidogenic phase is characterized by a high organic fraction and a BOD_5/COD ratio greater than 0.4. It can be easily biodegraded and it is weakly acidic, consequently mobilizing heavy metals. An older leachate in the methanogenic phase is not easily biodegraded as a young leachate. It contains refractory organic compounds, high concentration of ammonia and is characterized by higher pH value. Table 2.2 presents characteristics of typical young and older leachates reported by Pouliot (1999). It shows that young leachate contains high concentration of organic compounds and lower pH value than older leachate.

Table 2.2 Characteristics of young and older leachate

Components/Characteristics	Young leachate	Older leachate
Water	95 %	99 %
Dissolved and suspended inorganics	3 %	1 %
Dissolved and suspended organics	2 %	0.5 %
COD	23 000 ppm	3 000 ppm
BOD_5	15 000 ppm	180 ppm
pH	5.2 – 6.1	7.2 - 8

Source: Pouliot (1999)

Organic pollutants in leachate are originates from domestic sewage (raw or treated), urban run-off, industrial (trade) effluents and farm wastes (Leuntech, 2012). Therefore, organic compounds that are commonly found in raw leachate included aromatic compounds, organic phosphates, polycylic aromatic hydrocarbons (PAH), polychlorinated biphenyls and phenol. Miller and Clesceri, (2003) stated that concentration of organics in leachate for instance volatile acids are usually high, as ammonia, suspended solids and several chemical compounds typically elevated above water quality standard. Organic content is measured in biological or chemical oxygen demand (BOD or COD). In addition, soluble metals such as copper, cadmium, zinc, chromium, nickel and lead are also contained in leachate (Thorneby *et al*., 2006). Meanwhile, Ghassemi *et al*. (1983) showed that the highest concentration of organic in 11 United State landfills leachates is acetic acid, butyric acid, methylene chloride, 1-1 dichloroethane and trichlorofluoromethane. While, the highest leachate concentration of inorganics are iron (Fe), calcium (Ca), magnesium (Mg), cadmium (Cd) and arsenic (As). Therefore, the treatment systems to be applied in leachate treatment should efficiently reduce organic pollutants, heavy metals and nitrogen/phosphorus compounds. Landfill sites in Malaysia are normally situated near major towns and rivers. Direct discharge of leachates to these rivers will enhance the transport of organic contaminants such as PAHs to the coastal environments and open oceans. Besides that, the volume of leachate produced in a landfill varies greatly depending on the amount of precipitation it receives, which in turn is dependent on location and season changes.

2.3 Characteristics of leachate

The complexity and variability of landfill behavior resulted in diverse composition and concentrations of leachate. According to LaGorga (1996), the most concerned contaminants in leachate when developing a leachate treatment method are

BOD, COD, nitrogen, and heavy metals. This is because the disposal of such contaminants can affect water quality and aquatic life. In addition, Mulligan (2002) stated that the parameters include nutrient (example phosphorus and potassium), levels of BOD, COD, ammonia, iron and also toxic compounds.

2.3.1 pH

In addition, pH that is a chemical component of the wastewater has also direct influence on wastewater treatability — regardless of whether treatment is physical/chemical or biological. pH is an indicator of the aggressiveness of the leachate and aerobic versus anaerobic conditions in the refuse. The acidity or alkalinity of wastewater affects both treatment and the environment. High acidity of leachate makes them difficult to treat. However, within aging municipal solid waste landfills this may not be a problem as the pH returns close to neutral after the initial stage of acidogenic leachate decomposition. Furtheremore, in one article entitled "Removal to sewer system" (2009) stated that the highest permitted pH of permitted sewer discharges is at pH 9 to 10.

2.3.2 BOD and COD

The risks from waste leachate are due to its high concentrations of organic contaminant and ammoniacal nitrogen. According to Kjeldsen *et al*. (2002) in general, landfill leachates may contain very high concentrations of dissolved organic matter and inorganic macrocomponents. The concentrations of these components may typically be up to a factor 1000 to 5000 higher than concentrations found in groundwater. Organic matter in a substrate such landfill leachate, is transformed biologically when a variety of microorganisms interface with the organic matter. The organic material that is dissolved

in landfill leachate will produce methane, which in theory will be released in weakly ventilated area such as in the treatment plant of landfill leachate.

Kang *et al*. (2002) stated that organic concentration in leachate is measured as COD and BOD. COD test measures contaminants that can be readily oxidized. COD indicates those compounds that are chemically oxidizable. High COD in influent can signal abnormal event such as slug loading of BOD or industrial discharge. Leachate COD is a measure of all oxidizable matter in the leachate. The COD test determines the amount of oxygen required to chemically oxidize any oxidizable material present in waste water. Moreover, BOD test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material, the oxygen used to oxidized inorganic material such as sulfides and ferrous iron and also measures the amount of oxygen used to oxidize reduced forms of nitrogen. Cheremisinoff (1994) stated BOD by definition is the quantity of oxygen required for stabilization of the oxidizable organic matter present after five days of incubation at 20°C. In wastewaters, effluents and polluted water BOD test is used to determine the relative oxygen requirements. Leachate BOD is a measure of the biodegradable organic mass. In addition, BOD indicates the compounds that can be biologically degraded. The BOD test estimates the amount of oxygen required by aerobic microorganisms to oxidize biodegradable material in polluted waste-waters over a fixed period of time (normally 5 days), at constant temperature (20 °C) in the dark. The permitted COD and BOD concentration in Malaysia's waste water is less than 400 mg/l and 20 mg/l, respectively (Environmental Quality Act, [EQA], 2009).

The BOD/COD ratio can be considered as a measure of the biodegradability of the organic matter and hence of the maturity of the leachate and the landfill. The organic content of waste site leachate affects the degree of biological treatability. Leachate with higher BOD/COD ratios or higher organics contents should be more

capable of being treated (Johansen & Carlson, 1976). In general, the higher the ratio of BOD to COD, the more likely the organics can be biologically degraded. Johansen and Carlson (1976) also reported that the BOD/COD ratio of landfill leachate varies from 0.6 to 0.2, compared with about 0.50 for municipal wastewater, where the lower ratio associated with decreased degradability.

2.3.3 Nitrogen

Nitrogen is an essential nutrient for biological growth, normally comprising about 12-14 percent of the mass of cell protein. Nitrogen in wastewater can exist in four forms: organic nitrogen, ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen. Nitrogen compounds are becoming increasingly important in wastewater management, because of the many effects that nitrogenous material can have on the environment. Nitrogen, in its various forms can deplete oxygen due to nitrification, fertilize aquatic plant growth, exhibit toxicity toward aquatic life, affect chlorine disinfections efficiency and present a public health hazard. The presence of nitrogen compound in an effluent causes pollution in the receiving waterway, and provision must therefore be made for the removal of these materials in wastewater treatment. Nitrogen compounds have special polluting effects in addition to those of exerting oxygen demand and stimulating eutrophication (Aofah, 2004). Ammoniacal nitrogen, (NH_3-N) is generated because of slow leaching and the release of soluble nitrogen from solid waste in landfills, which may last for several decades (Aziz *et al*., 2004). NH_3-N is one of the most important contaminants to remove in order to reduce toxicity to water life. Landfill leachate treatment generally focuses on the removal of organic nitrogenous and carbonaceous matter and ammonia nitrogen. Most of the nitrogen in solid waste bioreactors is in the form of ammonia and is produced from the degradation of proteins and amino acids

(Kjeldsen *et al*., 2002). Ammonia in landfill leachate is transformed biologically and chemically through the processes shown below (Figure 2.1).

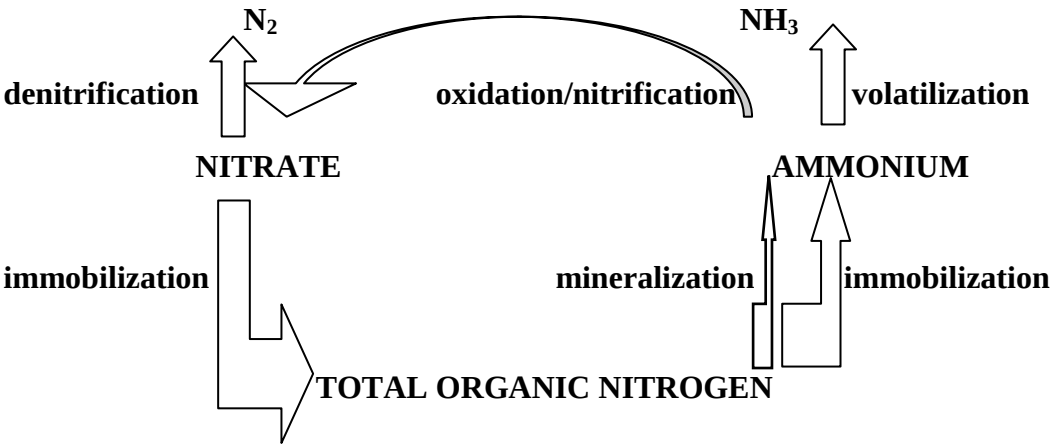


Figure 2.1 Compost Nitrogen Cycle

Source: LaGorga (1996)

Several researchers have identified ammonia as the most significant long-term component of leachate (Christensen *et al*., 1998; Kruempelbeck & Ehrig, 1999; El-Fadel, *et al*., 2002), as there is no mechanism for its degradation in anaerobic landfills (Bilgili, *et al*., 2007). According to Umar *et al*. (2010), the increase in landfill age can cause the increasing in concentration of ammonia nitrogen. This is due to hydrolysis and fermentation of nitrogenous fractions of biodegradable refuse substrates. Besides that, ammonia seems to be the constituent that lasts longer in landfill leachate and may be used to determine the remaining pollution potential in the landfill. Total solids include all of the solid constituents of a wastewater. Total solids are the total of the organic and inorganic solids or the total of the suspended and dissolved solids. In an average domestic wastewater, total solids are about half organic and half inorganic, and about two-thirds in solution (dissolved) and one-third in suspension. The organic solids, which are subject to decay, constitute the main problem in wastewater treatment.

2.3.4 Heavy metals

Heavy metal contaminations in the landfill leachate were resulted from industrial activities, and mining was one of them (Kurniati *et al*., 2014). The most common heavy metal contaminants are lead (Pb), chromium (Cr), cadmium (Cd), copper (Cu), mercury (Hg) and zinc (Zn) (Kurniati *et al*., 2014). At the earlier stages of landfill, concentration of heavy metals is generally higher. This is due to higher metal solubility as a result of low pH caused by production of organic acids (Kulikowska & Klimiuk, 2008). However, at later stages, as pH decreased metal solubility also decreased. As a result, rapid decreased in concentration of heavy metals except lead occurred. This is because lead is known to produce very heavy complex with humic acids (Umar et al., 2010).

All the contaminants present in leachate make them a potential hazardous waste from landfill sites. For instance, leachates are difficult waste streams to treat because they contain very high ammoniacal nitrogen concentrations, usually very acidic, and they are often anoxic. Besides that, if leachates are received in large volumes relative to the incoming sewage flow, the lack of phosphorus occured can result in nutrient starvation for the biological communities that perform the sewage treatment processes. Hence, it is important that leachates are properly treated to prevent from causing pollution to groundwater, health problems and effect the environment.

2.4 Treatments of leachate

The generated leachate can cause considerable environmental problems and must be collected and appropriately treated before being discharged in the environment. The applicability of leachate treatment in a particular case depends on the volume and characteristics of the leachate and discharge limits for the determined effluent (Marttinen *et al*., 2002). Besides that, strength of leachate and temperature, e.g., which

affect physical parameters and chemical and biological reaction rates, consequently are the capacity requirement of a leachate treatment system. Bilgili *et al*. (2007) and Marttinen *et al*. (2002) stated landfill leachate treatment generally focuses on the removal of organic nitrogenous and carbonaceous matter and ammonia nitrogen. Additionally, Marttinen *et al*. (2002) also reported that the usual prerequisite before leachate can be discharged into natural water is the removal of organic material that is based on chemical oxygen demand (COD), biological oxygen demand (BOD) and ammonium from the leachate.

According to Bodzek *et al*. (2006), leachate can be managed at landfill sites in many ways: treatment or pre-treatment, recirculation (spraying) at the site to speed up the process of waste methanation and evaporation of leachate to the solid phase using biogas. It can also be discharged into a sewer system or transferred to a wastewater treatment plant. Meanwhile, Renou *et al*. (2008) have reviewed and elaborated the evolution of landfill leachate treatments. They reported that leachate treatment can be divided up into two phases, which are conventional treatments and new treatments (which involved the use of membrane processes) (Figure 2.2). Conventional landfill leachate treatments can be classified into three major groups:

(a) recycling and combined treatment with domestic sewage,

(b) chemical and physical methods: chemical oxidation, adsorption, chemical precipitation, coagulation/flocculation, sedimentation/flotation and air stripping, and

(c) biodegradation: aerobic and anaerobic processes.

Leachate transfer which consists of the combination treatment of leachate between domestic sewage and recyling. These treatments were preferred for its easy maintenance and low operating costs (Ahn *et al*., 2002). However, this option is questionable since it may reduce treatment efficiency and increase the effluent concentrations (Cecen, & Aktas, 2004).

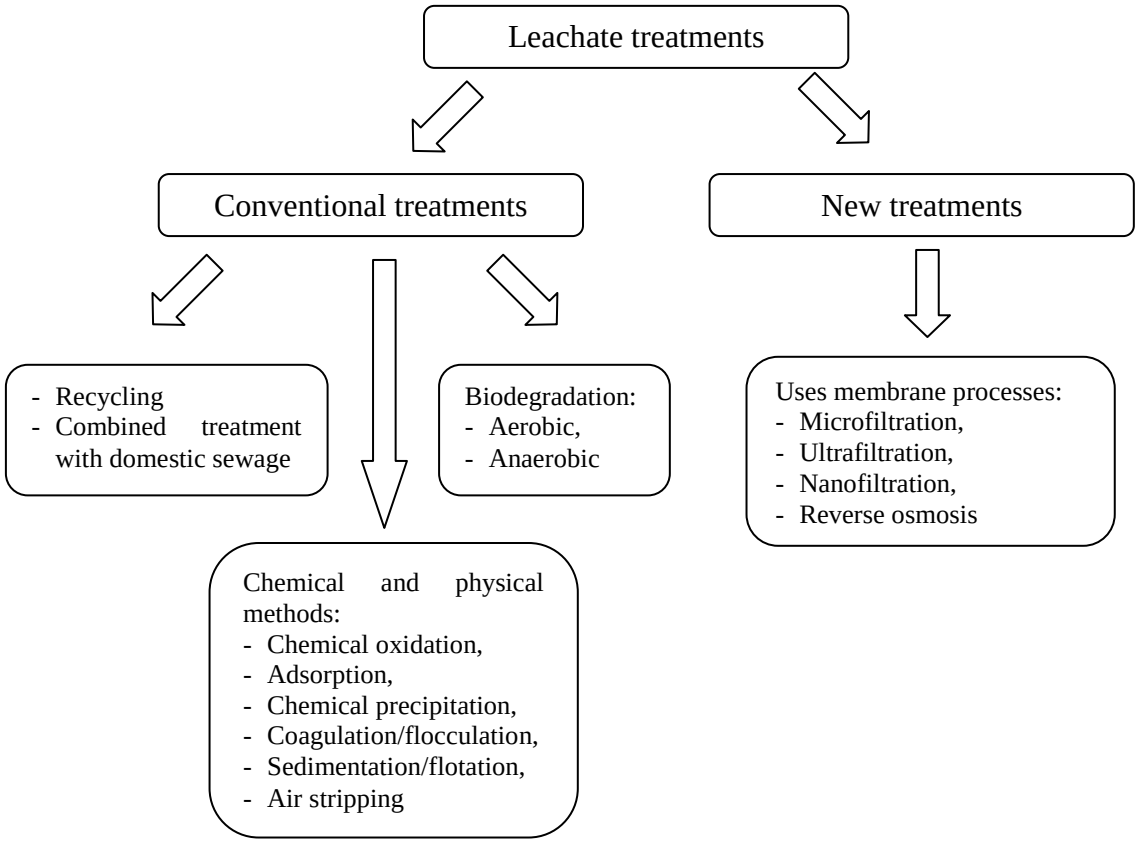


Figure 2.2 Current leachate treatments

Source: Renou *et al*. (2008)

2.4.1 Physical/Chemical treatments

Physical/chemical treatments for the landfill leachate are used as an addition at the treatment line (pre-treatment or last purification) or to treat a specific pollutant (stripping for ammonia) (Renou *et al*., 2008).

2.4.1.1 Chemical treatment

Chemical treatment is a widely used process for the destruction or separation of hazardous constituents in wastewater. This can be done by neutralization of acidic or alkaline wastewater until a suitable pH is obtained. Chemical treatment techniques commonly involved precipitation/coagulation/flocculation which is useful for the removal of heavy metal. Oxidation-reduction or redox processes is used for converting

toxic pollutants to harmless or less toxic materials that are more easily removed (Cheremisinoff, 1994). Among the chemical methods applied for leachate treatment are chemical precipitation, chemical oxidation and coagulation–flocculation. Chemical precipitation is widely used as pre-treatment in order to remove high strength of ammonium nitrogen (NH^{4+}-N) (Li *et al*., 1999). Chemical oxidation is a widely studied method for the treatment of effluents containing refractory compounds such as landfill leachate. They focused on advanced oxidation processes (AOP) that used a combination of strong oxidants, e.g. O_3 and H_2O_2, irradiation, e.g. ultraviolet (UV), ultrasound (US) or electron beam (EB), and catalysts, e.g. transition metal ions or photocatalyst (Wang *et al*., 2003). Meanwhile, Renou *et al*., (2008) stated that coagulation and flocculation may be used successfully in treating stabilized and old landfill leachates. It is also widely used as a pre-treatment prior to biological or reverse osmosis step or as a final polishing treatment step in order to remove nonbiodegradable organic matter (Tatsi, *et al*., 2003; Amokrane *et al*., 1997; Zamora *et al*., 2000).

2.4.1.2 Physical treatment

Physical treatment commonly used for separating pollutants from wastewater such as, evaporation is the process that heat the liquid, vents the vapors to the atmosphere, and concentrates the pollutants into slurry. Solvent extraction is a process whereby a dissolved or adsorbed substance is transferred from a liquid or solid phase to a solvent that preferentially dissolves that substance (Cheremisinoff, 1994). Adsorption and air stripping are the conventional landfill leachate physical treatments. Adsorption by activated carbon in columns or in powder form has been used along with biological treatment for effective treatment of landfill leachate. They provide better reduction in COD levels than the chemical methods, no matter what the initial organic matter concentration (Cecen, & Aktas, 2004; Cecen, & Aktas, 2001; Morawe *et al*., 1995;

Cecen *et al*., 2003). Air stripping is the most common method for eliminating a high concentration of NH^{4+}-N which is usually found in landfill leachates.

2.4.2 Membrane technology

The new treatments of leachate that uses membrane processes have emerged in the last 20 years. The main membrane processes applied in landfill leachates treatment are microfiltration, ultrafiltration, nanofiltration and reverse osmosis. As mentioned by Renou *et al*., (2008), microfiltration (MF) remains an effective method to eliminate colloids and the suspended matter like, for instance, in pre-treatment for another membrane process (UF, NF or RO) or in partnership with chemical treatments. However, it cannot be used alone. In the review by the same author reported that ultrafiltration (UF) is effective to eliminate the macromolecules and the particles, but it is strongly dependant on the type of material constituting the membrane. Study by Bohdziewicz *et al*. (2001) reported that UF has been applied to biological post-treatment of landfill leachate which demonstrated that 50% of the organic matter could be separated by the UF step alone. Another method that uses membrane processes is membrane bioreactors that involved the combination of membrane separation technology and bioreactors. Pirbazari *et al*. (1996) have reported the use of hybrid technology known as the ultrafiltration-biologically active carbon (UF-BAC) process that amalgamates adsorption, biodegradation and membrane filtration for the treatment of landfill leachate.

Nanofiltration (NF) is a type of membrane method that meets multiple water quality objectives, such as control of organic, inorganic, and microbial contaminants. Several researches have used this method to treat landfill leachate (Marttinen *et al*., 2002; Trebouet, *et al*., 1999; Rautenbach, & Mellis, 1994; Trebouet *et al*., 2001; Linde *et al*., 1995). They obtained nearly 60–70% COD and 50% ammonia removal by NF.

One of the most promising and efficient methods among the new processes for landfill leachate treatment is reverse osmosis (RO). RO has demonstrated promising performances on the separation of pollutants from landfill leachate (Linde *et al*., 1995; Bilstad, & Madland, 1992). Eventhough many physical/ chemical methods have been successfully applied for leachate treatment but these methods needs high maintenance and very costly. LaGorga (1996) reported that the physical-chemical processes are best suited for removing heavy metals while, biological processes are best suited for transforming or removing organic matter and ammonia from landfill leachate.

2.4.3 Biological treatments

Biological treatment process consists of controlling the environment required for optimum growth of microorganisms involved. These microorganisms are used to convert the colloidal, dissolved carbonaceous organic matter and inorganic elements such as Nitrogen, Potassium, Sulphur and Magnesium into cell tissue or/and into the various gases (Wiszniowski *et al*., 2006). Therefore, biological treatment can be used for the destruction and conversion of organic chemical and biological pollutants to CO_2, H_2O and minerals and synthesis of new microbial biomass (Equation 1).

$$\underset{\text{(Organic matter)}}{CHONS} + O_2 + \text{Nutrients} \longrightarrow CO_2 + NH_3 + \underset{\text{(New bacterial cells)}}{C_5H_7NO_2} \qquad \text{(Equation 1)}$$

According to Alley (2007), inorganic chemicals cannot be destroyed by biological treatment, but they can potentially be converted in valence or oxidative state to compounds that are permissable. In biological treatment using bacteria, the organic or hydrocarbon food serves as an energy source, or electron donor, to the bacteria and either, oxygen, nitrite and nitrate, sulphate or carbon dioxide serves as the electron

acceptor. A bacterium uses enzymes to gather and retrieve food in the form of hydrocarbon.

Since the early 1900s, microbial biomass has been used to degrade contaminants, nutrients, and organics in wastewater (Brown, 2007). Since then, biological treatment becomes more feasible and more likely to be accepted by the public since it is a green technology. Green technology refers to the processes that efficiently destroy contaminants instead of concentrating them. Application of biological treatment is in line with the National Green Technology Policy of Malaysian Government. The policy which focused on 4 pillars, namely Energy, Environment, Economy and Social shall be a driver to accelerate the national economy and promote sustainable development (National Trade Promotion Agency of Malaysia, 2010). Hence, biological treatment is selected instead of physical and chemical treatment.

Biological treatment is widely used for the removal, as well as partial or complete stabilization of biologically degradable substances present in wastewaters. Organic content of the waste can be eliminated by each of four tests: the BOD test which measures the biodegradable organic carbon and, under certain conditions, the oxidizable nitrogen present in the waste; the COD test measures the total organic carbon with the exception of certain aromatics; the TOC test measures all carbon as CO_2; and the TOD test measures organic carbon and unoxidized nitrogen and sulfur (Cheremisinoff, 1994). Biological processes based upon suspended-growth biomass, such as conventional activated sludge processes, were proven to be effective for the removal of organic carbon and nutrients content. Abdullah and Mohd. Yusof (1989) stated waste of organic nature generally require a biological treatment technique. It is supported by Aziz *et al*., (2012) who affirmed that biological processes are extremely efficient in removing organic biodegradable compounds and nitrogenous matter from young leachates, when BOD_5/COD has high value (>0.5).

In the landfill leachate treatment, biological processes (suspended/attached growth) is commonly used for the removal of the bulk of leachate containing high concentrations of BOD. This is due to its reliability, simplicity and high cost-effectiveness (Renou *et al*., 2008). The same author also reported that biological treatment of leachate is divided to anaerobic and aerobic biological processes.

The anaerobic digestion is the oldest process used in wastewater treatment. The anaerobic process involves biological decomposition of organic and inorganic matter in the absence of the molecular oxygen. The end products of the anaerobic process include methane (CH_4) and carbon dioxide (CO_2) (Wiszniowski *et al*., 2006). Pokhrel and Viraraghavan, (2004) stated that an anaerobic digestion treatment of leachates allow to end the process initiated in the landfill site. Therefore, it particularly suitable for dealing with high strength organic effluents discharged from young landfill site. Anaerobic digestion treatment of leachates applied suspended-growth biomass processes such as: digester, sequencing batch reactor and up-flow anaerobic sludge blanket (UASB) reactor; and attached-growth biomass processes which include anaerobic filter, hybrid bed filter and fluidized bed reactor (Renou *et al*., 2008). However, in contradiction of aerobic processes, anaerobic digestion conserves energy and produces very few solids, but suffers from low reaction rates (Berrueta & Castrillon, 1992).

In contrast, an aerobic treatment allows a partial reduction of biodegradable organic pollutants and should also achieve the ammonium nitrogen nitrification. Aerobic biological treatments are either based on suspended-growth biomass or attached-growth system where, in this study refers as free-cell mycelia and immobilized mycelia. Renou *et al*. (2008) also elaborated that aerobic biological processes that based on suspended-growth biomass are such as aerated lagoons, conventional activated sludge processes and sequencing batch reactors (SBR). Aerated lagoons have generally been viewed as an effective and low-cost method for removing pathogens, organic and

inorganic matters. Meanwhile, activated sludge processes are extensively applied for the treatment of domestic wastewater or for the co-treatment of leachate and sewage (Hoilijoki *et al*., 2000). The processes were proven to be effective for the removal of organic carbon, nutrients and ammonia content. Additionally, sequencing batch reactor (SBR) is reported to be ideally suited to nitrification–denitrification processes since it provides an operation system compatible with concurrent organic carbon oxidation and nitrification (Diamadopoulos *et al*., 1997).

Meanwhile, aerobic biological processes that are based on attached-growth systems suffer main problems of sludge bulking or inadequate separability (Dollerer & Wilderer, 1996) in conventional aerobic systems. Example of attached-growth systems are biofilters and the moving-bed biofilm reactor (MBBR). Trickling filters method has been investigated for the biological nitrogen lowering from municipal landfill leachate. Since then, biofilters remain an interesting and attractive option for nitrification due to low-cost filter media (Jokela, *et al*., 2002). Meanwhile, moving-bed biofilm reactor (MBBR) process is based on the use of suspended porous polymeric carriers, kept in continuous movement in the aeration tank, while the active biomass grows as a biofilm on the surfaces of them (Renou *et al*., 2008).

In general, biological processes are preferred for the treatment of leachates with high ratio of biochemical oxygen demand and chemical oxygen demand (BOD/COD) (Mulligan, 2002) and volume (Marttinen *et al*., 2002). According to Mulligan (2002), biological processes-aerobic, anaerobic, or a combination of both-are frequently selected as secondary treatment. Salem *et al*. (2008) reported that aerobic biological processes have been the most successful and reliable treatment for the landfill leachate. In addition, Lema *et al*. (1988) stated due to its reliability, simplicity and high cost-effectiveness, biological treatment (suspended/attached growth) is commonly used for the removal of the bulk of leachate containing high concentrations of BOD. Besides

that, Renou *et al*. (2008) reported biological processes have been shown to be very effective in removing organic and nitrogenous matter from immature leachates when BOD/COD ratio has a high value (>0.5). It is supported by Deng (2007), which stated that biological methods are typically applied for treatment of young leachates (e.g., from landfills of less than 1–2 years age), characterized by high 5-day biochemical oxygen demand (BOD5)/chemical oxygen demand (COD) ratios (>0.6) and high concentrations of low molecular weight organics. Meanwhile, Lema *et al*. (1988) acknowledge that both biological and physical–chemical methods have been applied to remove chemical oxygen demand (COD) and ammonia-nitrogen (NH_4-N) from landfill leachates.

Waites *et al*. (2001) stated that basic principle of aerobic treatment is that the waste-water is brought into contact with a mixed microbial population of aerobic organisms and oxygen. Soluble, suspended and colloidal biodegradable materials that contribute to the BOD are then metabolized:

$$\text{Aerobic microbes} + \text{BOD} + O_2 \rightarrow \underset{\text{(biomass)}}{\text{new cells}} + CO_2 + \text{residual BOD} + H_2O$$

Throughout the process, part of the biodegraded material is converted into CO_2 (mineralization) and a proportion becomes new biomass (assimilation). Although biological nutrient removal (BNR) had been perceived as emerging and costly, these processes are now efficient and cost effective. Among the biological technologies, bioremediation has evolved as the most promising one because of its economical, safety and environmental features (Saval, 2000).

2.5 Bioremediation

Bioremediation is an environmental clean-up technique that is currently being investigated for use on a wide variety of chemicals. Bioremediation involved the use of

naturally occurring microorganisms in order to enhance biodegradation, or normal biological breakdown. According to Alper (1993), bioremediation is at least six times cheaper than incineration, and three times cheaper than confinement. Through bioremediation, organic contaminants become actually transformed, and some of them are fully mineralized. Baker and Herson (1994) elaborated that bioremediation technologies (Table 2.3) can be classified as ex situ or in situ. *Ex situ* technologies are treatments that involve the physical removal of the contaminated material to another area for treatment (example: bioaugmentation and composting). In contrast, *in situ* technologies involve treatment of the contaminated material in place (example: bioventing, biostimulating and phytoremediation).

Table 2.3 Bioremediation Treatment Technologies

Bioaugmentation	Addition of bacterial cultures to a contaminated medium; frequently used in bioreactors and ex situ systems.
Biofilters	Use of microbial stripping columns to treat air emissions.
Biostimulation	Stimulation of indigenous microbial populations in soils and/or ground water; may be done in situ or ex situ.
Bioreactors	Biodegradation in a container or reactor; may be used to treat liquids or slurries.
Bioventing	Method of treating contaminated soils by drawing oxygen through the soil to stimulate microbial growth and activity.
Composting	Aerobic, thermophilic treatment process in which contaminated material is mixed with a bulking agent; can be done using static piles, aerated piles, or continuously fed reactors.
Landfarming	Solid-phase treatment system for contaminated soils; may be done in situ or in a constructed soil treatment cell.

Source: Baker & Herson (1994)

Bioremediation, in which hazardous waste products are degraded or detoxified by microorganisms, is a potential cost-effective technology for cleanup of environmental waste (Davis *et al*., 1993). In addition, Cooksun (1995) stated

bioremediation is the application of biological treatment, to the cleanup of hazardous chemicals. It requires the control and manipulation of microbial processes in surface reactors or in the subsurface, in situ treatment. Normally, the target compounds are hazardous chemicals that are to be remediated by biological methods. Bioremediation of chemical contaminants and waste categories had been carried out (Cai *et al*., 2007; Jeyasingh & Philip, 2005; Ding *et al*., 2001; Fu & Viraraghavan, 2001). Bioremediation has the potential to permanently eliminate contaminants through biochemical transformation, avoid harsh chemical and physical treatments, operate in situ, and be cost effective (Ding *et al*., 2001). The aerobic catabolism of aromatic compounds has been extensively investigated for a variety of microorganisms and for different natural and xenobiotic compounds (Cai *et al*., 2007). One of recent and promising bioremediation technique that use of plant to remediate pollutant from nature is called phytoremediation. Kurniati *et al*., (2014) stated that phytoremediation is an economically opportunity for pollutant removal that based on plant's ability to extract, filter, absorb, stabilize, accumulate and volatilize pollutant. In addition, Israa *et al*., (2014) stated that phytoremediation is an environmentally friendly engineering technology that has been successful in cleaning up the environment in a cost effective way without destroying the site.

Bioremediation is more intricate because it uses a catalyst (enzyme) that is supplied by microorganisms to catalyze the destruction of a specific hazardzous compound. This process is called as bioaugmentation that involve the addition of bacterial cultural products, containing different strains of microorganism and/or enzymes to wastewaters hence can help improve the performance of wastewater treatment system (Zouboulis *et al*., 2001). Enzymes are classified broadly as hydrolytic, oxidizing or reducing, depending on the type of reaction they control. The transformation takes place as the enzyme encounters its substrate (the target pollutant)

and splits the substrate into component parts or removes part of the molecule. The transformation process occurs very rapidly, leaving the enzyme unaltered and ready to deal with further molecules of substrate (Trombly, 1995).

2.5.1 Fungi in bioremediation

Bioremediation that involves the degradation of organic materials in natural environments is mediated primarily by two groups of microorganisms: bacteria and fungi (Baker & Herson, 1994). Currently, majority of bioremediation systems used bacteria. Kerry (1990) stated that a strain of *Corynebacterium* isolated from petroleum-contaminated soils was capable of degrading hydrocarbon. An engineered bacterium, *Burkholderia cepacia* showed an ability to degrade toluene (Barac *et al*., 2004). Meanwhile, Perriello (2000) reported using alkaline-utilizing bacteria that include *Pseudomonas, Variovorax, Nocardia, Chryseobacterium, Comamonas, Acidovorax, Rhodococcus, Aureobacterium, Micrococcus, Aeromonas, Stenotrophomonas, Sphingobacterium, Shewanella, Phyllobacterium, Clavibacter, Alcaligenes, Gordona, Corynebacterium* and *Cytophaga* to degrade pollutants comprising petroleum compounds. Abu Hasan *et al*., (2012) also reported that *Bacillus cereus* was the most ffective microbe for the removal of ammonia (NH_4^+-N) and manganese (Mn^{2+}) from water using a biological aerated filter under various operating conditions. Bacteria represent a widely diverse group of prokaryotic organism. Bacteria are found in all environment containing living organisms. Biochemically, bacteria show amazing metabolic versatility, and with the ability to adjust rapidly to a variety of environments – makes them very useful in bioremediation. However, most bacteria are only able to grow at a limited pH values ranging from 6.0 to 8.0 as opposed to fungi which are slightly more tolerant to acidic conditions. Most fungi are robust organisms therefore they are generally more tolerant to high concentrations of polluting chemicals than

bacteria (Gadd, 2001). In addition, they can break down tough debris and can also attack organic residues that are too dry, acidic, or low in nitrogen. This explains why fungi have been investigated extensively for their bioremediation capacities. Mollea *et al*., (2005) reported some characteristics of filamentous fungi (e.g. a specific bioactivity and growth morphology) enable them to be better potential degraders than bacteria.

Many studies have shown the breadth and efficiency of different fungi in degradation of a variety of compounds. For example, *Phanerochaete* can breakdown diesel fuel. A paper by Stajich (2007) also shows that crude extract from *Agaricus bisporus* (button mushrooms) can remove up to 90% of phenol from a polluted solution. Fungi are a diverse group of microorganisms ranging from unicellular yeasts to macroscopic mushrooms. Fungal cells generally have a cell wall of 80-90% chitin incorporating proteins, lipids, polyphosphates, and inorganic ions (Madigan & Martinko, 2003). Most fungi obtain nutrients by absorption across their cell walls and cytoplasmic membranes (Gadd, 2001). They release enzymes that are able to breakdown organic materials. According to Fu and Viraraghavan (2001) fungi can grow in a medium with wastewater as this medium is mainly composed of carbon source, nitrogen source and other nutrients. Davis *et al*. (1993) reported that lignin-degrading fungi *Phanerochaete sordida* showed potential in the solid-phase bioremediation of creosote-contaminated soils, whilst Mollea *et al*., (2005) stated that *Phanerochaete chrysosporium,* supported on wheat straw, has possibility to be used as biodegrading agent for highly naphthalene contaminated soil. Complex materials such as spent compost of oyster mushroom, *Pleurotus pulmonarius* are known to degrade various organopollutants (Law *et al*., 2003).

Trombly (1995) suggested that enzyme manipulation held great promise for improving bioremediation, whilst Chen *et al*. (1999) noted that the ability to design enzymes for remediation purposes remains an overwhelming task. Davis *et al*. (1993)

stated that degradation of xenobiotics under aqueous conditions, both by fungal cultures and by enzymes preparations *in vitro*, suggests that bioremediation using lignin-degrading fungi has potential. Enzyme, known as a biocatalyst, is used to stimulate and accelerate natural biological reactions by reducing the energy of activation. Waites *et al*. (2001) stated, if compared to chemical processes, enzyme-based processes are 'environmentally friendly' as enzymes are biodegradable. In addition, certain enzymes are not restricted to aqueous environments and can operate in two-phase water-organic solvent systems and in non-aqueous organic media, particularly hydrophobic solvents. Ho and Rashid (2008) reported the application of 'EZ-Enzyme' in bioremediation of oily sludge is promising where it helps to accelerate the stabilization of sludge within a shorter period of time. According to Hamman (2004), extracellular lignin modifying enzymes (LMEs) that have very low substrate specificity make them able to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin. Besides that, extracellular peroxidases (Mn-dependent peroxidase, MnP and lignin peroxidise, LiP) and laccases have been shown to be able to oxidize recalcitrant compounds *in vitro* (Novotny *et al*., 2004).

2.5.2 Bioremediation of leachate

Bioremediation of leachate has been reported by very few researchers. Jemec *et al*. (2012) stated currently biological treatment (bioremediation) still remains the most widely applied technique to treat landfill leachates. Bioremediation of leachate involve the treatment for mineralization of most organic compounds in leachate by microorganisms. Microorganisms can degrade organic compounds to carbon dioxide under aerobic conditions and to a mixture of carbon dioxide and methane under anaerobic conditions (Azni Idris *et al*., 2009). Besides that, characteristics of microorganisms which are ubiquitous, self-replicating, adaptable to a variety of leachate

compositions, and are active at moderate reaction conditions also suitable to be used in leachate treatment. A study by Ding *et al*. (2001) reported eight effective microorganisms (EM) that include five bacteria (*Pseudomonas* sp., *Nitrobacter* sp., *Nitrococcus* sp., *Thiobacillus* sp., and *Siderococcus* sp.) and three yeast strains (*Pachysolen* sp., *Rhodotorula* sp., and *Coccidiascus* sp.) could remove 25% and 40% of chemical oxygen demand (COD) from leachate in fine sand and sabulous clay columns, respectively. Biological treatment of medium-age landfill leachate on a membrane bioreactor with a mixed bacterial culture termed as bacteria-based membrane bioreactor (BMBR) and with mixed yeast culture termed as yeast-based membrane bioreactor (YMBR) found that the average COD and total Kjeldahl nitrogen (TKN) removal efficiency without ammonia stripping ranged between 52–66% and 14–28%, respectively. However, the performance of the BMBR and YMBR in terms of COD removal showed no significant difference but in terms of BOD_5 removal, YMBR showed better removal efficiency (Wichitsathian *et al*., 2004).

2.5.3 Fungal remediation of leachate

Researchers are now focusing on white rot fungi for use in bioremediation since these organisms have the ability to degrade a wide range of environmental pollutions (Fu, & Viraraghan, 2001; Paszczynski, & Crawford, 1995; Pointing, 2001; Shah, & Nerud, 2002). Recently, work on leachate treatment has received more attention by several researchers. Study by Saetang and Babel (2009) showed that immobilized *Trametes versicolor* on polyurethane foam could reduce biological oxygen demand and chemical oxygen demand of 52% and 42% respectively, with glucose 3 g/L in concentrated leachate. Besides that, Kim *et al*. (2003) reported the treatment of landfill leachate using a combined process of white rot fungus *P. chrysosporium* and the natural zeolite Clinoptilolite. Clinoptilolite was used in a pretreatment step as a sink for

ammonia nitrogen, thus the combination process achieved 81.5, 65, and 59% of ammonia nitrogen, soluble COD and color removal respectively. Biological treatment of landfill leachate, in order to detoxify the effluent by selected strains of white rot fungi (*T. trogii*, *P. chrysosporium*, *Lentinus tigrinus* and *Aspergillus niger*) were carried-out by Ellouze *et al*. (2008). Their observations obtained COD removal efficiencies for *P. chrysosporium*, *T. trogii* and *L. tigrinus* of 68, 79 and 90%, respectively, when landfill leachate underwent a two-fold dilution. Figure 2.3 shows the photos of several fungi that were used in leachate treatment.

Trametes versicolor *Trametes trogii* *Lentinus tigrinus*

Phanerochaete chrysosporium *Aspergillus niger*

Figure 2.3: Fungi used to treat leachate

The great potential of fungi in leachate treatment is due to their ability to produce various extracellular ligninolytic enzymes such as laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) which are involved in the degradation of lignin and their natural lignocellulosic substrates (Kalcíková *et al*., 2014). Moreover, ligninolytic enzymes also capable to degrade various pollutants such

as phenols, pesticides, polychlorinated biphenyls, chlorinated insecticides, dyes and a range of other compounds (Wesenberg *et al*., 2003). Ellouze *et al*. (2009) found that the reduction of COD was accompanied by important enzymes (ligninolytic enzymes) secretion by each fungus. *Phanerochaete chrysosporium* secreted MnP and LiP at high levels. The maximum production reached 115 and 80U/L, respectively. For *T. trogii*, the production of laccase was high (4000U/L) but MnP and LiP were secreted at low levels (48 and 52U/L, respectively). In addition, *L. tigrinus* produced high amounts of laccase (980U/L), while the amount of MnP produced did not exceed 30U/L. This may prove that the enzymatic system of these fungi was involved in the organic compounds degradation (Sayadi & Ellouze, 1995). Furthermore, previous works that showed involvement of enzymes in the leachate degradation was supported by Ellouze *et al*., (2009) who stated the biodegradation capacity of organic pollutants by white-rot fungi is correlated with their ability to secrete extracellular enzymes such as LiP, MnP, and Lac.

Investigation on treatment of landfill leachate was done using immobilized white rot fungi, namely, *T. versicolor* BCC 8725 and *Flavodon flavus* BCC 17421 (Saetang, & Babel, 2010). They found *T. versicolor* achieved 69 and 57% reduction of BOD and COD, respectively, at optimum conditions when using glucose as a co-substrate. Moreover, for *F. flavus*, BOD and COD reduction of 66 and 52 % respectively were obtained when using glucose as a co-substrate. Table 2.4 shows the summarization of previous researches that used fungi on leachate and other pollutants treatments.

Table 2.4 Treatments of pollutant by fungi

Fungi	Type of pollutant	Reference
P. chrysosporium	Xenobiotic compounds	Paszczynski & Crawford (1995)
P. chrysosporium, *Pleurotus ostreatus* and *Coriolus versicolor*	Oil contaminated soil	Yateem *et al*. (1998)
P. chrysosporium	Leachate	Kim *et al*. (2003)
Trametes trogii, *P. chrysosporium*, *Lentinus tigrinus* and *Aspergillus niger*	Leachate	Ellouze *et al*. (2008)
Trametes versicolor	Leachate	Saetang & Babel (2009)
T. versicolor BCC 8725 and *Flavodon flavus* BCC 17421	Leachate	Saetang & Babel (2010)

CHARACTERIZATION OF LEACHATES COLLECTED FROM LANDFILL IN SELANGOR

3.0 INTRODUCTION

Landfilling is still a most common disposal alternative of municipal solidwaste in most countries like China (Ding *et al.,* 2001), and Kuwait (Al-Muzaini, 2006) where about 90% of all the domestic wastes is disposed to landfill. Furthermore, Jemec *et al.* (2012) reported that according to EUROSTAT statistics for the year 2007, approximately 106 million tons (which yielding 522 kg/capita) of municipal waste was landfilled in the whole European. Meanwhile in Malaysia, landfilling and disposing of wastes in non sanitary landfill had been and is expected to remain as the most common method for the disposal of municipal solid wastes. According to Yahaya (2011), there are still 179 landfills with only 10% or less is sanitary landfill. A major problem arising from landfills is the discharge of leachate. Due to high amounts of precipitation, large quantities of leachate (liquid discharge from solid waste) from landfills in tropical climates are to be expected. According to Edi Munawar and Fellner (2013), annual leachate generation rates of more than 1,000 litres per m^2 are frequently observed in tropical countries.

Landfills can be either non-sanitary or sanitary landfill. According to Vesilind *et al*. (2002), "dump" or "tip" is the placement of solid waste on land. While, sanitary landfills are engineered operations, designed and operated according to acceptable standards. Most of the solid waste in Malaysia is disposed at non-sanitary landfills or open dump. As most of the landfills are dumpsites, they do not have the properties of sanitary landfill such as bottom liners and leachate collection system and they are built without any environmental impact assessment (EIA) study. Preliminary study was conducted in order to get a base data of leachate characteristics collected from different

landfills that include either closed or active and non-sanitary or sanitary landfill. Furthermore, it is important to characterize the landfill leachate since the success of landfill leachate treatment is obstructed by the difficulty in identifying and quantifying its typical characteristics (Chu *et al*., 1994).

Landfill leachate is formed by water passing through the waste layers and thus contains various types of pollutants. The subsequent movement of the landfill leachates into the surrounding soil, ground water or surface water could lead to severe pollution problems. Landfill leachate is a complex mixture of inorganic and organic substances. Ellouze *et al*. (2009) stated that landfills generated large amount of leachates that contained high concentrations of organics and ammoniacal nitrogen. Biological activity within the landfill influences chemicals concentration of the landfill (El-Fadel *et al*., 2002). Kjeldsen *et al*. (2002) summarized that the most common constituents of leachates based on several biological and chemical analyses performed on landfill leachates that come from different industrial origins. These include dissolved organic matter, inorganic macrocomponents e.g., Ca^{2+}, Mg^{2+}, K^{+}, Fe^{2+}, etc., heavy metals, and xenobiotic organic compounds originating from household or industrial chemicals e.g., aromatic hydrocarbons, phenols, chlorinated aliphatics, pesticides, etc. (Baun *et al*., 2004). However, in general, leachate may contain high concentrations of dissolved organic matter and inorganic macrocomponents which may vary according to varieties of influencing factors. Jemec *et al*. (2012) stated that the constituents of leachate depend on the stabilization stage of the landfill and seasonal variation. They reported that based on physicochemical characterization, the properties of leachates collected at different periods of the year vary considerably. In addition, Vesilind *et al*. (2002) reported the degree of compaction and composition of solid waste, climate, site hydrogeology, season, and age of the landfill are among the major factors that directly affect leachate composition. This is supported by Kim *et al*. (2003) who stated that the characteristics

of leachate depends on factors such as hydrogeology, waste composition, amount of rainfall, the landfill method, and the age of the landfill (e.g. active or closed landfill).

Leachate composition is an indication of the type of waste disposed and the processes occurring within the landfill (Slack *et al*., 2005). Al-Muzaini (2006) stated the complex chemical and biological reactions that take place in a landfill site make it difficult to predict the quality of leachate at any given landfill site. However, Marttinen *et al*. (2002) stated that characteristic of the leachate is one of the factors used to determine the applicability of a treatment in most cases. This is supported by Miller and Clesceri (2003) who reported that one of the important parameters for waste treatment studies is the characterization of the leachate. Mulligan (2002) stated, the ideal conditions for the biotreatment to occur require characterization of the contaminants and treatability (=feasibility) studies. Treatability study' means "a study in which a hazardous waste is subjected to a treatment process to determine: (1) Whether the waste is amenable to the treatment process, (2) what pretreatment (if any) is required, (3) the optimal process conditions needed to achieve the desired treatment, (4) the efficiency of a treatment process for a specific waste or wastes, or (5) the characteristics and volumes of residuals from a particular treatment process (US Legal Definitions, 2013). Treatability studies are often the best method to determine if the process will work and under what conditions. During the treatability test, properties of the contaminants and pH are the environmental parameters that are most important.

Generation of contaminated leachate remains an unavoidable consequence of the practice of waste disposal in landfills. Landfill leachates usually contain not only high concentrations of organics and ammonia nitrogen, but also heavy metals and hazardaous chemicals. These may contaminate the surrounding environment. The characteristics of landfill leachates are different from one place to another due to several factors such as the composition and amount of the waste, climate of the sites, and age of the landfills.

Hence, treatment methods that have been successfully employed in one location may not be an efficient treatment elsewhere. Therefore, leachate characterization is a critical factor in establishing a corresponding effective treatment process. Hence, to treat leachate, it is very important to determine the characteristic of that leachate.

Thus, the objective of this study is to determine the level of selected parameters in the raw leachate collected from sanitary and non-sanitary landfills in Selangor and to compare the changes of leachate composition for both aged, old leachate (from closed landfills) and young leachate (from active landfills).

3.1 MATERIALS AND METHODS

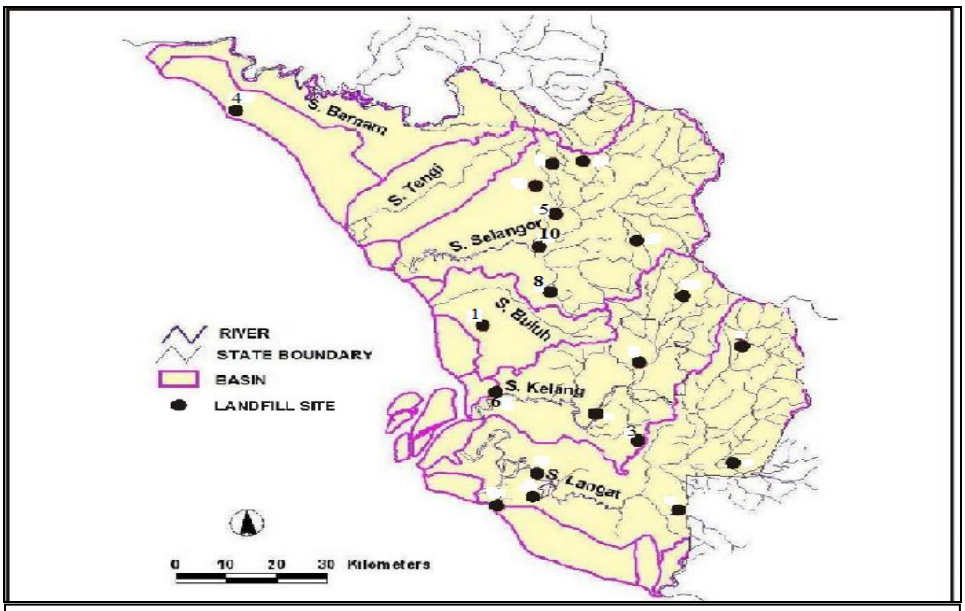
3.1.1. Landfills description

Leachate samples were collected from ten landfill sites in Selangor listed in Table 3.1. Leachate was collected from Jeram, Kuala Selangor (No. 1); Tanjung 12, Sepang (No. 2); Air Hitam, Puchong (No. 3); Sungai Besar, Sabak Bernam (No. 4); Bukit Beruntung, Hulu Selangor (No. 5); Teluk Kapas, Rantau Panjang (No. 6); Teluk Gong, Pandamaran (No. 7); Kundang, Selayang (No. 8); Batu 20, Rawang (No. 9) and Kubang Badak, Kuala Selangor (No. 10) (Table 3.1 & Figure 3.1). The landfills received wastes around the municipality area. Among the ten landfills, seven are non-sanitary landfills (i.e. without liners and leachate collection system) that include five closed landfills (Nos. 6-10) and two active landfills (Nos. 4-5) which were operated by either private contractor or Majlis Daerah. Another three landfills are sanitary landfills (i.e. with liners and leachate collection systems) that are operated by private companie and include two active landfills (Nos. 1-2) and a closed landfill (No. 3) (Figure 3.1). Table 3.1 also depicted that the landfills were in

Table 3.1 General characteristics of the Municipal solid wastes (MSW) landfills included in the study.

Landfill Nos.	Location	Type of Landfill (closed/active)	Landfill area (acre)	Period of operation	Type of waste
1	*Jeram, Kuala Selangor	Sanitary (active)	160	2007-	Domestic waste (95%), Others (5%)
2	*Tanjung 12, Sepang	Sanitary (active)	160	2010-	Domestic waste (96%), Industrial (3%), Others (1%)
3	*Air Hitam, Puchong	Sanitary (closed)	100	1995-2006	Domestic waste
4	Sg Besar, Sabak Bernam	Non-sanitary (active)	10	NA	Domestic waste
5	Bukit Beruntung, Hulu selangor	Non-sanitary (active)	NA	NA	Domestic waste
6	Teluk Kapas, Rantau Panjang,	Non-sanitary (closed)	32.4	2000-2003	Domestic waste
7	Teluk Gong, Pandamaran	Non-sanitary (closed)	19.42	1986-2000	Domestic waste
8	Kundang, Selayang	Non-sanitary (closed)	NA	NA	Domestic waste
9	Batu 20, Rawang	Non-sanitary (closed)	NA	NA	Domestic waste
10	*Kubang Badak, Kuala Selangor	Non-sanitary (closed)	30	2006-2007	Domestic waste

Sanitary: Engineered landfill with liners and leachate collection system;
Non-sanitary: Uncontrolled landfills without liners and leachate collection system;
NA: Not available.
(Source: Department of Local Government, 2003; *Sanitary landfills (2009))



1: Jeram, Kuala Selangor **2: NA** **3: Air Hitam, Puchong**
4: Sg. Besar, sabak Bernam **5: Bukit Beruntung, Hulu Selangor**
6: Teluk Kapas, Rantau Panjang **7: NA** **8: Kundang, Selayang**
9: NA **10: Kubang Badak, Kuala Selangor**

NA: Not available

Figure 3.1. Landfill sites included in the study.

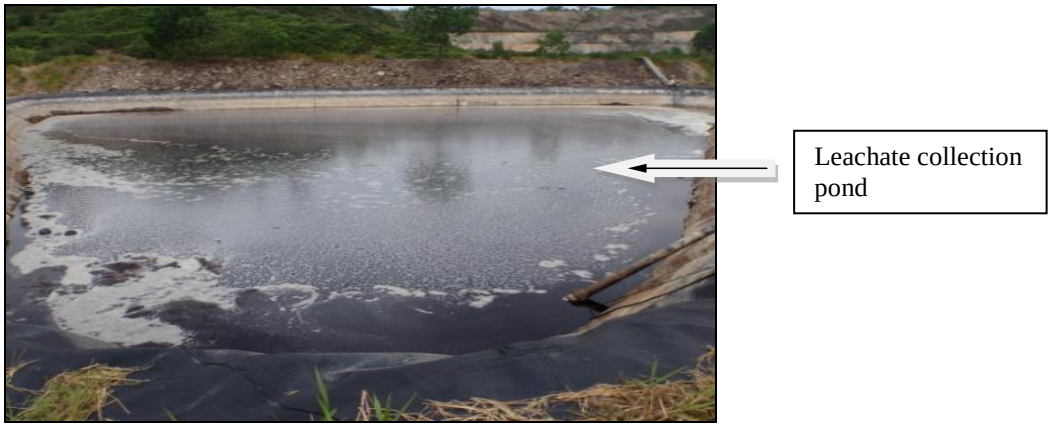
Source: Wan Zuhairi Yaacob (2009).

various range of operation duration. It is between three years to 27 years. The sources of solid waste in Malaysia usually generated by residential, commercial, institutional, construction, municipal services, treatment plant site, industrial and agriculture. Unfortunately, most of the wastes that are dumped at non-sanitary landfill with lack of record on the total amount and type of waste due to the uncontrolled manner of waste disposal during the early 1980's except for sanitary landfills (Table 3.1). Samples were collected from each landfill.

3.1.2. Sampling procedure

Leachate samples were collected from existing ponds. Leachate collection ponds servicing the whole landfill were sampled at all the sanitary landfills (Nos. 1-3) (Figure 3.2a and 3.2b). At the non-sanitary landfills no such collection ponds were installed hence, samples were collected from leachate flowing out below the Landfills Nos. 4-10

(Figure 3.2c). At all landfills, the sampling bottles were lowered into the pond in order to collect leachate. All samples were collected in plastic bottles. The samples were packed in cool boxes (8–15 °C) and were transported to the laboratory for analysis.



3.2a Closed Air Hitam sanitary landfill

3.2b Active Jeram sanitary landfill

3.2c Closed non-sanitary landfill Batu 20, Rawang

Figure 3.2: Example of sanitary landfills and non-sanitary in the study

3.1.3 Analysis of leachate

The leachate was characterized based on several pollution parameters as required by EQA (2009) i.e. Biological oxygen demand (BOD), Chemical oxygen demand (COD), Total carbon (TOC), Total nitrogen (TN), Total suspended solid (TSS), Ammoniacal-nitrogen (NH_3-N), pH and also heavy metals such as: magnesium, lead, copper, iron, zinc and cadmium. The techniques used for sampling and analyses were in accordance with the Standard Method for the Examination of Water and Wastewater (APHA, 1998). The Hach DR 2800 spectrophotometer (USA) was used for the determination of chemical concentration. The experiment was replicated three times to obtain an average. All the experiments were undertaken at 20 ± 2° C. The achieved parameter values were compared with the Malaysian Standard "Environmental Quality (Control of Pollution from Solid Waste Transfer Station and Landfill) Regulations 2009 under the Laws of Malaysia – Malaysia Environmental Quality Act 1974" (EQA, 2009).

3.1.3.1 pH

The pH of leachate was determined using a pH meter (model IQ160). This analysis was done immediately after sampling.

3.1.3.2 Total suspended solid

Total suspended solid are the portion of the solids retained by a filter. The procedure for measuring total suspended solids was a simple gravimetric analysis involving the difference in weights before and after a sample of water was passed through a Whatman filter paper. The filter paper was prewashed with distilled water and oven dried for one hour at 103°C, then cooled in a dessicator before weighing. This was repeated until a constant weight was obtained. Each leachate sample was mixed by shaking and 100 ml of leachate was measured in a graduated cylinder. The leachate was then filtered through the filter. The filter paper was then oven dried at 103 °C–105 °C

for at least an hour, and cooled in a dessicator before weighing. The total suspended solid was obtained by subtracting the weight of the filter paper.

3.1.3.3 Biological oxygen demand (BOD_5)

The procedure is based on the consumption of oxygen by microorganisms that are present in the sample. Dilution method (Standard methods 5210 B) was used to determine the concentration of dissolved oxygen in the samples. This method requires the addition of dilution water which consists of phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride solutions. Sample was first filled in BOD bottles without trapping any air bubbles under the stopper. Then, the bottle was filled with dilution water to just below the lip of the bottle. The dilution water was allowed to flow down the sides of the bottle to prevent air bubbles from becoming trapped in the bottle. The glass stopper was placed and the contents were mixed by inverting the bottle several times. The initial dissolved oxygen (DO) was measured using a probe and meter. Then the stopper was replaced carefully and dilution water was added to the lip of the BOD bottle to make a water seal. An aluminium foil was placed over the lip of the bottle. The bottle incubated at the specified temperature 20 (±1) °C for five days.

Dissolved oxygen was measured before and after incubation, and the BOD was calculated from the difference between initial and final DO. Since initial DO was determined shortly after the dilution was made, all oxygen uptake occurring after this measurement was included in the BOD measurement.

3.1.3.4 Chemical oxygen demand (COD)

In this experiment, reactor digestion method and colorimetric determination was used. The chemical oxygen demand (COD) is the amount of oxygen consumed by organic matter when oxidized with boiling acidified potassium dichromate. This method involved the addition of 2 ml sample into the COD digestion reagents vials. The sample was heated for two hours with a strong oxidizing agent, potassium dichromate.

Oxidizable organic compounds react, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). The COD reagent also contains silver and mercury ions. Silver is a catalyst and mercury is used to complex chloride interferences. Then the vial was cooled to room temperature before preceded to Colorimetric Determination Method 8000.

3.1.3.5 Total nitrogen (TN)

Total nitrogen in the leachate was determined using TNT Persulfate Digestion Method. Digestion was required for determining total nitrogen. In this method, all forms of nitrogen were converted to nitrate by an alkaline persulfate digestion. Then, sodium metabisulfite was added after digestion to eliminate halogen oxide interferences. Nitrate then reacts with chromotropic acid under strongly acidic conditions to form a yellow complex with an absorbance maximum at 410 nm.

3.1.3.6 Ammoniacal nitrogen (NH_3-N)

Ammoniacal nitrogen in the sample was detected by Nessler method. The analysis was done by measuring 25 ml sample in a mixing graduated cylinder or volumetric flask. Then three drops of Mineral Stabilizer (EDTA), three drops of Polyvinyl alcohol and 1.0 ml of Nessler reagent were added. After addition of each chemical the flask was stopped and inverted several times to mix. After that, a one minute reaction was allowed and absorbance was measured at 425 nm.

3.1.3.7 Total organic carbon (TOC)

The total organic carbon (TOC) was determined by first sparging the sample under slightly acidic conditions to remove the inorganic carbon. In the outside vial, organic carbon in the sample was digested by persulfate and acid to form carbon dioxide. During digestion, the carbon dioxide diffuses into a pH indicator reagent in the inner ampoule. The absorption of carbon dioxide into the indicator forms carbonic acid. Carbonic acid changes the pH of the indicator solution which, in turn, changes the

color. The amount of color change was related to the original amount of carbon present in the sample. Test results were measured at 426 nm.

3.1.3.8 Heavy metal

Heavy metals such as magnesium, lead, copper, iron, zinc and cadmium were analyzed using the Hach DR 2800 spectrophotometer (according to the APHA, 1998).

3.2 RESULTS AND DISCUSSION

3.2.1 Preliminary study of leachate characteristic collected from sanitary and non-sanitary landfills

Leachate was characterized based on its pH value, BOD_5, COD, Ammoniacal nitrogen, Total suspended solids, Total nitrogen and Total carbon. According to Bilgili *et al*., (2007) leachate quality was investigated by measuring pH, alkalinity, total dissolved solids (TDS), COD, total Kjeldahl nitrogen (TKN), and ammonia nitrogen (NH_3-N). From this work, it was found that the characterization of the landfill leachates in terms of stated general chemical parameters showed huge differences among the landfills (Table 3.2). This could be due to the method of leachate collection, age of the landfill and amount of waste. For instance, the total nitrogen concentrations varied from 6.0 to 1700 mg/L, the total suspended solids varied from 10 to 3000 mg/L, and correspondingly the observed concentrations of ammoniacal nitrogen were from 0.94 mg/L up to the extremely high concentration of 3200 mg/L in the leachate from Landfill No. 3.

The concentration of total organic carbon was 12–45000 mg/L and was not detected in the leachate from Landfill No. 9, while the pH-values varied from 6.29 to 8.39. The value of BOD_5 was lower for all the leachate collected from non-sanitary landfills than in leachate from sanitary landfills. The values of BOD_5 for leachate from closed non-sanitary landfills were 171 ± 18.36 mg/L (Landfill No. 6), 369 ± 32.97 mg/L

(Landfill No. 7), 81 ± 6.24 mg/L (Landfill No. 8), and from closed sanitary landfill is 2497 ± 221.31 mg/L (Landfill No. 3). Leachate from non-sanitary Landfill No. 4 has BOD_5 value of 1160 ± 98.49 mg/L, while leachate from sanitary Landfill No. 1 and Landfill No. 2 have BOD_5 value of 11360 ± 703.42 mg/L and 1971 ± 16.46 mg/L respectively. Similar patterns were shown for the COD value for leachate from non-sanitary and sanitary landfill.

Leachates from the non-sanitary landfills (Nos. 4–10) generally had lower values than sanitary for all tested parameters, except for the pH-values. This may be due to the method of leachate collection and other different factors such as amount and composition of waste. However, these results are within the ranges generally observed in landfills (Kjeldsen *et al*., 2002). According to El-Fadel *et al*. (2002), leachate quality is difficult to forecast due to a variety of influencing factors such as waste composition and landfill operations. The value of BOD_5 and COD for the leachate from sanitary landfill demonstrated higher concentration than leachate collected from uncontrolled landfills. This occurrence arised since in un-engineered (uncontrolled) landfills, production of leachate may comes from groundwater entering the waste, some additional leachate volume is produced during waste decomposition, and some additional surface water will sometimes run onto waste from its surroundings (Landfill Leachate Treatment Expert Website, [LLTEW], 1995).

In addition, Chu *et al*. (1994) stated that chemical properties of leachate samples from different landfills vary widely. They were affected by the amount of waste disposal on landfill, composition, and moisture content of the refuse; hydrogeology and climate of the site; age and height of the landfill; and season of the year. Bolton and Evans (1991) reported research has shown that the composition of landfill leachate from the same source, as well as from different sources, is extremely varied.

Table 3.2 Characteristics of landfill leachates with respect to general chemical parameters.

On top of that, it is also found that leachate produced from landfills is a high strength of organic wastewater which, when discharged directly to a municipal wastewater treatment plant, may cause corrosion of the pump station, difficulty in maintaining constant effluent chlorine residual, and sludge bulking and settling problems (Deng, 2007). Biological activity within landfill influence chemical concentration levels of the landfill. Particularly, at the onset of biodegradation processes the high organic and moisture contents resulted in an extremely strong leachate, which can affect the leachate treatment facility (Bilgili *et al*., 2007).

3.2.2. Comparison of leachate characteristics collected from active and closed landfills

Besides comparing the characteristics of leachate from different type of landfills, the characteristics of leachate from active and closed landfills were also compared. The significance of the comparison was to identify the status of the pollutant resulting from the landfills even though it has been closed for several years.

Characteristics of the leachate content is one of the important criteria to be determined before establishing the most suitable method for treating and disposing of any given pollutant. Besides that, an extensive characterization for the leachate is required in order to design a leachate treatment system for a particular landfill site. These analytical methods are required to assess the polluting strength of the waste. According to the EQA Standard 1974 (2009) and Waites *et al*. (2001), usually the tests include the determination of BOD, COD, TSS and TS. However, other tests may also be performed in order to determine the levels of specific components such as nitrogen, phosphorus, and heavy metals. From the results displayed in Table 3.3, it shows that the characteristics of leachate from two active landfills i.e. sanitary (No.1) and open dump (No.4) while, two were closed landfills i.e. sanitary (No.3) and Landfill No.10 (non-

sanitary). The composition of leachate shows almost all the studied parameters of leachate from closed landfills were lower than leachate from active landfills except pH and ammoniacal nitrogen that were higher. These may be due to biological and chemical composition in the landfill. Kjeldsen *et al*. (2002) stated that even after a landfill stops accepting waste and a final cover is placed over the landfill, the waste will continue to decompose.

Measurement of BOD and COD reveals the organic acid content in leachate. The value of BOD_5 for the leachate of closed landfills (Landfill No.3: 2497 ± 221.31mg/l and Landfill No.10: 925 ± 6.00 mg/L) were lower than leachate of active landfills (Landfill No.1: 11360 ± 703.42 mg/l and Landfill No.4: 1160 ± 98.49 mg/l). Similar findings were obtained for the COD value where the values for leachate from closed landfills i.e. Landfill No.3: 4000 ± 312.77 mg/l and Landfill No.10: 2880 ± 128.55 mg/L were lower than COD value for leachate from active landfills i.e. Landfill No.1: 16000 ± 1130.63 mg/l and Landfill No.4: 2982 ± 308.64 mg/l. Ratios of leachate BOD_5/COD can be used to predict the effectiveness of various biological and physical-chemical processes for leachate treatment (Chian & De Walle, 1976). Table 3.3 shows that the BOD_5 to COD ratio was reduced from 0.71 ± 0.08 of active-landfill (Landfill No. 1) to 0.62 ± 0.09 of closed-landfill (Landfill No. 3). This represents the decrease in biodegradability of the leachates with respect to their age and also the 5 stages of biodegradation, which are initial adjustment (stage 1), transition phase (stage 2), acid phase (stage 3), methane fermentation (stage 4) and maturation phase (stage 5). However this small amount of differences may be due to the factor that the closed landfill was only stopped receiving the waste just three years before sample collection.

Table 3.3 Comparison of leachate characteristics from active and closed landfills based on selected pollution parameters.

This study revealed that leachate from closed landfills of both sanitary and non-sanitary has lower biological oxygen demand (BOD_5) and chemical oxygen demand (COD) content compared to leachate from active landfills. This finding is parallel with report by Kang *et al*. (2002) which stated that, organic concentration (measured as BOD and COD) decreased as landfilling age increased. Also this is supported by Avezzu *et al*. (1995) which found out that leachate BOD and COD concentration steadily declined with age. According to Tatsi and Zouboulis (2002) higher proportions of organic materials existing in 'fresh' leachates are biodegradable and can be removed by biological processes. Another consequence is that biological reactions which take place in 'fresh' leachates are expected to produce an acidic pH value and an unpleasant smell. In addition, Miller and Clesceri (2003) stated that for waste sites (landfills) of longer standing (inactive or closed), COD and BOD levels in leachate may be less than the value for young leachate (which collected from active landfill). However, they generally exceed levels found in wastewaters, thus cannot be discharged to the surrounding environment.

Considering individual parameters, Table 3.3 shows the range of pH was from 8.05 to 8.30. The pH for the leachate from active sanitary landfill (Landfill No. 1) was 8.05 ± 0.05 while the pH value for the leachate from closed sanitary landfill (Landfill No. 3) was 8.19 ± 0.17. Meanwhile, the table also revealed that the content of ammonium-nitrogen for leachate collected from closed landfills (Landfill No.10: 650 ± 8.89 mg/L) was higher compared to the amount for leachate from active landfills (Landfill No.1: 21.3 ± 3.17 mg/L).

Results showed that the concentrations of all tested heavy metals in leachate landfill were below the standards levels (refer Table 3.3), except for Pb (0.5 ± 0.17 mg/L) and Fe (10.41 ± 0.74 mg/L) for Landfill No. 10. The comparison of heavy metals concentrations in leachate from active landfill (Landfill No. 1) and closed landfill

(Landfill No. 3) demonstrated that the concentration of heavy metals was higher in leachate from closed landfill than in leachate from active landfill except for Fe. The concentrations of Pb were 0.11 ± 0.01 mg/L for Landfill No. 3 and 0.06 ± 0.02 mg/L for Landfill No. 1; the concentrations for Zn were 0.19 ± 0.03 mg/L for Landfill No. 3 and 0.18 ± 0.03 mg/L for Landfill No. 1 and, the concentrations for Mg were 180.79 ± 5.31 mg/L for Landfill No. 3 and 23.01 ± 0.90 mg/L for Landfill No. 1. Meanwhile, the concentrations of Fe were 2.62 ± 0.11 mg/L for Landfill No.3 and 4.44 ± 0.11 mg/L for Landfill No.1.

The result obtained in this study showed that the composition of leachate for almost all the studied parameters from closed landfills were lower than leachate from active landfills except pH and ammoniacal nitrogen that were higher. These demonstrated that biodegradability within natural environment without any treatment of the leachates decrease with respect to their age. These observations are coherent with findings by El-Fadel *et al*. (2002) which stated that the BOD/COD ratio can be considered as a measure of the biodegradability of the organic matter, which typically decrease with time. Therefore, according to Tatsi and Zouboulis (2002), the observed decrease in BOD_5/COD ratio represents a more complete oxidation of organic carbon; hence, it becomes less readily available as an energy source for microbial growth. On the contrary, pH of leachate increased in the closed landfills, which were older. These results are consistent with several previous reports whereby, Faeiza *et al*., (2004) reported a study at Hong Kong detected that pH for a 3.5 years of closed landfills ranged from 7.2 to 8.0 and pH for a 1.5 year closed landfill was only at 5.8. The finding was supported by Chu *et al*. (1994) who stated that pH of leachate increased with time due to the decrease of the concentration of partially ionized free volatile fatty acid over the age.

The ammonia concentration in aged leachate showed higher concentration than in young (active) leachate that may be due to biological activities. According to Faeiza *et al*. (2004) considering the nitrogenous compounds, ammonia nitrogen was present in high concentrations, probably owing to the deamination of amino acids during destruction of original organic compounds. However, high level of ammonia (>5,000 mg/L) is toxic and will decrease the biological treatment efficiency. Instead of that, the result also shows that as landfills age, the organic content of leachate decreases and ammonia content increases. Tatsi and Zouboulis (2002) stated the great majority of total Kjeldahl nitrogen (TKN) content was found to be in ammoniacal form. Therefore, ammonia is the principal pollutant of 'old' leachate (from closed landfill) samples. On the other hand, Chu *et al*. (1994) has mentioned that, after a period of 3–8 years, the ammoniacal nitrogen reaches mean values between 500 and 1500 mg/l, and it will remain at this level for at least 50 years. Whereas, the result of this study for other parameters such as total suspended solids and total nitrogen varies in leachate. Similar results were found by Tatsi and Zouboulis (2002) where the concentration of total dissolved solids (TDS) fluctuates widely. However, Bilgili *et al*. (2007) stated that total solids (TS) concentration is expected to decrease as the leachate moves from acidogenic to methanogenic.

On the other hand, observations for heavy metal concentrations in leachate in this study are in contrast with the finding by Tatsi and Zouboulis (2002) which stated inorganic contaminants also follow the trend of decreasing concentrations with increasing of leachate age and stability. Lo (1996) reported the concentration of lead decreased with increasing age of landfill. Besides that, Tatsi and Zouboulis (2002) stated that the concentration of metals in leachate samples were affected by the initial amounts that existed in domestic solid wastes, but they can also be leached by degradation processes within the landfill. 'Fresh' leachate samples showed a higher

degree of metal solubilization due to lower pH values caused by the biological production of organic fatty acids. The difference of findings between this study and Tatsi and Zouboulis (2002) may be due to the different in climate and source of waste disposal. However, as the landfill age increased, consequently the pH values increases. This may be caused by a decrease in the concentration of free fatty acids which, due to anaerobic consumption.

Overall, characterization of leachate in this study showed that the principal pollutants in the leachate samples were organic and ammonia loads. This finding is supported by Ding *et al*. (2001) who stated that landfill leachates are highly contaminated and contains high concentrations of organic matter and toxic substances such as metals. Meanwhile, CAP (2001) reported that Malaysian solid wastes contain very high organic waste and consequently high moisture content and bulk density of above 200kg/m3. Leachate composition in landfill is influenced by several factors including site operations and management (such as refuse pretreatment and irrigation), refuse characteristics (such as composition and age), internal processes (such as biodegradation, hydrolisis and adsorption) and the corresponding landfill fermentation stage. Besides that, refuse age and the corresponding landfill fermentation stage are usually major determinants of leachate composition.

3.3 CONCLUSION

As a conclusion, the present study that monitored the main physico-chemical pollution parameters of leachate samples, collected from ten different sites in Selangor landfill revealed that the characteristics of the leachate in terms of general chemical parameters showed huge differences among the landfills. For instance, the total nitrogen concentrations varied from 6.0 to 1700 mg/L, the total suspended solids varied from 10 mg/L to 3000 mg/L, and correspondingly the observed concentrations of ammoniacal

nitrogen were from 0.94 mg/L up to the extremely high concentration of 3200 mg/L. The concentrations of total organic carbon, BOD_5 and COD were from 0–45000 mg/L, 56-11360 mg/L and 165-16000 mg/L, respectively, and the pH-values were varied from 6.29 to 8.39. Hence, reveals the difficulty in leachate treatment.

The study indicated that the age of the landfill, has a significant effect on leachate characteristic and composition. Our study showed that leachate from closed landfills of both sanitary and open dump has low biological oxygen demand (BOD_5) and chemical oxygen demand (COD) content compared to leachate from active landfills. However, pH of leachate increased in the older age of landfill. Similar pattern was showed by ammoniacal nitrogen where the ammonia concentration in aged leachate is higher compared to young leachate.

This study also noted that the principal pollutants in the leachate samples were organic and ammonia loads. With reference to the discharge limit of 400 mg/l for COD and 5 mg/l for NH_3, it is suggested that the leachates need further treatment before they can be discharged to the nearby environment. Since the result obtained in this study showed that the concentration of ammoniacal nitrogen and organic matter were high, hence biological method specifically fungal remediation was considered for leachate treatment.

SCREENING OF FUNGI AS CANDIDATE FOR LEACHATE BIOREMEDIATION

4.0 INTRODUCTION

Biological treatment methods use microorganisms to remove or at least reduce the toxicity of a waste stream. Typically, contaminants are transformed or removed from landfill leachate through biological treatments in combination with physical-chemical treatment. Ellouze *et al*. (2009) reported that the treatment of landfill leachates is very complicated, expensive and required various process applications due to their high concentration of COD and nitrogen. Ding *et al*. (2001) stated that external treatment of landfill leachate by previous researchers require physical, chemical and biological processes for the removal of high-strength organic and inorganic materials. Biological treatment converts the contaminants into other chemicals with a far lower contaminating potential, and will remove those contaminants from the waste. Therefore, biological methods are best suited for transforming or removing organic matter and ammonia from landfill leachate. Organic matter in a substrate, such as landfill leachate, is transformed biologically by the interactions with a variety of microorganisms (Lema *et al*., 1988). Aerobic biological processes have been the most successful and reliable treatment methods for landfill leachate (Salem *et al*., 2008). Due to their reliability, simplicity and high cost-effectiveness, biological treatments (suspended/attached growth) are commonly used for the removal of the bulk of the leachate containing high concentrations of biological oxygen demand (BOD) (Leonowicz *et al*., 1991).

Various microorganisms have been used for removing pollutants from the environment. Most research had focused on bacteria, with fungal applications only attracting interest within the past two decades. Fungi are a diverse group of microorganisms ranging from unicellular yeasts to macroscopic mushrooms (Madigan

& Martinko, 2003). Some characteristics of filamentous fungi (e.g. a specific bioactivity and growth morphology) potentially make them better degraders than bacteria (Chiara *et al*., 2005). The biodegradation abilities of some white rot fungi are promising. Researchers are now focusing on white rot fungi for use in bioremediation since these organisms have the ability to degrade a wide range of environmental pollutions (DeMarco *et al*., 2003; Paszczynski, & Crawford, 1995; Pointing, 2001; Shah, & Nerud, 2002). Based on non-specific nature of the lignin oxidation system, white-rot fungi are normally capable of oxidising a wide spectrum of xenobiotic compounds. White-rot fungi are characterized by their ability to degrade lignin, which is a high-molecular weight complex polymer in wood (Matsubara *et al*., 2006).

Most fungi obtain nutrients by absorption across their cell walls and cytoplasmic membranes. They release a variety of enzymes that are able to breakdown organic materials. For examples, *P. chrysosporium* that produced manganese peroxidase (MnP) and lignin peroxidise (LiP) was able to remove 68% of COD in 50% of diluted leachate (Ellouze *et al*., 2009). A study by Sayadi and Ellouze (1995) using *Phanerochaete chrysosporium* indicated that the culture conditions which yield high levels of LiP activity lead to high levels of olive mill wastewaters decolorization. Meanwhile, Pointing (2001) reported high decolorization efficiencies (up to 85% chloro-guaiacol degradation) was demonstrated by laccase of *Trametes versicolor*. The ability of fungi in pollutants degradation may be due to the facts that fungi can grow in a medium with wastewater. The wastewater medium is mainly composed of carbon source, nitrogen source and other nutrients needed for fungi growth (Fu & Viraraghavan, 2001). The ability of fungi in leachate treatment has been shown by *Trametes versicolor* (Saetang & Babel, 2009) and *Phanerochaete chrysosporium* with combination process of natural zeolite Clinoptilolite (Kim *et al*., 2003).

The strains that were used in this study can be characterized according to the following:

Pleurotus species produce manganese peroxidase (MnP). These fungi are able to depolymerize synthetic lignin *in vitro* and oxidize non-phenolic compounds via peroxidation of lipids (Martinez *et al*., 1996). *P. ostreatus*, which produces lignin peroxidase (LiP), MnP and laccase can degrade polycyclic aromatic hydrocarbons (PAHs), 2, 4, 6-trinitrotoluene (TNT), RDX and polychlorinated biphenyl (PCB) (Matsubara *et al*., 2006). *P. eryngii*, which produces laccase and aryl-alcohol oxidase (AAO) has the capacity to remove lignin (Martinez *et al*., 1996) and *P. sajor-caju*, which produces lacc*ase* was able to decolorized azo dyes such as acid red 18, acid Black 1, and direct blue 71 (Murugesan *et al*., 2006). *Trichoderma* species which produces laccase was able to oxidize aromatic compounds and transform to phenolic compounds (Chakroun *et al*., 2010). *Rhizopus* species are able to remove hydrocarbon (Mancera-López *et al*., 2008) and also metal (Faryal & Hameed, 2005). *Aspergillus* and *Penicillium* species can remove both soluble and insoluble metal species from solution and are also able to leach metal cations from solid waste (Faryal & Hameed, 2005). *P. sanguineus*, which produces laccase able to decolourize all dyes (Pointing, & Vrijmoed, 2000). *Trametes* species which produces laccase can degrade PAHs, pentachlorophenol, PCB, 3, 4-dichloroaniline, dieldrin and also can oxidize acrylamide and 4-hydroxybenzoic acid (Matsubara *et al*., 2006). *Fomitopsis* species has showed a high ability to degrade 1, 1, 1-trichloro-2, 2-bis (4-chlorophenyl) ethane (DDT) (Purnomo *et al*., 2008). *S. commune*, which produces LiP, MnP and laccase can degrade TNT and textile dyes (Matsubara *et al*., 2006). *Ganoderma* species, which produces MnP and laccase, can degrade pentachlorophenol (Matsubara *et al*., 2006).

However, study of using local fungi (including white-rot fungi) for leachate bioremediation is still lacking. This study focuses on a screening method for selecting

potential local fungi for the use in the bioremediation of leachate. Therefore, the aim of this study is to investigate the fungi capability to grow in leachate, hence potential to be used in leachate bioremediation.

4.1 MATERIALS AND METHOD

4.1.1 Leachate sample

Raw leachate was collected from the pond of untreated leachate at the sanitary landfill No. 1 in Selangor. Leachate samples were stored in 50-litre polyethylene containers at 8-15 °C.

4.1.2 Fungal strains

Twelve fungal strains used in this study were *Pleurotus eryngii* (DC.) Quel.(White rot fungi, WRF), *Trichoderma* sp. (WRF), *Rhizopus* sp., *Aspergillus* sp., *Pycnoporus sanguineus* (L.) Murrill (WRF), *Penicillium* sp., *Pleurotus ostreatus* (Jacq.) P. Kumm. (WRF), *Trametes menziesii* (Berk.) Ryvarden (WRF), *Fomitopsis feei* (Fr.) Kreisel, *Schizophyllum commune* (Fr.) (WRF), *Pleurotus sajor-caju* (Fr.) Singer (WRF) and *Ganoderma australe* (Fr.) Pat. (WRF). These strains consisting of micro- and macrofungi were chosen since they were potential candidate for bioremediation.

All the strains were obtained from the Mycology Laboratory, Institute of Biological Sciences, University of Malaya, Malaysia. 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 ºC.

4.1.3 Preparation of leachate medium

The leachate medium was prepared using malt extract agar (MEA-Oxoid CM0059) medium dissolved with 50% or 100% leachate collected from Jeram sanitary

landfill. Characteristics of the leachate are as follows: BOD_5: 11360 ± 703.42 mg/L, COD: 16000 ± 1130.62 mg/L, TSS: 130 ± 13.45 mg/L, NH_3N: 21.3 ± 3.17 mg/L, TOC: 4700 ± 145.26 mg/L, TKN: 98 ± 13.45 mg/L and pH: 8.17 ± 0.05. The 50% leachate medium contained 50 g MEA, 500 ml leachate and 500 ml distilled water. The 100% leachate medium contained 50 g MEA and 1000 ml leachate only. Then the media were sterilized in an autoclaved at 121 ºC for 20 minutes.

4.1.4 Effect of pH of leachate medium on growth of fungi

Leachate medium dissolved with 50% leachate was used in order to study the effect of pH of leachate medium on fungal growth. Two different leachate medium were used which are unadjusted medium and adjusted medium to pH 6.0 before autoclaving using a pH meter (model IQ160). The pH of the unadjusted medium was 8.17. The pH was adjusted to pH 6.0 because after autoclaving the pH medium will change to the raw leachate pH value that is in the range of pH 8.0 to 8.25.

4.1.5 Effect of leachate concentration on growth of fungi

The effect of leachate concentration on the growth of fungal was studied using malt extract agar (MEA) medium and malt extract agar (MEA) medium dissolved with 50% or 100% leachate. The pH of malt extract agar (MEA) medium, malt extract agar (MEA) medium dissolved with 50% and or malt extract agar (MEA) medium dissolved with 100% leachate medium before autoclaving was 5.26, 6.43 and 6.85, respectively. Plates without addition of leachate were also run as controls.

4.1.6 Growth of fungi on leachate medium

In order to obtain fungal growth rate data for each of the twelve fungal species and to test the effect of pH leachate medium and the effect of leachate concentration for

each of the fungi, the following method was used: A 6-mm^2 diameter mycelia plug of colonized malt extract agar (MEA) was taken from the periphery of a 7-day old inoculums culture and placed in the middle of the leachate medium agar described previously. The inoculated leachate-incorporated media were incubated at 28±2 ºC and colony radius from the edge of the MEA piece was measured every 48 hours to obtain the fungal growth rate for each fungal species in the different treatments for 30 days (Figure 4.1).

A 6-mm^2 piece of colonised malt extract agar (MEA) cut from the edge of fungal colony.

⇩

Central inoculation of each ME agar plate supplemented with leachate.

⇩

Plates incubated at 25 ºC

⇩

Colony radius from the edge of the MEA piece every 48 hrs for 30 days was measured

⇩

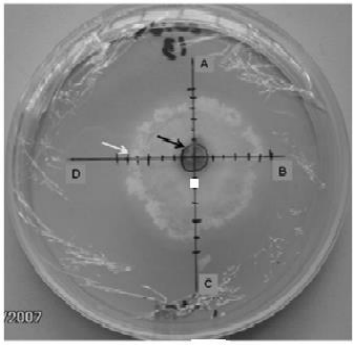


Figure 4.1. Flow chart and method to determine growth of fungi on Petri dish.

The photo ilustrated the petri dish used for fungus growth bioassay. Black arrow indicates the edge of initial inoculums. White arrow indicates the edge of fungi radial growth. Letter A, B, C, D correspond to the four segments used for growth measurements.

4.1.7 Statistical analysis

Data were analysed for any significant differences between treatments using analysis of variance (ANOVA). To prove whether differences between individual treatments were significant ($P<0.05$), the test for least significant differences (LSD) was used (SPSS for Windows Version 14.0).

4.2 RESULTS AND DISCUSSION

Besides bacteria, fungi are also one of the most efficient decomposer organisms. Most fungi are robust organisms therefore; they are generally more tolerant to high concentrations of polluting chemicals than are bacteria (Gadd, 2001). In addition, they can break down tough debris and also can attack organic residues that are too dry, acidic, or low in nitrogen. This explains why fungi have been investigated extensively for their bioremediation capacities. Stajich (2007) reported many studies had shown the breadth and efficiency of different fungi in the degradation of a variety of compounds. As example, *P. sordida* had showed potential in the solid-phase bioremediation of creosote-contaminated soils (Davis *et al*., 1993) and crude extract from *A. bisporus* able to remove 90% of phenol from a polluted solution (Stajich, 2007).

Twelve species of fungi were screened for the ability to grow on MEA in the presence of 50% and 100% leachate. The effect of pH of leachate medium suitable for fungal growth was compared.

The effect of pH of leachate medium on the growth of twelve fungi species was studied by comparing their growth rate on adjusted and unadjusted pH of MEA medium containing 50% leachate. The pH of adjusted leachate medium before autoclaving was kept at pH 6.0, while unadjusted leachate medium at pH 8.17. From the result, as depicted in Fig. 4.2, it shows that at pH 6.0 the growth for almost all the fungi was better compared to the growth at pH 8.17. All the fungi demonstrated better growth on

medium with pH 6.0; however, *Rhizopus* sp, *P. ostreatus* and *F. feei* did not grow on the unadjusted pH medium. The growth of *T. menziesii*, and *G. australe* at pH 6.0 and pH 8.17 showed no significant difference ($p>0.05$) (Appendix 3) with growth rates varying from 7.0 ± 0.2 mm/day at pH 6.0 compared to 7.4 ± 0.5 mm/day at pH 8.17 for *T. menziesii*, and 6.1 ± 1.3 mm/day and 6.9 ± 0.3 mm/day for *G. australe*. The growth of other fungi was superior on the adjusted medium with pH 6.0 compared to on the unadjusted medium with pH 8.17. *Trichoderma* sp. showed tremendous high growth on medium with pH 6.0 with growth rates of 16.8 ± 0 mm/day compared to 1.1 ± 0.1 mm/day on medium with pH 8.17.

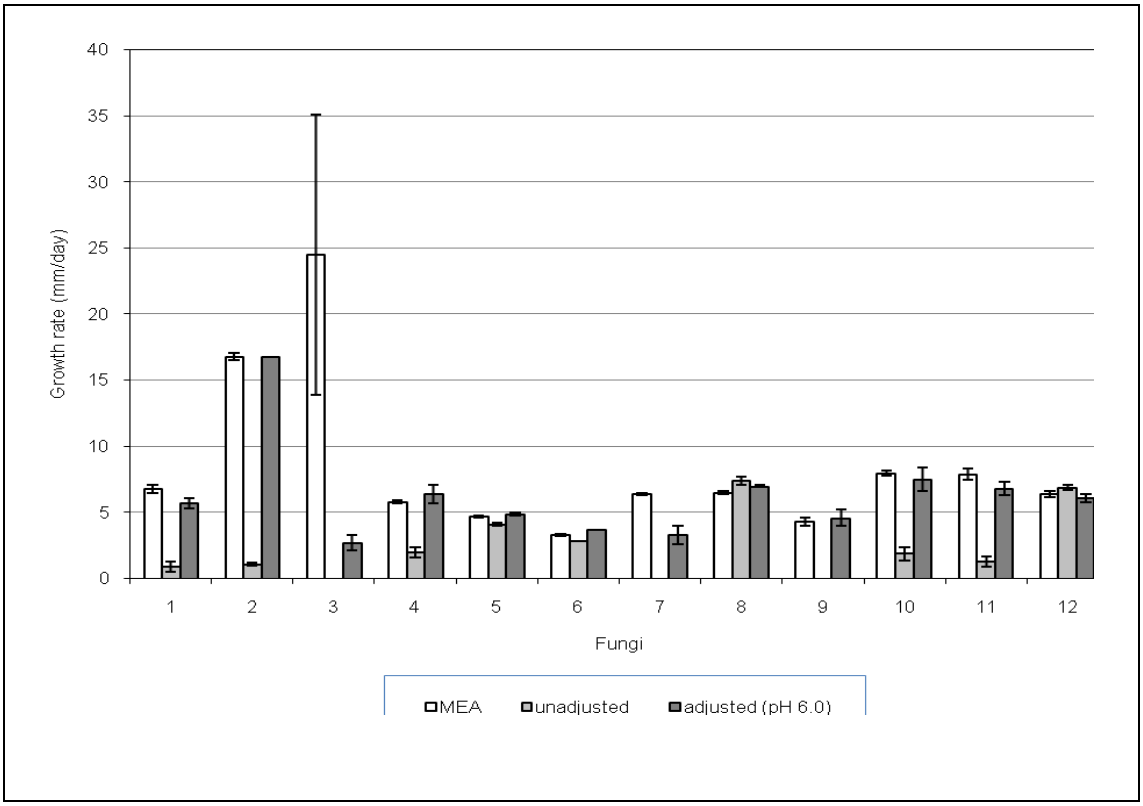


Figure 4.2 Growth rates of twelve different fungi on leachate medium containing 50% leachate with pH adjusted to 6.0 and unadjusted pH at 28±2 ºC.

1, *Pleurotus eryngii*; 2, *Trichoderma* sp.*;* 3, *Rhizopus* sp.*;* 4, *Aspergillus* sp.; 5, *Pycnoporus sanguineus*; 6, *Penicillium* sp.;7, *Pleurotus ostreatus*; 8, *Trametes menziesii*; 9, *Fomitopsis feei*; 10, *Schizophyllum commune*; 11, *Pleurotus sajor-caju*; 12, *Ganoderma australe*. Results are expressed as means ± standard deviation. Values are means of triplicates from three separate runs; n=3.

Study on the ability of fungi to grow on MEA in the presence of 50% and 100% leachate showed that only four species, namely *G. australe*, *T. menziesii*, *P. sanguineus*, and *Penicillium* sp.demonstrated prominent mycelial growth on MEA incorporated with either 50% or 100% leachate as illustrated in Fig. 4.3 after twelve days of incubation at 28±2 ºC.

Mycelium density of *G. australe* on 50% leachate medium

Mycelium density of *G. australe* on 100% leachate medium

Figure 4.3 Growth of *Ganoderma australe* on malt extract agar incorporated with 50% and 100% leachate after twelve days of incubation at 28±2 ºC.

Fig. 4.4 shows the growth rates of the twelve fungal species on malt extract agar and malt extract agar dissolved with 50% or 100% leachate. The growth rate of each fungal species was determined by measuring the radial growth every 48h for a period of 30 days after incubation at 28±2 ºC. The result confirmed that all twelve fungi species were able to grow well on MEA medium without leachate. However, the results showed no mycelia growth on malt extract agar containing 100% leachate, except for *G. australe*, *T. menziesii*, *P. sanguineus*, and *Penicillium* sp. The growth rates of these fungi were 6.1 ± 0.5 mm/day, 5.9 ± 1.0 mm/day, 2.8 ± 0.6 mm/day and 2.3 ± 0.5 mm/day, respectively. Meanwhile, nine of the twelve fungi revealed the ability to grow on MEA medium incorporated with 50% leachate with *G. australe* showed the most rapid growth, followed by *T. menziesii* and *P. sanguineus* with growth rates of 6.9 ± 0.3 mm/day, 5.8 ± 0.2 mm/day, and 4.1 ± 0.1 mm/day, respectively. The growth rates of

other fungi were 2.9 ± 0.1 mm/day for *Penicillium* sp., 2.0 ± 0.4 mm/day for *Aspergillus* sp., 1.9 ± 0.5 mm/day for *Schizophyllum commune*, 1.3 ± 0.4 mm/day for *Pleurotus sajor-caju*, 1.1 ± 0.1 mm/day for *Trametes menziesii*, and 0.9 ± 0.4 mm/day for *Pleurotus eryngii*.

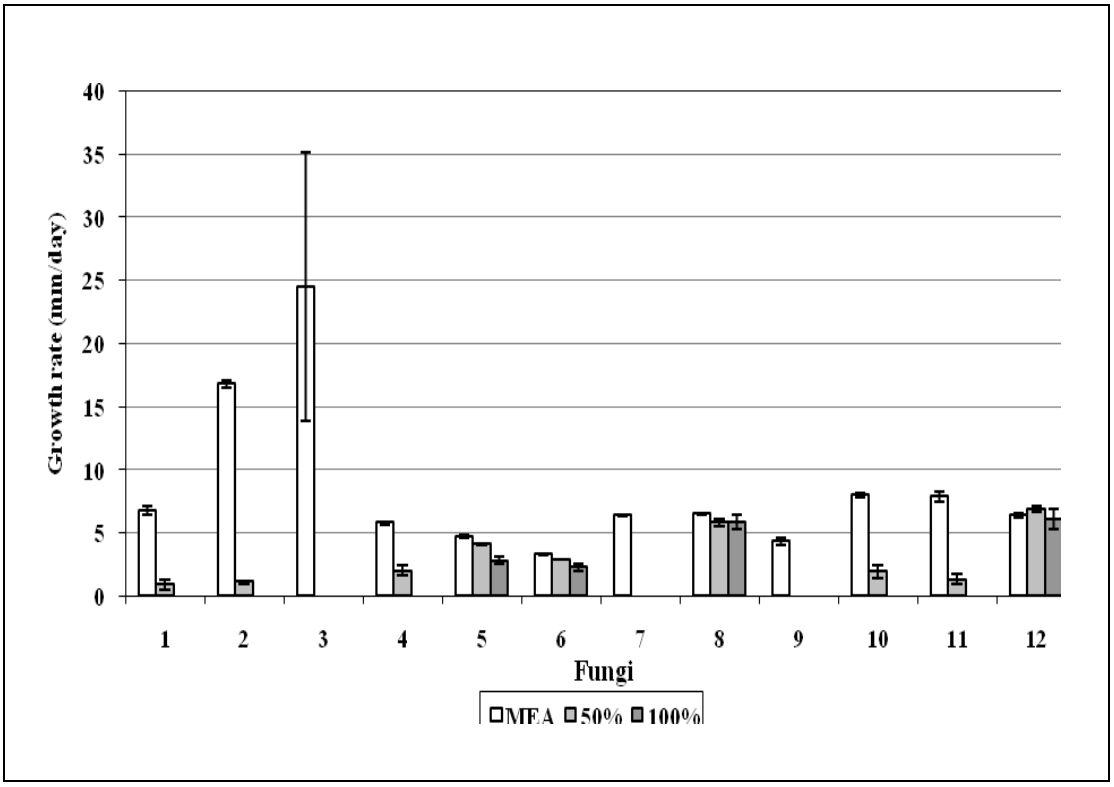


Figure 4.4 Growth rates of twelve different fungi on MEA, MEA with 50% leachate and MEA with 100% leachate at 28º C.

1, *Pleurotus eryngii*; 2, *Trichoderma* sp.*;* 3, *Rhizopus* sp.*;* 4, *Aspergillus* sp.; 5, *Pycnoporus sanguineus*; 6, *Penicillium* sp.*;*7, *Pleurotus ostreatus*; 8, *Trametes menziesii*; 9, *Fomitopsis feei*; 10, *Schizophyllum commune*; 11, *Pleurotus sajor-caju*; 12, *Ganoderma australe*. Results are expressed as means ± standard deviation. Values are means of triplicates from three separate runs; n=3.

The effect of leachate concentration on the growth of *Penicillium* sp., *T. menziesii* and *G. australe* showed no significant different (P>0.05) (Appendix 4) with the comparison growth rates was as follows: for *Penicillium* sp., 2.9 ± 0.1 mm/day on MEA medium incorporated with 50% leachate and 2.3 ± 0.5 mm/day on MEA medium incorporated with 100% leachate; for *T. menziesii*, 5.8 ± 0.2 mm/day on MEA medium incorporated with 50% leachate and 5.9 ± 1.0 mm/day on MEA medium incorporated

with 100% leachate; for *G. australe*, 6.9 ± 0.3 mm/day on MEA medium incorporated with 50% leachate and 6.1 ± 0.5 mm/day on MEA medium incorporated with 100% leachate. However, the growth of *P. sanguineus* on different concentration of leachate showed slight, but significant ($P<0.05$) different (Appendix 4) with growth rates on MEA medium incorporated with 50% leachate was 4.1 ± 0.1 mm/day and 2.8 ± 0.3 mm/day on MEA medium incorporated with 100% leachate. This result suggested that the concentration of leachate did not effect the growth of *Penicillium* sp., *T. menziesii* and *G. australe*, but slightly affect the growth of *P. sanguineus*.

The growth of fungal species on malt extract agar supplemented with leachate revealed that, out of twelve fungi species studied, only two fungi species namely *G. australe,* and *T. menziesii* demonstrated the ability to grow well on the MEA (-with both 50% and 100% leachate concentrations). Besides that, both fungi species were not affected by the pH of the medium eventhough, wood rotting fungi like *T. versicolor*, *Pholiota mutabilis*, *P. ostreatus*, *Phlebia radiate* and *P. chrysosporium* prefer a slightly lower pH range 3.5-5.5 (Leonowicz *et al*., 1991). Thus, *G. australe*, and *T. menziesii* were the most potential to be used in the bioremediation of leachate. Both species are white rot fungi. According to Hestbjerg *et al*. (2003), white-rot fungi are a physiological grouping of fungi that can degrade lignin (-and lignin- like substances). White-rot fungi are also recognized for their capacities to adapt severe environmental constraints (Coulibaly *et al*., 2003). Researchers are now focusing on white rot fungi for use in bioremediation since these organisms have the ability to degrade a wide range of environmental pollutions. Fu and Viraraghan (2001) reported many fungal strains which including, *S. commune*, *Pycnoporus cinnabarinus* and *T. versicolor* were capable of decolorizing dye wastewater while, *P. chrysosporium* had been used in degradation xenobiotic compounds (Paszczynski & Crawford, 1995). The used of white-rot fungi including *Coriolopsis polyzona*, *P. chrysosporium*, *P. ostreatus*, and *T. versicolor* in the

removal of polychlorinated biphenyls (PCBs) has been described by Pointing (2001). Coulibaly *et al*. (2003) also reported the growing interest of fungi for the wastewater biotreatment such as for the removal or destruction of metals, inorganic nutrients and organic compounds. In addition, Shah and Nerud (2002) discussed the applications of white-rot fungi in dyes decolorization were due to their ability to produce various ligninolytic enzymes.

The biodegradation abilities of some white rot fungi are promising since they are known for their superior ability to produce a large variety of extracellular proteins, organic acids and other metabolites. Eugenio *et al*. (2008) and Ehlers and Rose, (2005) reported on the involvement of white-rot fungi in the transformation of a large amount of organopollutants structurally related to lignin. This property is based on the white-rot fungi capacity to produce one or more extracellular lignin-modifying enzymes (LMEs) (Wesenberg *et al*., 2003). Hamman (2004) stated that extracellular LMEs (lignin peroxidase, LiP; Mn- dependent peroxidase, MnP, and laccase, LAC) that have very low substrate specificity make them able to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin. According to Novotny *et al*. (2004), these enzymes are secreted by white-rot fungi such as *P. chrysosporium* and *T. versicolor*). The high growth on state medium adjusted to pH 6.0 makes *Trichoderma* sp. a potential candidate too. However, besides being able to grow in leachate, other criteria such as type of enzymes produced by the fungi must also be considered. DeMarco *et al*. (2003), reported that *Trichoderma* only produces hydrolases extracellular enzymes such as cellulase, amylase and proteases that are used to control plant pathogens but not for pollutant degradation.

Through intensive studies of ligninolytic fungi, it has been determined that these organisms produce extracellular enzymes with very low substrate specificity. This makes them suitable for the degradation of many different compounds, notably

organopollutants with structural similarities to lignin such as PAH, PCBs, TNT, DDT ("The landfills leachate", 2008). Four main genera of white rot fungi that have shown potential for bioremediation are *Phanerochaete*, *Trametes*, *Bjerkandera*, and *Pleurotus*. This is supported by Pointing (2001), who stated that white-rot fungi have been shown to degrade a wide variety of environmental pollutants, including pentachlorophenol (PCP).

4.3 CONCLUSION

Based on these results, it is clear that the growth of both *T. menziesii*, and *G. australe*, is not affected by the pH of leachate. Out of twelve different fungi, *G. australe* and *T. menziesii* showed the highest growth rate on MEA incorporated with both 50% and 100% leachate. The highest growth rates of both fungi were 5.9 ± 1.0 mm/day on MEA incorporated with 100% leachate for *T. menziesii*, and 6.9 ± 0.3 mm/day on MEA incorporated with 50% leachate for *G. australe*. In addition, from the result obtained in this study, it was concluded that the pH of leachate medium and the concentration of leachate only reduced slightly the growth of most promising fungi on leachate medium which are *T. menziesii* and *G. australe*. Both fungi showed well growth on pH 6.0 and pH 8.17 of leachate medium, and also on leachate medium dissolved with 50% and 100% leachate.

This finding suggested that white rot fungi *T. menziesii* and *G. australe* are of current interest to be used for the bioremediation of a broad spectrum of persistent xenobiotics, thus can also be used to treat wastewater including landfill leachate. Therefore, white rot fungi, *G. australe* and *T. menziesii* were selected to be used in leachate treatment.

TREATMENT OF LEACHATE USING MYCELIA OF SELECTED WHITE-ROT FUNGI

5.0. INTRODUCTION

Landfill leachates have been treated by means of several processes such as physicochemical processes (Kim *et al*., 2003) that provide better treatment efficiency in treating older landfill leachate compared to biological processes (Ehrig 1984), but generally have higher operating cost. Meanwhile, biological processes were proven to be suitable for the treatment of younger leachate whereby the organic content are mainly composed of readily biodegradable volatile fatty acids (Zouboulis *et al*., 2001; Chian & DeWalle, 1976). However, Kim *et al*. (2003) noted a wide variety of organic compounds and high levels of ammonia nitrogen in landfill leachates making the efficiency of the biological process unpredictable.

White-rot basidiomycetous 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) and *P. chrysosporium*, *Pleurotus* sp., and *T. versicolor* in mineralizing polycyclic aromatic hydrocarbons (PAH) (Pointing, 2001). Besides that, Polak and Jarosz-Wilkołazka (2010) also stated that the use of fungal cultures to transform various chemical compounds had been reported in several studies. White rot fungi were able to produce extracellular lignin peroxidase (LiP) and manganese – dependent peroxidase that is essential for lignin degradation (Leonowicz *et al*., 1999). These enzymes are able to oxidize a variety of high-priority aromatic pollutants, such as polycyclic aromatic hydrocarbons, chloromatics and polyaromatic dyes (Kotterman *et al*., 1996). 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 (2009) have applied immobilized *T. versicolor* to treat landfill leachate in column experiments since the immobilization is expected to improve the mass transfer of oxygen and nutrients for fungal mycelia by providing a large attaching surface area. Besides that, Saetang and Babel (2010) stated that the advantagesof using immobilized microorganism for pollutant degradation is due to their economically cheaper, easier to handle and the immobilized fungus is reusable for several batches. Mohammadi and Nasernejad (2009) reported, on the limitation of free cells of *P. chrysosporium* for use in biodegradation process of recalcitrant compounds while cell immobilization offers a suitable alternative. They found that immobilized *P. chrysosporium* showed significant biodegradation capacity on anthracene (compound of PAH family) compared to free cells, which is related to the production of extracellular ligninolytic activities, in free cells and immobilized cells.

During the last 20–25 years, the cell immobilization technology has attracted the attention of several research groups. According to Ramakrishna and Prakasham, (2010), immobilization of cells is the attachment of cells or their inclusion in distinct solid phase that permits exchange of substrates, products, inhibitors, etc., but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. This process eliminates most of the constraints faced with the free-cell systems such as facilitates operation of microbial fermentation on continuous mode without cell washout and the whole-cell immobilization process decouples microbial growth from cellular synthesis of favored compounds. Meanwhile, Beshay (2003) illustrated that immobilization of whole cells for the production of extracellular enzymes offers many advantages such as the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous continuous operation over a prolonged period and enhance reactor productivity.

The objectives of this part of study are to compare the remediation of leachate ability by free and immobilized mycelia of two selected white-rot fungi, *G. australe* and *T. menziesii*.

5.1. MATERIALS AND METHOD

5.1.1. 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 ºC.

5.1.2. 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 ºC at 150 rpm.

5.1.3. Leachate sample

The raw leachate used in this experiment was collected from the pond of active and untreated leachate at landfill No. 3 in Selangor (refer Table 3.1 at page 45). 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 Hach DR 2800 spectrophotometer.

Comparison of the leachate characteristics with industrial effluent standards in Malaysia was as previously shown in Table 3.3 (page 56). Results for initial analysis showed that most of the parameters well exceed the standards. 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 3.3).

5.1.4. Treatment of leachate with free fungal mycelium in batch culture at aerobic condition

Ganoderma australe and *T. menziesii* capable of good growth in leachate were selected in this study. In this experiment, mycelial broth (10 ml) was transferred into 250-ml Erlenmeyer flasks containing 100 ml leachate prepared as follows:

i) 50% leachate: 50 ml leachate and 50 ml distilled water.

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 ºC and 150 rpm for 30 days. Three important leachate components: BOD_5, COD and ammoniacal nitrogen (NH_3N) together with pH were measured before and after 30 days of incubation. Three flasks were replicated for each treatment. Previous experiment on effect of leachate concentration on growth of fungi showed no significant differences in growth of fungi at 50% leachate medium and 100% leachate medium. Therefore, 100% leachate medium was used in the experiments.

Figure 5.1 Free fungal mycelium

5.1.5. Preparation of immobilized fungal mycelium

5.1.5.1. Sterilization of 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. 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.

5.1.5.2. Immobilization of fungal mycelium on Ecomat

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.

Figure 5.2 Immobilization of fungal mycelium on Ecomat

5.1.6. 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 single culture mycelium of *G. australe* or *T. menziesii* or coculture mycelia of *G. australe* and *T. menziesii*. The excess GYMP medium was poured-off and 125 ml of 50% and 100% leachate (as mentioned in Section 5.1.4) were added into each 250 ml Erlenmeyer flask. Dilution was done using distilled water. The flasks were then agitated on an orbital shaker for 28 days at 28±2 ºC at 150 rpm for 4 weeks. Every week (day 7, 14, 21, and 28), three flasks of each different cultures were collected, filtered using Whatmann filter paper and the supernatant was measured for pH, BOD_5, COD and ammoniacal nitrogen (measured as mentioned in Section 5.1.8). All processes were done under sterile conditions at ambient temperature. Three flasks were replicated for each treatment. The experimental procedures used are outlined in Figure 5.3.

5.1.7. Treatment of leachate using immobilized *G. australe* mycelium on Ecomat in column

The column experiment was using 50% and 100% leachate. The leachates were treated by immobilized *G. australe* mycelium in column. The leachates were passed through the column packed with Ecomat containing immobilized mycelia of selected fungal species which are *G. australe*. Immobilized mycelia on Ecomat was arranged and packed in column chromatography. The column chromatography with 40 mm diameter, 500 mm height in size and 40/38 socket size were used. A total of 30 pieces (since the column can support until 30 pieces only) of Ecomat-immobilized mycelium were arranged and packed into each column. Three columns were used which two column representing treatment column (containing 50% and 100% leachate with

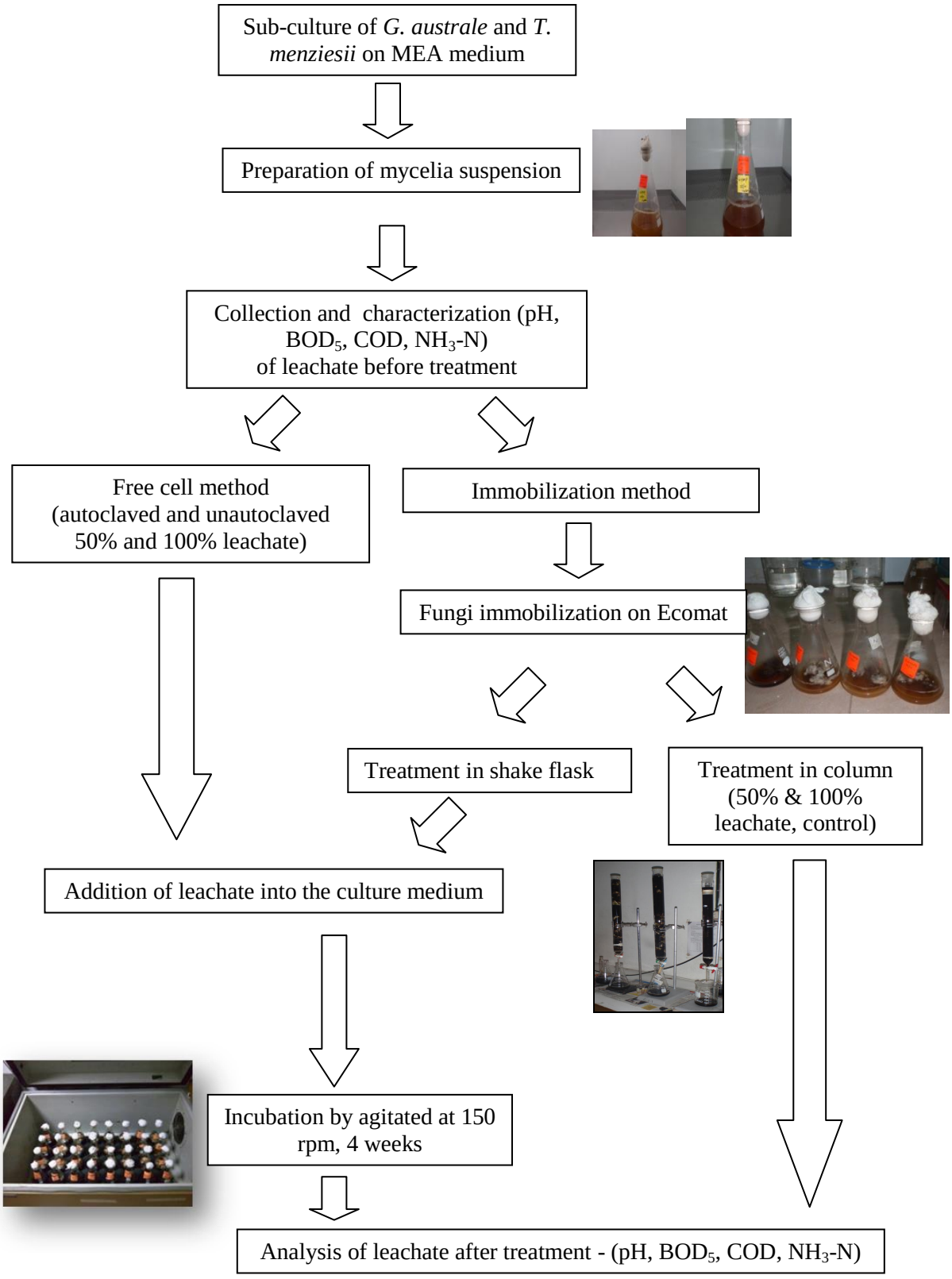


Figure 5.3 Flow chart of experimental procedures for leachate treatment with fungal mycelia

Ecomat-immobilized mycelium) and one untreated (control) column (containing 100% leachate without fungal mycelium). After the set up, 1000 ml of leachate medium was passed through the column at the flow rate adjusted to 20 ml per minute (Noorlidah, *et al*., 2013). The columns were operated at room temperature. The medium was passed thru for ten cycles where after two successive cycles pH, BOD_5, COD and ammoniacal nitrogen were measured as in Section 5.1.8. The experimental set-up of column of immobilized *G. australe* on Ecomat is as shown in Figure 5.4.

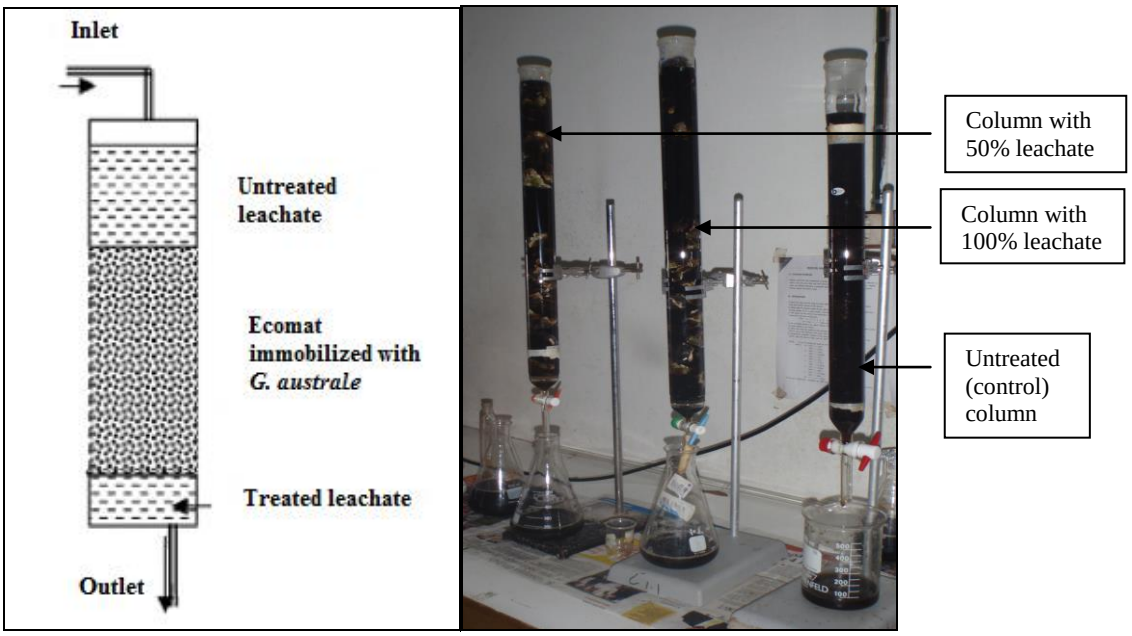


Figure 5.4 Experimental set-up of column of immobilized *G. australe* on Ecomat for the leachate treatment

5.1.8. Leachate analysis.

The degradation of leachate was determined by measuring the removal percentage of leachate contaminants denoted as BOD_5, COD, NH_3N and changes of pH. These contaminants were analysed in accordance with the Standard Method for the Examination of Water and Wastewater (APHA, 1998) using Hach DR 2800 spectrophotometer. Removal of leachate components (BOD_5, COD, and NH_3N) were investigated after the fungal treatment and the results were compared with the initial value (Saetang & Babel, 2009).

The calculation of percentage removal of leachate contaminant is as follows:

$$\% \text{ of removal} = \frac{\text{Initial value of contaminant - Final value of contaminant}}{\text{Initial value of contaminant}} \times 100\%$$

5.1.9. Statistical analysis.

Due to vared initial levels of contaminants, treatment effects were evaluated in terms of the percentage of the contaminant remaining after 30 days of treatment. To avert any potential effects on analytical procedures, concentrations before treatment were considered as initial levels. In order to reduce nonnormality and the possible influence of outliers, the percentage contaminant remaining in the samples from each treatment were averaged to give a mean value for each parameter. Evaluation of the depletion of each target contaminant was performed for overall treatment effects by analysis of variance (ANOVA). Statistical tests were performed using SPSS software (SPSS Version 14.0, Chicago, IL).

5.2. RESULTS AND DISCUSSION

For this study, two white-rot fungi, *G. australe* and *T. menziesii* were investigated for their ability to degrade BOD, COD, and ammoniacal nitrogen from leachate. Different biological methods were studied at aerobic condition. The treatments were included using free cell mycelia in batch culture, treatment using immobilized mycelium in culture flask and treatment using immobilized mycelium in column.

5.2.1 Treatment of leachate with free cell mycelia of *Ganoderma australe* and *Trametes menziesii* in batch cultures at aerobic condition

Percentage removal of leachate BOD_5 and COD after 30 days treatment with *G. australe* is as shown in Table 5.1. From the results, it show that using unsterilized leachate resulted in better percentage of BOD_5 removal where in LM-100, 85.24% of BOD_5 was removed compared to 48.40% in SLM-100. In addition, when compared the

percentage removal of leachate BOD_5 at different leachate concentration, it results suggested that diluted (50%) leachate demonstrated slightly higher than concentrated leachate (100%) where in LM-50, 89.24% of BOD_5 was removed compared to 85.24% in LM-100. Statistical analysis revealed that BOD_5 removal showed significant difference ($p<0.05$) among all the treatments (Appendix 7). Meanwhile, when using *G. australe*, COD removal of leachate only resulted in LM-50 with 24.72%. Thus, this study revealed that the highest percentage removal of BOD_5 (89.24%) and COD (24.72%) was achieved when *G. australe* was cultured in unsterilized medium with 50% leachate (LM-50). Hence, free cell mycelia of *G. australe* showed most promising ablity to treat BOD_5 and COD of unsterilized medium of 50% leachate.

Meanwhile, percentage removal of leachate BOD_5 and COD after 30 days treatment with *T. menziesii* (Table 5.2) showed that unsterilized leachate resulted in better percentage in BOD_5 removal. The percentage removal of BOD_5 in LM-100 was 81.39% while, in SLM-100 was 63.65%. Similar pattern of BOD removal is shown in diluted (50%) leachate where percentage removal of BOD_5 in LM-50 was 86.14% while in SLM-50 was 50.38%. Nevertheless, the concentration of leachate medium demonstrated small significant differences in percentage of BOD_5 removal where the percentage removal of BOD_5 at LM-50 (86.14%) and LM-100 (81.39%) with $p= 0.033$ only (Appendix 13). On the other hand, COD removal was obtained in SLM-100, LM-50 and LM-100 with the percentage of 63.65%, 2.97%, and 11.23%, respectively.

Similar findings with *G. australe* were obtained where the highest percentage removal of BOD5 (86.14%) and COD (56.49%) was acquired when *T. menziesii* was cultured in unsterilized medium with 50% leachate (LM-50). Based on BOD5 removal by both free cell mycelia, *G. australe* demonstrated a higher percentage of removal with 73.18% for SLM-50, 48.93% for SLM-100, 89.33% for LM-50, and 85.28% for LM-

TABLE 5.1. Percentage removal of leachate BOD_5, COD, NH_3-N and pH changes by free cell mycelia of *G. australe* after 30 days incubation in submerged cultures at various treatments

Treatments	Parameters	Levels in Untreated Leachate	Levels in Treated Leachate	Percentage Removed (-), Increased (+)
SLM-50	BOD_5 (mg/l)	11820.00	3168.00 ± 13.11	-73.18[b]
	COD (mg/l)	16627.00	23942.00 ± 2133.39	+44.00
	NH_3-N (mg/l)	34.00	35.20 ± 3.36	+2.94
	pH	7.95	7.84 ± 0.02	-
SLM-100	BOD_5 (mg/l)	12100.00	6098.00 ± 130.28	-48.93[a]
	COD (mg/l)	16730.00	23313.00 ± 1324.36	+39.35
	NH_3-N (mg/l)	33.00	28.00 ± 1.97	-15.15
	pH	7.93	7.66 ± 0.10	-
LM-50	BOD_5 (mg/l)	11560.00	1272.00 ± 43.55	-89.33[c]
	COD (mg/l)	15320.00	11533.00 ± 926.71	-24.72
	NH_3-N (mg/l)	28.00	30.30 ± 1.47	+8.21
	pH	7.92	6.46 ± 0.25	-
LM-100	BOD_5 (mg/l)	11850.00	1742.00 ± 443.18	-85.28[c]
	COD (mg/l)	16127.00	17211.00 ± 1738.84	+6.72
	NH_3-N (mg/l)	21.30	22.10 ± 1.90	+3.76
	pH	7.98	6.73 ± 0.40	-

SLM-50: sterilized medium with 50% leachate; SLM-100: sterilized medium of 100% leachate; LM-50: unsterilized medium with 50% leachate; and LM-100: unsterilized medium of 100% leachate. – indicates reduced (removed); + indicates increased.

values expressed are means ± S.D. of triplicate measurements.

values in the same column with different letters (a-c) were significantly different ($p<0.05$).

100 compared to *T. menziesii* with 50.38% for SLM-50, 63.65% for SLM-100, 86.14% for LM-50 and 81.39% for LM-100. The highest percentage removal of BOD_5 is 89.33% showed by *G. australe* in unsterilized medium with 50% leachate (Table 5.1). Meanwhile, the pH value was reduced in all the treatments compared to raw leachate for both mycelia cultures. In contrast, concentration of ammoniacal nitrogen showed an increase in all treatments for both mycelia cultures. This indicated that no removal of ammoniacal nitrogen shown in all treatments and the increasing of ammoniacal nitrogen may be due to the breakdown of organic compound by *G. australe* that produce ammonia, NH_3.

In this study, treatment of leachate with *T. menziesii* and *G. australe* using free fungal mycelia in Erlenmeyer flask at different treatments (SLM-50, SLM-100, LM-50, LM-100) demonstrated promising percentage removal of leachate BOD_5. The percentage removal of leachate BOD_5 by free-cell mycelium of *T. menziesii* was 50.38%, 63.65%, 86.14% and 81.39, respectively. While, the percentage removal of leachate BOD_5 by free- cell mycelium of *G. australe* was 73.18, 48.93, 89.33 and 85.28%, respectively. The highest percentage (89.33%) of leachate BOD_5 removal was obtained using *G. australe* in unsterilized medium of 50% leachate. Meanwhile, COD removal showed variations among the treatments (SLM-50, SLM-100, LM-50, LM-

TABLE 5.2. Percentage removal of leachate BOD_5, COD, NH_3-N and pH changes by free cell mycelia of *T. menziesii* after 30 days incubation in submerged cultures at various treatments

Treatments	Parameters	Levels in Untreated Leachate	Levels in Treated Leachate	Percentage Removed (-), Increased (+)
SLM-50	BOD_5 (mg/l)	11820.00	5861.00 ± 65.02	-50.38[a]
	COD (mg/l)	16627.00	22897.00 ± 3076.77	+37.71
	NH_3-N (mg/l)	34.00	63.16 ± 8.82	+85.76
	pH	7.95	7.75 ± 0.20	-
SLM-100	BOD_5 (mg/l)	12100.00	4396.00 ± 221.74	-63.65[b]
	COD (mg/l)	16730.00	16233.00 ± 580.56	-2.97
	NH_3-N (mg/l)	33.00	36.60 ± 4.45	+10.91
	pH	7.93	7.80 ± 0.17	-
LM-50	BOD_5 (mg/l)	11560.00	1600.00 ± 313.21	-86.14[d]
	COD (mg/l)	15320.00	6665.00 ±224.22	-56.49
	NH_3-N (mg/l)	28.00	31.72 ± 2.06	+13.29
	pH	7.92	6.17 ± 0.17	-
LM-100	BOD_5 (mg/l)	11850.00	2204.00 ± 245.50	-81.39[c]
	COD (mg/l)	16127.00	14316.00 ± 1972.04	-11.23
	NH_3-N (mg/l)	21.30	25.68 ± 0.97	+20.60
	pH	7.98	7.31 ± 0.84	-

SLM-50: sterilized medium with 50% leachate; SLM-100: sterilized medium of 100% leachate; LM-50: unsterilized medium with 50% leachate; and LM-100: unsterilized medium of 100% leachate.
– indicates reduced (removed); + indicates increased.
values expressed are means ± S.D. of triplicate measurements.
values in the same column with different letters (a-c) were significantly different ($p<0.05$).

100) where for *T. menziesii*, COD removal occurred at SLM-100, LM-50 and LM-100 while, for *G. australe* only at LM-50 the removal of COD take placed. Overall, the highest leachate COD removal (56.49%) was shown by *T. menziesii* in unsterilised medium of 50% leachate. The removal of COD content in landfill leachate has been reported by Kim *et al*. (2003). In their study, the treatment of landfill leachate using a combination process of white-rot fungus *P. chrysosporium* and the natural zeolite Clinoptilolite demonstrated 4.7 % reduction of leachate COD content. In another study, Coulibaly *et al*. (2002) reported 72 % of COD was removed from domestic wastewater after the pretreatment by fungal biomass of *A. niger* strain under transient conditions. In addition, this study also found that treatment of leachate by *G. australe* and *T. menziesii* at various conditions demonstrated better BOD_5 and COD removal in unsterilised leachate than sterilised leachate.

However, in other work done by Kissi *et al*. (2001) reported unsterilised and sterilized olive mill waste (OMW) (20%) treated with *P. chrysosporium* at the same treatment conditions found that after 15 days, the reductions in COD content were 69% and 74%, respectively. However, in this study it was 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 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. Consequently, Saetang and Babel, (2009) indicated that eventhough white rot fungi can treat diluted leachate better, but the species still can treat concentrated leachate. This revealed that white rot fungi can be used for treatment of waste water (i.e. leachate) with high BOD and COD value.

5.2.2 Treatment of leachate using immobilized mycelia of *Ganoderma australe* and *Trametes menziesii* on Ecomat in batch cultures

Immobilization of cells is the attachment of cells in distinct solid phase that permits exchange of substrates, products, and also inhibitors. Table 5.3 depicted the result of leachate degradation by immobilized *G. australe* incubation in flask containing liquid growth medium (GYMP) incorporated with 50% leachate and 100% leachate for 28 days. The result showed that the percentage removal of BOD_5 in 100% and 50% leachate increased every week. However, the percentage removal of BOD_5 in 100% leachate was higher (93.09%) compared to 50% leachate (81.57%) after 28 days of incubation. This indicates that *G. australe* treatment was more efficient at lower organic concentration of leachate; though the fungal was also able to treat concentrated leachate.

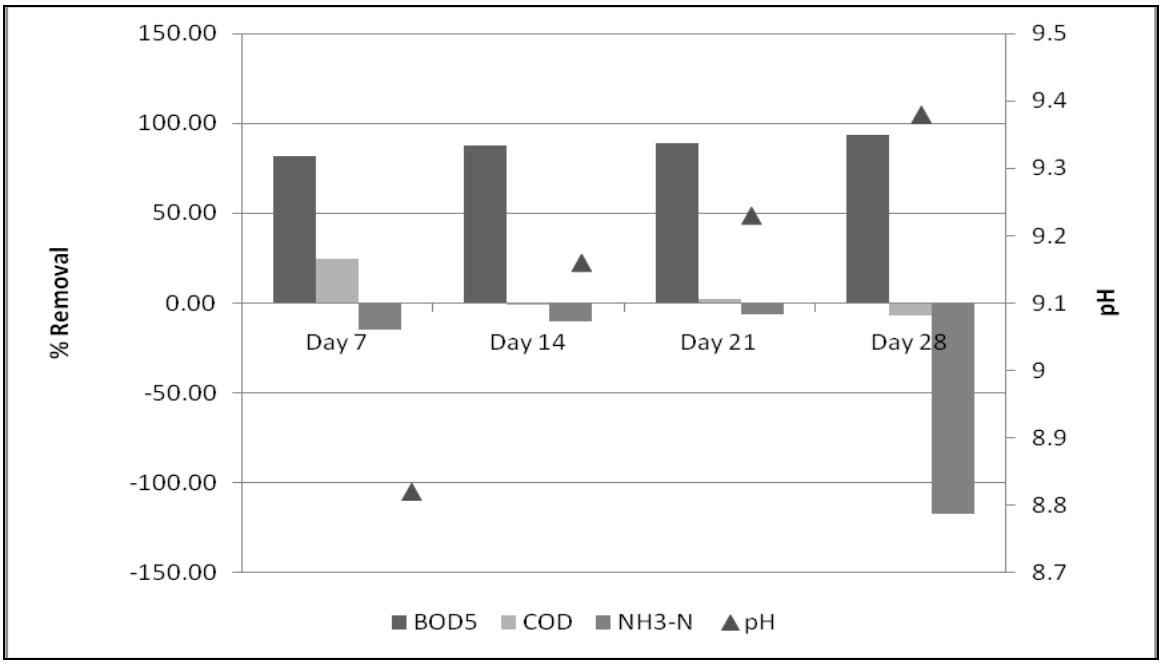
Table 5.3 also shows that percentage removal of COD was higher in 50% leachate compared to 100% leachate. The percentage removal of COD in 50% leachate increased every week with the highest percentage of 44.60% in week four (after 28 days of incubation time). However, the percentage removal of COD by 100% shows slight decreased after two weeks of incubation. At the end of 28 days of incubation, the percentage of COD removal was 17.84%. In addition, Table 5.3 also demonstrated that pH of the leachate medium increased drastically after one week incubation in both leachate concentrations. However, in 50% leachate the pH value from week 1 to week 2 decreased as this is possibly due to the organic compound produced by fungi. In addition, Table 5.3 also reveals that the concentration of ammoniacal nitrogen for both leachate concentrations where the concentration of ammoniacal nitrogen increased until week three in both leachate concentrations before it decreased in week four. The increment of ammoniacal nitrogen content shows that no removal had occurred in

TABLE 5.3. Percentage removal of 100% and 50% leachate BOD_5, COD, NH_3-N and pH changes by *Ganoderma australe* immobilized on Ecomat at weekly intervals for 28 days incubated at room temperature, shaking at 150 rpm.

leachate content. The production of extracellular enzymes by viable fungi could be the cause of this increase.

Referring to Figure 5.5, it shows that the result of leachate treatment by immobilized *T. menziesii* incubation in flask containing 100% leachate for 28 days. At weekly time intervals (day 7, 14, 21, and 28), the value of BOD_5, COD, pH and ammoniacal nitrogen were analyzed. The result revealed that the value of BOD_5 was reduced as the incubation time increased. It shows that the removal of BOD_5 increased until Day 28 with the highest percentage of 93.48%. It was observed that significant removal of BOD_5 occurred at Day 7, 82.00% and gradually increased at Day 14 with BOD_5 of 87.78% and 89.14% respectively. Similarly, the removal of COD occurred at Day 7 at 24.66% and at Day 21 with COD removal of 2.11%. Figure 5.5 also shows that the value of NH_3-N increased through-out the experiment until at Day 28 and indicating that no removal of NH_3-N occurred. The value of pH shows that the longer the incubation time, the leachate growth medium will became more alkaline.

FIGURE 5.5 Percentage removal of 100% leachate BOD_5, COD, NH_3-N and pH changes by *T. menziesii* immobilized on Ecomat at weekly intervals for 28 days incubated at room temperature, shaking at 150 rpm.



These fungi – *G. australe* and *T. menziesii* were able to degrade BOD and COD, but the respective percentages of degradation were being influenced by the culture technique employed (free or immobilized mycelium). According to Mtui and Nakamura (2002) immobilized mycelia can enhance enzyme production by facilitating mycelia-fluid contact, hence improving the mass and O_2 transfer rates. The ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very high dilution rates, are few of the many advantages of immobilized cell systems (Ramakrishna, & Prakasham, 2010). The importance of the microbial cell density in attaining higher volumetric productivities has developed the application of continuous fermentations since the free-cell systems cannot operate under chemo static mode that decouples specific growth rate and dilution rates.

This study revealed that after 28 days, the percentage removal of BOD_5 in 100% leachate increased from 85.28% by free cell 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. 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. Other study by Lapadatescu *et al*. (1997) revealed that the immobilization of *Bjerkandera adusta* on polyurethane foam for the production of enzymes by fungi is influenced by their cultivation method. Beshay (2003) reported that the production of alkaline protease by *T. turnirae* is not good when cultivated in submerged cultures, since the enzyme titters are relatively low. However, immobilization of *T. turnirae* cells in Ca-alginate beads showed a significant increase in

the production of protease enzyme. In another study by Lapadatescu *et al*. (1997) that compared two strains of *P. chrysosporium* BKM-F-1767 and INA-12 as free cells or under immobilized cell culture conditions, they found that when the fungus was immobilized, lignin and Mn peroxidase production was increased 2- to 3-fold and productivity 3- to 4-fold, respectively. According to Omar, *et al*. (1992) this could be attributed to the effect of shear forces and/or culture techniques on fungal morphology and fungal metabolism (Bonnarme *et al*., 1991).

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% and 50% leachate every week. However, the percentage removal of BOD_5 in 100% leachate was higher (93.09 %) compared to 50% leachate (81.57%) after 28 days of incubation. This indicated that *G. australe* can work better at lower organic concentration; though the fungi are also able to treat concentrated leachate. This finding is in accordance with study conducted by Saetang and Babel (2009) using immobilized *Trametes versicolor* which obtained higher color removal efficiency in diluted leachate than in concentrated leachate. In addition, 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.

The value of pH shows that the longer the incubation time, the leachate growth medium became more alkaline. The pH of leachate medium increases drastically after one week incubation in both leachate concentrations. Rodriguez *et al*. (2004) stated that the pH increase is an indicator of ammonia production. It was revealed by the increased of ammoniacal nitrogen concentration in all treatments. The concentration of ammoniacal nitrogen increased until week three in both leachate concentrations before

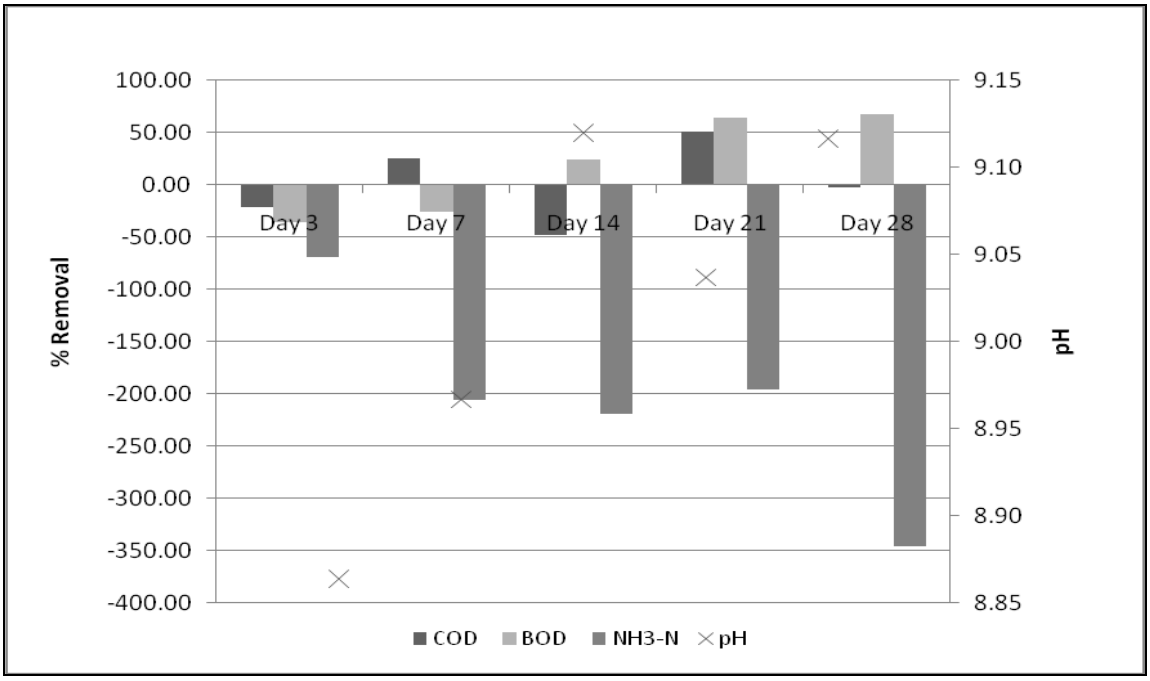
it decreased in week four. The increment of ammoniacal nitrogen content indicated that no removal of ammoniacal nitrogen had occurred in all treatments. Besides that, this could be caused by the production of ammonia, NH_3 due to the breakdown of organic compound by microorganism.

5.2.3 Treatment of leachate by immobilized coculture mycelia of *Ganoderma australe* and *Trametes menziesii* on Ecomat in batch culture

Immobilization of coculture mycelia of *G. australe* and *T. menziesii* on Ecomat was used to remediate 100% leachate in culture flask for 28 days incubation. Percentage removal of BOD_5 and COD by coculture mycelia of immobilized *G. australe* and *T. menziesii* were calculated after 3, 7, 14, 21 and 28 days of incubation. The content of leachate BOD_5 was removed only after 7 days incubation with 25.39% percentage removal. From 14 days incubation onwards, the percentage removal of leachate BOD_5 showed gradual increment. After 21 days of incubation, the removal of leachate BOD_5 has increased to 64.11% and achieved the highest percentage removal of BOD_5 (67.66%) after 28 days of incubation (Figure 5.6). Figure 5.6 also revealed that the percentage removal of leachate COD showed non-consistent trend throughout the incubation period. The removal of leachate COD occurred after 7 days of incubation with the percentage removal was 25.72%. On the other hand, result showed that ammoniacal nitrogen was not removed by these culture combination. Figure 5.6 showed that the content of ammoniacal nitrogen increased until the end of the incubation period. This result was slightly similar to the pH of leachate where it also showed an increment at the end of the incubation period (Figure 5.6). This study shows that the ability of immobilized coculture mycelia of fungi in removing the important leachate parameter was not as significant as applying individual fungal culture. This may be due to the

possible competition among the cultures for their survival since both are white-rot fungi.

FIGURE 5.6 Percentage removal of 100% leachate BOD_5, COD, NH_3-N and pH changes by coculture mycelia of immobilized *G. australe* and *T. menziesii* after treatment for 28 days, incubated at room temperature, shaking at 150 rpm.



Immobilization of coculture mycelia of *Ganoderma australe* and *Trametes menziesii* on Ecomat was used to remediate 100% leachate in culture flask for 28 days. The incubation showed that the percentage removal of BOD_5 only occurred after 7 days incubation and achieved the highest percentage removal of BOD_5 (67.66%) after 28 days of incubation. However, the percentage removal of leachate COD showed non-consistent trend which only occurred after 7 days of incubation. On the other hand, ammoniacal nitrogen and pH of leachate increased until the end of the incubation day. The result of using cocultute mycelia was slightly similar to the ability of applying individual fungal culture. As a consequence, this study shows that the ability of immobilized combination fungal culture in removing the important leachate parameter

was not as better as applying individual fungal culture. This observation is contrary with Anastasi *et al*. (2008) who demonstrated a good degradative capability of a consortium of three basidiomycetes that have been isolated from compost as sterile mycelia and identified as basidiomycetes morphophysiologically (i.e. *T. versicolor, Bjerkandera* sp. and *Lopharia spadicea*) against the complex aromatic molecule Poly R-478 (83% decolorization in 7 days). This differences was obtained possibly due to the type of waste where their waste is chemical compound whereas in this study the target waste is organic compound. Study on the benefits of mixed cultures under immobilized state to accelerate the fermentation processes by Dincbas *et al*. (1993) using mixed cultures of plasmid-free and plasmid-containing *E. coli* HB101 in alginate matrix found that the stability of the plasmid-recombinant cells was enhanced in co-immobilized state.

The degradation of some leachates content is effective by certain white rot fungi. From this study, it was found that the fungal *G. australe* and *T. menziesii* displayed a good degradative capability against the complex leachate content. Treatment of leachate with free mycelia of *G. australe* and *T. menziesii* exhibited promising BOD_5 removal only. The highest BOD_5 by *T. menziesii* was 86.50 % in 50% unsterilised leachate medium. Meanwhile, the highest BOD_5 removal by *G. australe* was achieved in 50% unsterilised leachate medium. The percentage removal was 89.10%. Furthermore, immobilization of *G. australe* and *T. menziesii* revealed higher percentage removal of BOD_5 and COD. The percentage removal of BOD_5 on 100 % leachate medium after 28 days incubation by immobilized *G. australe* and *T. menziesii* were 93.10 % and 93.62 %, respectively. In addition, the percentage removal of COD was 17.80 % and -2.97 % by using immobilized *G. australe* and *T. menziesii*, respectively.

The application of free cell mycelia and immobilized mycelia of *G. australe* and *T. menziesii* demonstrated that these fungi only showed the ability to treat leachate

organics that represented by BOD_5 and COD. Table 5.4 summarized the percentage removal of 100% leachate BOD_5 and COD by *Trametes menziesii*, *Ganoderma australe* and coculture mycelia of immobilized *G. australe* and *T. menziesii* after 28 days of incubation. Results show that immobilized mycelia of *G. australe* demonstrated better percentage removal of leachate organics (BOD_5 and COD) compared to the other methods.

Table 5.4 Percentage removal of 100% leachate BOD_5 and COD by *Trametes menziesii*, *Ganoderma australe* and coculture mycelia of immobilized *G. australe* and *T. menziesii* after treatment for 28 days, incubated at room temperature, shaking at 150 rpm

	Percentage removed (-), Increased (+)		
	Trametes menziesii	***Ganoderma australe***	**Coculture of immobilized *G. australe* and *T. menziesii***
i) Free cell method			
BOD_5	-81.39	-85.28	-
COD	-11.23	+6.72	-
ii) Immbolization method – in flask			
BOD_5	-93.48	-93.09	-67.66
COD	+7.12	-17.84	2.35

– indicates reduced (removed); + indicates increased.

These observations are coherent with worked by Mohammadi, and Nasernejad (2009) which obtained the residual anthracene concentration for the free cells after 7 days of the incubation was 54%, while a significant decrease in residual anthracene was observed by day 7 of the incubation for immobilized cells. This result could be due to the levels of extracellular enzymes produced by the fungi. Novotny *et al*. (2004) reported a comparison of cultures of *Irpex lacteus* on free cells and immobilized cells showed differences between the production of extracellular ligninolytic activities, in free cells and immobilized cells. The results showed a significant reduction in the

synthesis of MnP in free cells and immobilized cells where the highest activity of MnP was produced by the bagasse-immobilized *P. chrysosporium* at 7 days of the incubation (76Ul-1).

5.2.4. Treatment of leachate using Ecomat-immobilized mycelium of *G. australe* in column.

Leachate treatment using immobilization method in culture flask indicated that *G. australe* shows better BOD removal in leachate remediation compared to *T. menziesii*. Hence *G. australe* was selected for the treatment of leachate in column packed with Ecomat-immobilized *G. australe* mycelium was done on 50% (diluted) and 100% (raw) leachate. Leachate was run through the column continuously at constant flow of 20 ml/min (Noorlidah *et al*., 2013) and was recycled for 10 times to facilitate attachment of fungi at room temperature. Analysis was done repeatedly after 2 cycles for a total of 10 cycles and the leachate parameters are as shown in Table 5.5 and Table 5.6. BOD_5 content in 100% leachate was almost the same for all intervals however, after the last cycle (I5) the content of BOD_5 was slightly decreased at 3440 mg/l compared to control. On the other hand, the content of BOD_5 in 50% leachate (Table 5.6) showed an increment compared to untreated leachate at all intervals. Based on this result, it can be suggested that the BOD_5 removal only occurred at cycle 4 and cycle 10 of 100% leachate with the percentage removal was 9.02% and 1.43% respectively (Table 5.5). This may be due to the short exposure of leachate to the immobilized mycelia at the flow-rate used. Fungal mycelia require adaptation for the enzymes to act (Saetang & Babel, 2009).

For 100% leachate (Table 5.5), the removal of COD increased gradually from cycle 2 (2.36%), to cycle 4 (5.23%). After that the percentage removal of COD decreased to 1.23% after sixth cycle and -3.08% after eighth cycle. However , after last

TABLE 5.5. Percentage removal of BOD_5, COD, NH_3N and pH changes in raw leachate (100%) after treatment by Ecomat-immobilized *G. australe* in column packing at room temperature

Cycle/Interval	Parameters	Levels in Untreated Leachate Control	Levels in Treated Leachate	Percentage Removed (-), Increased (+)
C2/I1	BOD_5 (mg/l)	3490.00	3495.00 ± 5.00	+0.14
	COD (mg/l)	3250.00	3175.00 ± 8.66*	-2.36
	NH_3-N (mg/l)	26.60	29.20 ± 1.04*	+9.77
	pH	8.05	8.07 ± 0.03	+
C4/I2	BOD_5 (mg/l)	-	3175.00 ± 13.23	-9.02
	COD (mg/l)	-	3080.00 ± 26.46*	-5.23
	NH_3-N (mg/l)	-	27.40 ± 0.72	+3.01
	pH	-	8.11 ± 0.12	+
C6/I3	BOD_5 (mg/l)	-	3485.00 ± 26.46	+0.14
	COD (mg/l)	-	3210.00 ± 8.66	-1.23
	NH_3-N (mg/l)	-	16.40 ± 0.13*	-38.34
	pH	-	8.15 ± 0.22	+
C8/I4	BOD_5 (mg/l)	-	3495.00 ± 27.84	+0.14
	COD (mg/l)	-	3350.00 ± 32.79*	+3.08
	NH_3-N (mg/l)	-	18.60 ± 0.24*	-30.08
	pH	-	8.22 ± 0.04	+
C10/I5	BOD_5 (mg/l)	-	3440.00 ± 36.06	-1.43
	COD (mg/l)	-	2625.00 ± 13.23*	-19.23
	NH_3-N (mg/l)	-	23.60 ± 1.85*	-11.28
	pH	-	8.28 ± 0.07	+

– indicates reduced (removed); + indicates increased.

values expressed are means ± S.D. of triplicate measurements.

* shows significant difference ($p<0.05$) with untreated leachate.

cycle the removal percentage increased to 19.23%. Table 5.6 shows result for 50% leachate where the percentage removal of COD at cycle 2 was 16.77% and after that it showed slight increased at cycle 4 (22.15 %). After 6 and 8 cycles the percentage removal of COD remained quite constant at 41.26% and 36.00% respectively. Finally, during the last cycle, the percentage removal of COD increased slightly to 49.38%. Therefore, it can be seen that COD removal is higher than BOD removal in most cases.

The degradation of leachate ammoniacal nitrogen (NH_3-N) by immobilized *G. australe* also occurred in the column. The content of leachate NH_3-N in untreated 100% leachate shows quite comparable with the content of treated 100% leachate after cycle 2 and cycle 4 (Table 5.5). Then, the content of NH_3-N decreased after cycle 6 (I3). Table 5.5 and Table 5.6 show the removal of NH_3-N occurred in both 50% and 100% leachate. The highest percentage of NH_3-N removal for both leachate concentration was acquired at cycle 6 with 43.61 % for 50% leachate and 38.34% for 100% leachate.

On the other hand, degradation of leachate by immobilized *G. australe* in column showed that the pH of leachate increases after each cycle for both diluted (Table 5.6) and raw leachates (Table 5.5). However, the increment was not significant ($p>0.05$) ranging from pH 8.05 to 8.35.

The treatment of leachate in column using immobilized *G. australe* mycelia on Ecomat revealed that BOD_5 removal not occurred in 50% leachate. Nevertheless, in 100% leachate the BOD_5 removal only occurred at cycle 4 and cycle 10 with the small percentage removal of 9.02% and 1.43%, respectively. Meanwhile, it can be seen that COD removal is higher than BOD removal in most cases. COD removal occurred at most of the cycles and 50% leachate demonstrated higher COD removal at all the cycles compared to 100% leachate. Similar results were found by Saetang and Babel (2009), who obtained much less (mg/mg removal) of BOD and COD when the leachate is diluted 5 times than that of the concentrated leachate. In addition, this result indicates that white rot fungi can degrade the organic compounds with high BOD and COD. From this study, comparing the percentage removal of BOD and COD for 100% and 50% leachate showed that COD removal is higher than BOD removal in most cases. This finding is almost parallel with the finding by Saetang and Babel (2009), which obtained higher COD removal compared to BOD for all cases in their study. The patterns of BOD and COD removal in this study that showed low value at the early

stage were also consistent with the finding by Saetang and Babel (2009), indicating that the fungi might took time to acclimatize new environment at the early stage and then only start degrading the pollutants.

TABLE 5.6. Percentage removal of BOD_5, COD, NH_3N and pH changes in diluted leachate (50%) after treatment by Ecomat-immobilized *G. australe* in column packing at room temperature

Cycle/Interval	Parameters	Levels in Untreated Leachate Control	Levels in Treated Leachate	Percentage Removed (-), Increased (+)
C2/I1	BOD_5 (mg/l)	3490.00	3505.00 ± 31.22*	+0.43
	COD (mg/l)	3250.00	2705.00 ± 13.23*	-16.77
	NH_3-N (mg/l)	26.60	16.00 ± 1.21*	-39.86
	pH	8.05	8.01 ± 0.03	-
C4/I2	BOD_5 (mg/l)	-	3525.00 ± 8.66*	+1.00
	COD (mg/l)	-	2530.00 ± 18.03*	-22.15
	NH_3-N (mg/l)	-	25.80 ± 2.10	-3.01
	pH	-	8.06 ± 0.12	+
C6/I3	BOD_5 (mg/l)	-	3510.00 ± 8.66	+0.57
	COD (mg/l)	-	2050.00 ± 18.03*	-41.26
	NH_3-N (mg/l)	-	15.00 ± 0.92*	-43.61
	pH	-	8.08 ± 0.15	+
C8/I4	BOD_5 (mg/l)	-	3505.00 ± 13.23	+0.43
	COD (mg/l)	-	2080.00 ± 13.23*	-36.00
	NH_3-N (mg/l)	-	16.70 ± 1.21*	-37.22
	pH	-	8.13 ± 0.09	+
C10/I5	BOD_5 (mg/l)	-	3510.00 ± 8.65	+0.57
	COD (mg/l)	-	1645.00 ± 8.66*	-49.38
	NH_3-N (mg/l)	-	23.80 ± 1.25*	-10.53
	pH	-	8.12 ± 0.12	+

– indicates reduced (removed); + indicates increased.

values expressed are means ± S.D. of triplicate measurements.

* shows significant difference ($p<0.05$) with untreated leachate.

Contradictary result was obtained for the degradation of leachate ammoniacal nitrogen by immobilized *G. australe* in column when compared to in batch cultures. The removal of ammoniacal nitrogen occured in both 50% (diluted) and 100% (raw) leachate with the highest percentage was acquired after cycle 8 (43.61%) for 50%

leachate and 38.34% after cycle 6 for 100% leachate. The removal of ammoniacal nitrogen demonstrated removal in the continuous process since nitrification requires a long retention time, and a plug-flow extended aeration tank is ideal for nitrification ("Nitrogen Removal", 2013). Besides that, continuous process that occurred in column increased aeration. Aeration brought a lot of air bubbles into the solution which might result in turbulence and agitation (Cheung *et al*., 1997). Therefore, in column the mass transfer of ammonia nitrogen in the solution was enhanced, which benefited the volatile of molecular ammonia. As a result, aeration enhanced the removal of ammonia (Lin *et al*., 2009).

In contrast, degradation of leachate by immobilized *G. australe* in column showed that the pH of leachate increased from one cycle to another in 100% leachate and 50% leachate. This result is similar as obtained in the earlier experiments where the value of pH shows increment when leachate was treated by immobilized *G. australe* in Erlenmeyer flask. In the column packing, immobilized mycelium of *G. australe* was applied since immobilization can eliminate most of the constraints faced with the free-cell systems such as it can facilitates operation of microbial fermentation on continuous mode without cell washout (Ramakrishna & Prakasham, 2010).

Treatment of leachate using selected fungi, *Ganoderma australe* and *Trametes menziesii* based on several important parameters; BOD_5, COD, NH_3N and pH in this study revealed the ability of these white-rot fungi to treat wastewater. Zheng and Obbard (2002) study the synergistic reaction between *P. chrysosporium* and soil indigenous microorganisms in the oxidation of low molecular weight PAH (i.e. acenaphthene, fluorine, phenanthrene, fluoranthene and pyrene) in a soil-slurry found that the oxidation was enhanced by up to 43% in the presence of fungus. Thanh and Simard (1973) reported the capacities of seventeen fungal biomasses to remove phosphates (84.1%), ammonia (73.3%), total nitrogen (68.1%) and chemical oxygen

demand (COD) (39.3%). They also reported that *Epicoccum nigrum*, *Geotrichum candidum* and *Trichoderma* sp. were the best in the removal of ammonia (84%), total nitrogen (86.8%) and COD (72.3%). It is supported by Coulibaly *et al*. (2003) who discussed the ability of fungi to produce a large variety of extracellular proteins (i.e. enzymes) and also their capabilities to adapt to severe environmental constraints. Therefore, fungi have been attracting a growing interest for biotreatment (removal or destruction) of wastewater ingredients such as metals, inorganic and organic compounds. Meanwhile, according to Tatsi and Zouboulis (2002) the main requirement to be considered in the treatment of 'old' (pond) leachate was clearly to remove approximately 96% of COD and also 95% of ammonia concentrations. The comparison of waste treatment by different fungi is tabulated in Table 5.7.

Table 5.7 Application of fungi in waste treatment

Fungi	Type of waste	Percentage removal (%)	Reference
P. chrysosporium and soil indigenous microorganisms	PAH	43.0	Zheng & Obbard, 2002
17 fungal biomasses	Phosphates	84.1	Thanh & Simard, 1973
	Ammonia	73.3	
	Total nitrogen	68.1	
	COD	39.3	
Immobilized *T. versicolor*	Leachate BOD	52.0	Saetang & Babel, 2009
	Leachate COD	42.0	
Ganoderma australe	Leachate BOD_5	85.28	Wan Razarinah, 2013
Trametes menziesii	Leachate BOD_5	81.39	
	Leachate COD	11.23	
Immobilized *G. australe*	Leachate BOD_5	93.09	
	Leachate COD	17.84	
Immobilized *T. menziesii*	Leachate BOD_5	93.48	
Coculture of immobilized *G. australe* and *T. menziesii*	Leachate BOD_5	67.66	

5.3. CONCLUSION

As a conclusion, free cell cultures of *T. menziesii* and *G. australe* used in this study were capable in removing BOD_5 with the highest (89.33%) shown by using *G. australe* in 50% unsterilised leachate. Besides that, dilution of leachate (50% or 100%) and sterility of the medium (sterilized or unsterilised) did not significantly increase the removal efficiency. This result indicates that using free cell cultures gave better BOD_5 removal on unsterilised concentrated leachate.

In addition, the percentage removal of BOD_5 and COD by immobilized *G. australe, T. menziesii* and coculture of *G. australe* and *T. menziesii* using 100% leachate after 28 days of incubation are 93.09% and 17.84%; 93.48% and -7.12%; and 67.66% and -2.35% respectively. The treatment of leachate in column using immobilized *G. australe* mycelia on Ecomat revealed that BOD_5 removal only occurred at cycle 4 and cycle 10 in 100% leachate with the percentage removal is 0.43% and 1.58%, respectively. However, COD removal demonstrated higher percentage than BOD removal in most cases. COD removal occurred at most of the cycles and 50% leachate demonstrated higher COD removal at all the cycles compared to 100% leachate. On the other hand, the degradation of leachate ammoniacal nitrogen was obtained when leachate was treated with immobilized *G. australe* in column. The removal of ammoniacal nitrogen occured in both 50% and 100% leachate with the highest percentage was after cycle 8 for 50% leachate (45.95%) and 30.90 % for 100% leachate.

Based on the result obtained, we can conclude that using immobilized *G. australe* give the best result in the removal of BOD_5 and COD for concentrated leachate. Therefore, in the next chapter, the potential *G. australe* was further evaluated for the production of ligninolytic enzymes which, is suggested may be involved in the bioremediation of leachate.

TREATMENT OF LEACHATE USING ENZYMES PRODUCED BY *G. AUSTRALE* AND *T. MENZIESII*

6.0 INTRODUCTION

The generated leachate in landfill can cause considerable environmental problem. Landfill leachates are usually high strength wastewaters, as well as the presence of several toxic/ hazardous components (Ehrig, 1984; Zoubolis *et al*., 2001). According to Mohammadi and Nasernejad (2009), environmental pollution with hazardous wastes containing recalcitrant xenobiotic chemicals hence become one of the major ecological problems. Several options have been applied for leachate treatment, presenting varying degree of efficiency. Biological processes based upon suspended-growth biomass, were proven to be effective for the removal of organic carbon and nutrients content (Zoubolis *et al*., 2001). However, several other problems have been encountered. Ahuja *et al*. (2004) reported factors that may limit the use of microbes for bioremediation are the methods that needed higher cost and longer time to produce microbial cultures. Therefore, bioaugmentation that involved the addition of microbial cultural product such as enzymes to wastewater has been applied in order to improve the performance of wastewater treatment system.

Bioaugmentation is the addition of microbial cultural product such as enzymes to wastewater. According to Trombly (1995), enzymes are classified broadly as hydrolytic, oxidizing or reducing, depending on the type of reaction they control. The transformation takes place as the enzyme encounter its substrate (the target pollutant) and splits the substrate into component parts or removes part of the molecule. This process occurs very rapidly, leaving the enzyme unaltered and ready to deal with further molecules of substrate. There are several benefits of using enzymes for environmental applications as discussed by Novo Nordisk (1995) which include: (i) they can function

either at mild, replacing harsh or work in extreme conditions, hence saving energy and preventing pollution; (ii) they are highly specific, which results in less unwanted side effects and byproducts in the production process; (iii) enzymes also able to treat waste consisting of biological material; and (iv) finally, enzymes themselves are biodegradable, so they are readily absorbed back into nature.

Microorganisms are the best source for the production of useful enzymes. Cell immobilization technology is pertinently suited to produce extracellular enzymes particularly for the continuous production of enzymes. Immobilization of various microbial cells, namely *Rhizopus chinensis* (Nakashima *et al*., 1990), *A. niger* (Jamil, & Omar, 1992), *Candida rugosa* (Ferrer, & Sola, 1992) and *Sporotrichum thermophile apinis* (Johri *et al*., 1990) were reported for the production of lipases and white rot fungi, *P. chrysosporium* for the production of lignin peroxidases (Ruckenstein, & Wang, 1994).

Ahuja *et al*. (2004) stated that enzymes are natural catalysts, which are commonly found in all living organisms. They may be used either for building more complex molecules from simple ones or for selective breakdown of a mixture of larger molecules. Novo Nordisk (1995) reported that over 1,000 different enzymes excreted by just one microorganism. This is supported by Steve (2008) who stated that fungal able to produce certain organic compound such as inducible enzymes. Enzymes from fungi have gained much attention lately, mainly due to its industrial importance. The used of fungal ligninolytic enzymes in bioremediation have been reported by several researchers. Gianfreda and Rao (2004) elaborated that several extracellular enzymes of fungi, either as cell-associated or cell-free enzymes, may behave as powerful catalysts in the bidegradation of harmful pollutant. Reddy, (1995) reported lignin-degrading white-rot fungi have the unique ability to degrade/mineralize a broad spectrum of structurally diverse toxic environmental pollutants. As an example, extracellular

peroxidases are important in degrading some, but not all, xenobiotic compounds. In other work, extracellular peroxidases and laccases have been shown to oxidize recalcitrant compounds (Novotny, *et al*., 2004) such as laccase of *P. sanguineus* exhibited the ability to degrade polycyclic aromatic hydrocarbons (Munusamy, *et al*., 2008). The reason of applying fungi instead of bacteria is because they possess a very powerful extra cellular oxidative enzymatic system: the lignin-degrading enzyme system (LDS), which has broad substrate specificity and is able to oxidize several environmental pollutants (Reddy, 1995; Cameron *et al*., 2000). As a consequence, a vast range of toxic environmental pollutants, including low soluble compounds, can be mineralized or degraded by white-rot fungi (Gianfreda & Rao, 2004).

Many reports (Eugenio *et al*., 2008) demonstrated that the complex ligninolytic machinery of basidiomycetous fungi is involved in many of these degradation processes. This enzymatic complex includes, among many others, enzymes such as lignin peroxidase, manganese peroxidase (Leonowicz *et al*., 1999) and laccase, which has been confirmed to be essential for ligninolytic activity in many white-rot fungi (Leonowicz *et al*., 1991). Furthermore, Wesenbertg *et al*. (2003) stated that white-rot fungi have the capacity to produce one or more extracellular lignin-modifying enzymes (LME) due to their lack of substrate specificity, hence also capable of degrading a wide range of xenobiotics. Gold and Alic (1993) demonstrated white rot fungi such as *P. chrysosporium* typically secrete one or more of the three principal ligninolytic enzymes, i.e. lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13) and phenol oxidase (Laccase) (LAC, E.C. 1.10.3.2). In this study, white rot fungi – *G. australe* and *T. menziesii* that showed the promising result in previous leachate treatment were used for the production of ligninolytic enzymes.

Therefore this study aimed to evaluate the importance of ligninolytic activities of selected fungi for degradation of leachate important components. Hence, the more specifice objectives of this study are:

i) to profile the type of ligninolytic enzymes produced by *G. australe* and *T. menziesii*.

ii) to determine the effect of inducers on yield of ligninolytic enzymes.

iii) to investigate the degradation rates of leachate important parameters by the extracellular enzymes.

6.1 MATERIALS AND METHODS

6.1.1 Fungal cultures and maintenance

Two fungal species from the previous work which are *G. australe* and *T. menziesii* were selected for this study. Each fungal culture was maintained on malt extract (MEA) (Oxoid) agar slants, and inoculum was prepared by sub-culturing onto MEA grown for 7 days at 28±2 ºC.

6.1.2 Preparation of mycelial suspension and inoculum

Mycelial suspensions of *G. australe* and *T. menziesii* were prepared by transferring four agar plugs (6- mm^2 diameter) of 7-day old fungal colony growing on MEA media in Petri plates into 250- ml Erlenmeyer culture flasks. The flasks contained 100 ml of Glucose-yeast-malt-peptone (GYMP) growth medium (as described in Section 5.1.2, page 79) under sterile conditions. 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 ºC at 150 rpm (Saetang & Babel, 2009).

6.1.3 Profiling of extracellular enzymes and the effect of inducers on enzymes yield.

Profiling of extracellular enzymes produced by *G. australe* and *T. menziesii* were carried out in GYMP liquid medium with 0.1% enzyme inducers. Two sets of GYMP liquid medium (Medium A and B) were used. Each medium consists of a group of inducers for the production of enzymes studied consisting of ligninolytic enzymes (lignin peroxidase, LiP; manganese peroxidase, MnP; and laccase, Laccase) and also protease, lipase and amylase enzymes. The inducers in medium A consists of (0.10%) Tween 80, skim milk and starch. Meanwhile, in medium B the inducers consist of (0.10%) skim milk, olive oil, veratryl alcohol, manganese chloride ($MnCl_2$), cuprum sulphate ($CuSO_4$) and starch. These inducers were selected since they show possibility to induce the production of enzymes needed. According to Levin *et al*. (2005) and Novotny *et al*. (2004) Tween 80 is an inducer for the production of Laccase, LiP, MnP and lipase, skim milk for the production of protease, starch for the production of amylase, olive oil for the production of lipase, veratryl alcohol for the production of LiP, $MnCl_2$ for the production of MnP and $CuSO_4$ for the production of Laccase. Then, the flasks containing GYMP liquid medium added with 0.1% enzyme inducers were then agitated on an orbital shaker for 10 days at 28±2 ºC at 150 rpm. At regular interval of two days after inoculation, three culture flasks were sampled and assayed for enzymes activities. A set of uninoculated medium without inducer in flasks was used as control.

6.1.4 Preparation of extracellular enzyme and crude enzyme of *G. australe*.

Extracellular enzyme is an enzyme that is secreted by a cell (e.g. fungi) and works outside of that cell, while crude enzyme is cell-free enzyme that was isolated from their originating cells. The extracellular enzymes were produced by *G. australe* in

flasks containing GYMP liquid medium (as mentioned at Section 6.1.2) added with 0.1% of skim milk, olive oil, veratryl alcohol, $MnCl_2$, $CuSO_4$ and starch. Then the medium was incubated at room temperature with agitation rate of 150 rpm for four days. After four days incubation, the medium was filtered and the supernatant (extracellular enzyme) was analyzed for enzymes activity. After that, the supernatant was freeze-dried until it became powder. Then the powdered enzyme (crude enzyme) was added with sodium buffer and the enzymes activity was analyzed again. The method of enzyme preparation was modified from the method of Gassara *et al*., (2010). Both type of enzymes preparation might contain all enzymes produced by *G. australe*.

6.1.5 Preparation of enzyme extract

The extracellular enzymes to be profiled were extracted with 50 mM sodium-phosphate buffer at pH 6.5 (10/1, v/w). Then, the mixture was placed in a shaker at 150 rpm for one hour. It was then filtered through Whatman filter paper (1.5 μm) and the filtrates were analysed for enzyme activity.

6.1.6 Enzyme assays

In this study, six types of enzymes that are lignin peroxidase, manganese peroxidase, laccase, protease, lipase and amylase were investigated. Lignin peroxidase, manganese peroxidase and laccase were studied because they have been confirmed to be essential for ligninolytic activity in many white-rot fungi (Leonowicz *et al*., 1991), hence capable of degrading a wide range of xenobiotics (Wesenbertg *et al*., 2003). Meanwhile, protease, lipase and amylase were chosen to be investigated in this study due to the leachate characteristic which has high organic compound. Protein, starch and fat that are present in the leachate can be degraded by protease, amylase and lipase, respectively. Karam and Nicell (1997) stated that proteases can solubilize proteins,

while amylases can be used to break down the long starch molecule into smaller fragments. Meanwhile, lipases can be used to break down fats such as by hydrolyzing the ester bonds and trans-esterify triglycerides (Ib, 1996; Bornscheur, 2002).

6.1.6.1 Lignin peroxidase Activity

Lignin peroxidase (LiP) activity was determined by monitoring the conversion of veratryl alcohol to veratryl aldehyde at 25 ºC by hydrogen peroxide (H_2O_2) (Tien & Kirk, 1984). The increase in absorbance was measured at 310 nm. The reaction was initiated by the addition of H_2O_2 at a final concentration of 0.5 mM. One unit of enzyme activity (U) was defined as the amount of the enzyme that can produce 1 μmol veratryl aldehyde from the oxidation of veratryl alcohol per minute.

6.1.6.2 Manganese peroxidase Activity

Manganese peroxidase (MnP) activity was measured under the conditions described by Singh (2008). The reaction mixture contained 2.40 ml of sodium lactate buffer (pH 4.5), 0.1 ml of $MnS0_4$ (6 mM), 0.2 ml of guaicol (6 mM), 0.2 ml of enzyme and 0.1 ml of H_2O_2 (3 mM). The reaction was initiated by the addition of H_2O_2 at final concentration of 0.1 mM. MnP activity was measured by oxidation of guaicol at $\lambda = 465$ nm after 1 min. One unit (U) of MnP activity was defined as the enzyme producing one unit of absorbance change/min/g of substrate.

6.1.6.3 Laccase Activity

Laccase activity was determined by the increase in the absorbance at $\lambda = 525$. This was due to the production of tetramethoxy-azo-bis-methylenequinone resulting from the reaction of laccase with syringaldazine (Harkin & Obst, 1973; Leonowicz & Grzywnowicz, 1981). The substrate was 0.1 mM syringaldazine in 50% ethanol (w/v). One unit (U) of laccase activity was defined as the amount of enzyme producing one unit change in absorbance/min at $\lambda = 525$.

6.1.6.4 Protease Activity

Protease activity was determined using azocasein as substrate. The reaction mixture which consists of 0.5 mL of enzyme was mixed with 0.5 ml of 0.5% azocasein. After 18 h of incubation at 37 º C, the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid. The supernatant was collected by centrifugation at 10,000 g for 10 min. Then, 1.0 ml of the supernatant was added to 0.1 ml of 0.5 M sodium hydroxide solution (Singh, 2008). The final volume was adjusted to 3.0 ml with distilled water. The absorbance was measured at λ = 595 nm. One unit of activity was defined as the amount of enzyme giving an increase of one absorbance unit/min.

6.1.6.5 Lipase Activity

Lipase activity was determined with p-nitrophenyl palmitate (pNPP) by the method reported by Savitha *et al*. (2007). The substrate for this reaction was composed of solution A and solution B. Solution A contained 40 mg of pNPP dissolved in 12 ml isopropanol. Solution B contained 0.1 g of gum Arabic and 0.4 ml of triton X-100 dissolved in 90 ml of water. The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring. The reaction mixture contained 1 mL of substrate, 0.5 mL of buffer (50 mM Tris-HCl buffer, pH 8.0), 0.1 mL of enzyme (the filtrate) and the volume was made up to 3 ml with distilled water. This was incubated at 40ºC for 45. The reaction was immediately stopped by the addition of 0.2 mL of isopropanol. The absorbance was measured at λ = 410 nm. One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per min.

6.1.6.6 Amylase Activity

Amylase activity was determined using 1% soluble starch in citrate-phosphate buffer (pH 6.5) as substrate. The reaction mixture which consists of 0.5 ml of enzyme was mixed with 0.5 ml of soluble starch. After 30 min of incubation at 40 º C, the

reaction was stopped by adding 1.0 ml of DNS reagent. Then, the mixture was boiled for 5 minute. Once cooled, 10 ml of distilled water was added. The absorbance was measured at λ = 540 nm. One unit of activity was defined as the amount of glucose produced per ml in the reaction mixture per min (Abe *et al*., 1988).

6.1.7 Remediation of leachate by crude enzyme

The application of crude enzyme in leachate remediation was based on method by Munusamy *et al*. (2008). The crude extracellular enzyme was added into the Erlenmeyer flask containing 100% leachate. Before the treatment, the leachate medium used in this study was filtered and the content of pH, BOD_5, COD and ammoniacal nitrogen were measured as in Section 5.1.8. The reaction mixture consists of 19.8 ml of 50 mM sodium citrate buffer (pH 6.5), 2.0 ml of crude enzyme and 0.2 ml of leachate. The combination of crude extracellular enzyme and leachate medium were incubated on an orbital shaker at 80 rpm. The measurement of pH, BOD_5, COD and ammoniacal nitrogen was studied at each interval as in Section 5.1.8.

Leachate remediation was investigated at different enzyme concentrations and time of leachate exposure to enzyme. At treatment using various enzyme concentrations, the time of leachate exposure to crude enzyme was maintained for 24 hours. Four different enzyme concentrations were used i.e. 0 U/ml, 5 U/ml, 10 U/ml and 20 U/ml. These enzyme concentrations were calculated based on the enzyme activity of LiP. Meanwhile, in order to get the best time exposure of leachate to crude enzyme, the enzyme concentration was maintained at 10 U/ml and four different time exposures were investigated which were 0, 4, 8 and 24 hours.

6.1.8 Remediation of leachate by immobilized *G. australe* followed by treatment with crude enzymes.

Experiments were carried-out in Erlenmeyer flask and consisted of two phases. In the first phase, in 250 ml Erlenmeyer culture flasks, 125 ml of leachate was treated with immobilized *G. australe* on Ecomat (as in Section 5.1.5). The flasks were then agitated on an orbital shaker for seven days at 28±2 ºC at 150 rpm. Then, in the second phase, the treated leachate from the first phase was collected and subsequently treated with 10 U/ml of crude enzyme for 4 hours on an orbital shaker at 80 rpm. All processes were done under sterile conditions at ambient temperature. Before the treatment, the leachate medium used in this study was filtered. The content of pH, BOD_5, COD and ammoniacal nitrogen was measured (as in Section 5.1.8) before and after the treatment. These concurrent methods were used based on the result of previous experiments. In Chapter 5, application of immobilized *G. australe* in flask demonstrated the best percentage removal of leachate organic (BOD_5, and COD) but not NH_3-N. Meanwhile, treatment of leachate with crude enzyme showed very promising result for NH_3-N removal. Therefore, combination of these methods were applied in order to achieve optimum removal of leachate BOD_5, COD and NH_3-N.

6.2 RESULTS AND DISCUSSION

The profiling of important enzymes for leachate remediation by *G. australe* and *T. menziesii* revealed that both fungal were able to produce important enzymes for pollutants degradation.

6.2.1 Profiling of extracellular enzymes and the effect of inducers on enzymes yield.

The profiling of *G. australe* and *T. menziesii* over a period of 10 days exhibited varying titers of enzymes. Profiling of extracellular enzymes was investigated in 10 days since previous work (Section 5.2.2) showed that between Day 7 and Day 14 promising percentage removal of leachate components (especially BOD_5 and COD) were obtained. Enzymes profile of *G. australe* in medium A and B are shown in Figure 6.1 and 6.3, respectively. Enzymes profile of *T. menziesii* in medium A and B was illustrated in Figure 6.2 and 6.4, respectively. Productivity of all the enzymes in medium A and medium B of both fungi demonstrated the highest productivity of LiP in medium A with 1.90 ± 0.09 U/ml and in medium B: 3.28 ± 1.19 U/ml; MnP (medium A: 24.74 ± 0.20 U/ml and medium B: 45.83 ± 1.81 U/ml ; Laccase (medium A: 14.17 ± 0.11 U/ml and medium B: 21.93 ± 0.79 U/ml); protease (medium A: 14.20 ± 0.17 U/ml and medium B: 8.36 ± 0.51 U/ml); lipase (medium A: 1.97 ± 0.01 U/ml and medium B: 1.49 ± 0.05 U/ml); and amylase (medium A: 1.08 ± 0.00 U/ml and medium B: 0.79 ± 0.02 U/ml). The results revealed that medium B produced higher productivity of most studied enzymes especially ligninolytic enzymes (LiP, MnP and Laccase) than medium A.

In addition, ligninolytic enzymes are the important enzymes for pollutant degradation. Due to that, it is important to find that the medium to produce these enzymes. Comparison of ligninolytic productivity in medium B by *G. australe* and *T. menziesii* was shown in Figure 6.3 and 6.4. Results show that the highest activity of LiP was 1.39 ± 0.60 U/ml by *G. australe* and 3.28 ± 1.19 U/ml by *T. menziesii*; MnP (45.83 ± 1.81 U/ml by *G. australe* and 27.22 ± 0.90 U/ml by *T. menziesii*) and Laccase were 21.93 ± 0.79 U/ml by *G. australe* and 14.04 ± 0.18 U/ml by *T. menziesii*. Based on

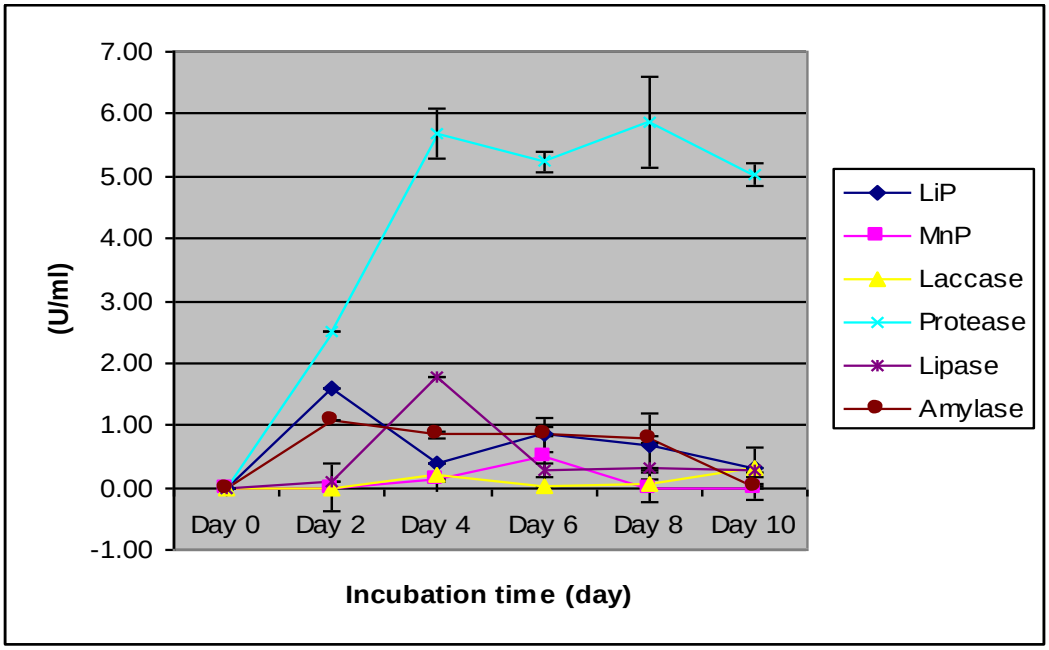


Figure 6.1: Profiling of enzymes produced by *G. australe* in Medium A incubated at room temperature and agitated at 150 rpm. The experiment was performed in triplicates.

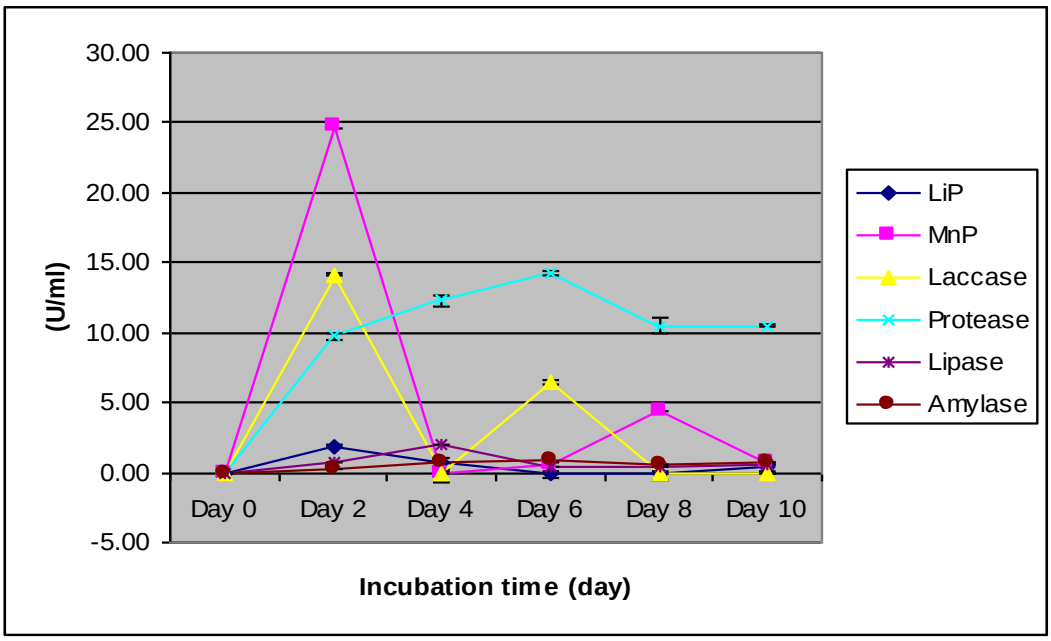


Figure 6.2: Profiling of enzymes produced by *T. menziesii* in Medium A incubated at room temperature and agitated at 150 rpm. The experiment was performed in triplicates.

these results, it was shown that *G. australe* is better in producing ligninolytic enzymes than *T. menziesii*.

A significant productivity of protease was exhibited by *T. menziesii* and *G. australe* in both medium. It was revealed that the productivities of protease by both fungi showed significantly higher in medium A than in medium B. The protease productivity on Day 4 of *T. menziesii* was 12.27 ± 0.33 U/ml in medium A and 6.02 ± 0.01 U/ml in medium B, while, for *G. australe* the productivity of protease was 5.70 ± 0.15 U/ml in medium A and 4.23 ± 0.42 U/ml in medium B. Both fungi produced very low titers of lipase and amylase in both medium. The productivity of lipase by *G. australe* in both medium ranged between 0.10-1.78 U/ml, while for *T. menziesii* the productivity of lipase in both medium ranged between 0.35-1.97 U/ml. Productivity of amylase exhibited by *G. australe* in both medium ranged between 0.04-1.08 U/ml and between 0.24-0.82 U/ml by *T. menziesii*. Moreover, production of ligninolytic enzymes for medium B of *G. australe* (Figure 6.3) exhibited a highest productivity on Day 4. The highest enzymes productivity obtained was 1.39 ± 0.60 U/ml for LiP, 45.83 ± 1.81 U/ml for MnP and 21.93 ± 0.79 U/ml for Laccase.

This fact is coherent with the observations by Anastasi *et al*. (2008) who reported about the production of laccase by *T. versicolor* to decolorize Poly R-478 and degrade PAH. Laccase and MnP are reported to be excreted at varying levels by different white-rot fungal cultures (Lema *et al*., 2000). Mileski *et al*. (1988) stated recently ligninases are also able to catalyze the initial oxidation of a number of environmentally persistent xenobiotics. However, *G. australe* demonstrated better production of ligninolytic enzymes than *T. menziesii*.

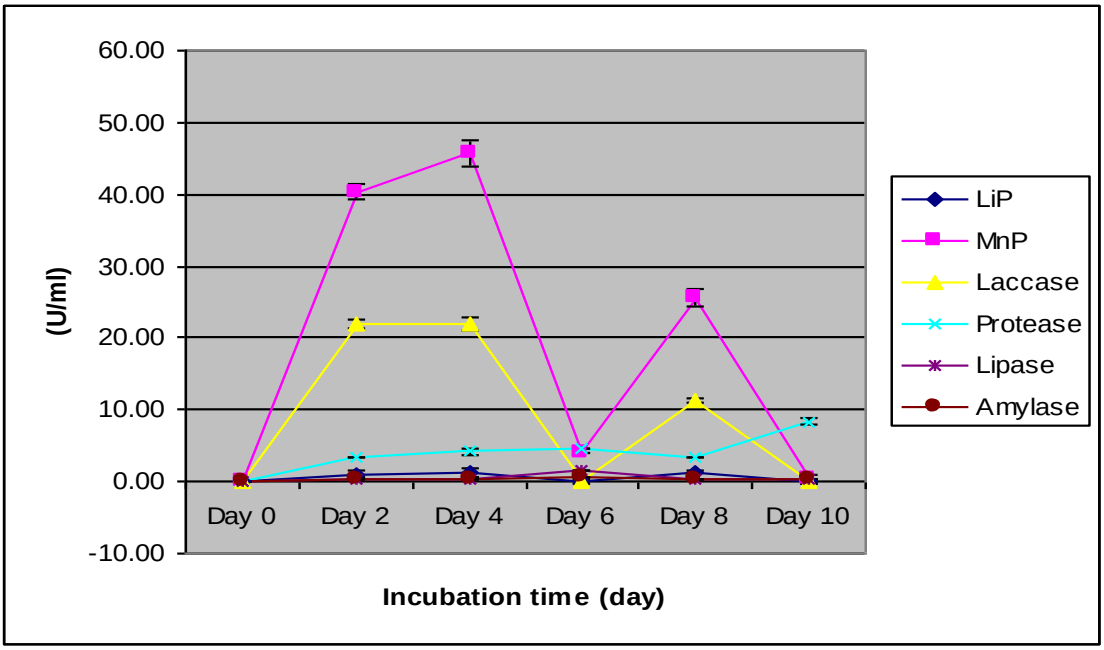


Figure 6.3: Enzymes profile of enzyme extract from *G. australe* in Medium B incubated at room temperature and agitated at 150 rpm. The experiment was performed in triplicates.

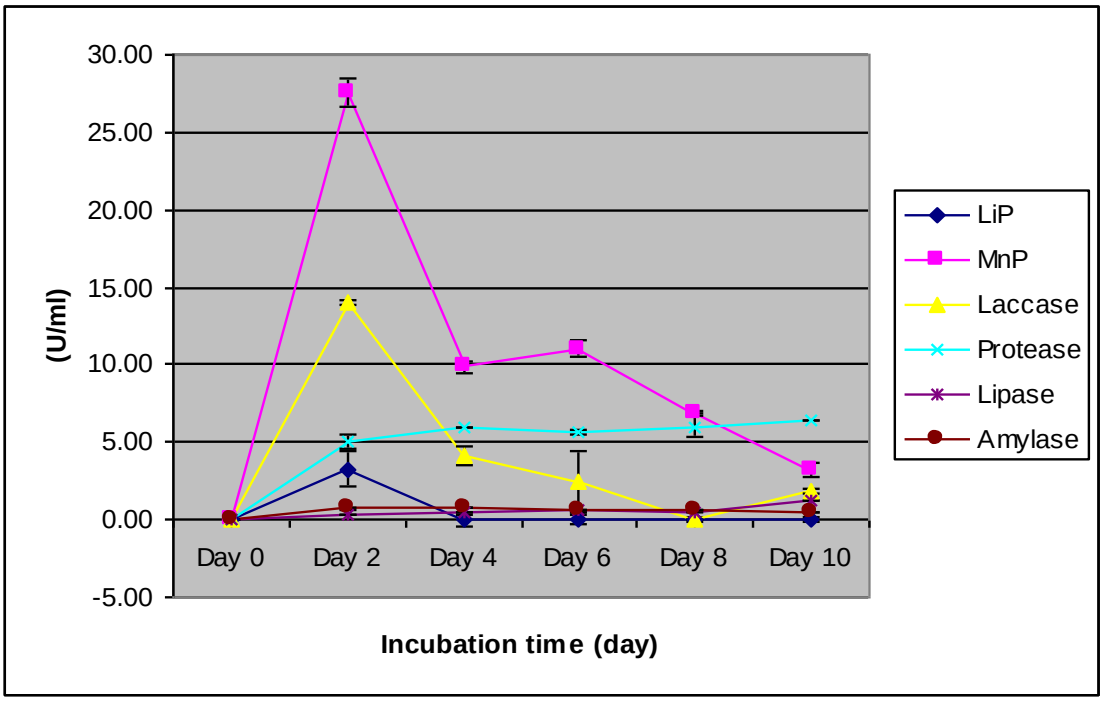


Figure 6.4: Enzymes profile of enzyme extract from *T. menziesii* in Medium B incubated at room temperature and agitated at 150 rpm. The experiment was performed in triplicates.

Comparison of enzymes production on two set of medium that consists of different enzyme inducers showed that medium B was better than medium A since it produced higher activity of most important enzymes especially ligninolytic enzymes. The enzymes inducer for Laccase, LiP, MnP and lipase in medium A is Tween 80. Whereas, in medium B enzyme inducer for lipase is olive oil, for LiP is veratryl alcohol, for MnP is $MnCl_2$ and for Laccase is $CuSO_4$. Meanwhile, skim milk and starch which may induce the production of protease and amylase respectively, contained in both medium.

This finding is consistent with Levin *et al*. (2005) who reported that copper had the highest positive influence on ligninolytic enzyme production. In some cases, copper has been reported to be a strong laccase inducer in several species, among them *P. chrysosporium* (Dittmer *et al*., 1997) and *T. versicolor* (Collins & Dobson, 1997). It is known that copper induces both laccase transcription and activity. Tween detergent in growth medium was reported to increase the production of extracellular ligninolytic enzymes (especially MnP) in submerged culture (Novotny *et al*., 2004). Meanwhile, Leonowicz *et al*., (1991) observed that the production of ligninase enzyme was highly stimulated by sunflower and olive oils. Sayadi and Ellouze (1995) claimed that veratryl alcohol is an inducer of ligninolytic enzymes. In addition, Kotterman *et al*. (1996) stated the expression of ligninolytic peroxidases in white-rot fungi is regulated by the presence of nutrients such as nitrogen and manganese. They demonstrated that the presence of manganese is known to induce the production of MnP in many white- rot fungi, and organic N nutrients stimulated LiP and MnP production by *Bjerkandera* sp. strain BOS55. Yateem *et al*. (1998) noted, the production of peroxidases enzymes was induced by high concentrations of carbon and nitrogen in white-rot fungal strains, and thus can positively affect the degradation rates.

6.2.2 Production of extracellular enzymes by *G. australe*.

Based on the previous result on profiling of extracellular enzymes, it was found that the extracellular enzymes by *G. australe* in liquid medium containing (0.1%) of skim milk, olive oil, veratryl alcohol, $MnCl_2$, $CuSO_4$ and starch produced a highest productivity of ligninolytic enzymes. In order to choose which type of enzyme preparation is best to be applied in leachate remediation, the enzyme activity of studied enzymes was compared between extracted enzyme and crude enzyme. The results in Figure 6.5 show that the enzyme productivity of extracted enzyme and crude enzyme was not significantly different ($P>0.05$) for Laccase, protease, and lipase (Appendix 32). No amylase enzyme was detected in both enzyme preparations. The result also shows that the enzyme productivity of LiP crude enzyme was higher (3.66 ± 2.19 U/ml) compared to enzyme extract (0.12 ± 0.24 U/ml). The highest enzyme productivity for both enzyme preparations was shown by protease. This enzyme shows similar pattern as LiP where productivity of protease crude enzyme was also higher (14.6 ± 1.98 U/ml) than enzyme extract (13.2 ± 0.40 U/ml). At the same time, MnP, Laccase and lipase show low enzyme activity in both enzyme preparations with the enzyme activity of MnP was 1.75 ± 0.35 for both preparation, Laccase (enzyme extract: 0.12 ± 0.24 U/ml and crude enzyme: 0.10 ± 0.42 U/ml) and lipase (enzyme extract: 1.04 ± 0.14 U/ml and crude enzyme: 0.55 ± 0.21 U/ml). The enzyme activity of enzyme extract and crude LiP shows slightly significant difference ($p=0.1$). However, the difference is not significant ($p>0.05$) for other studied enzymes (Appendix 32).

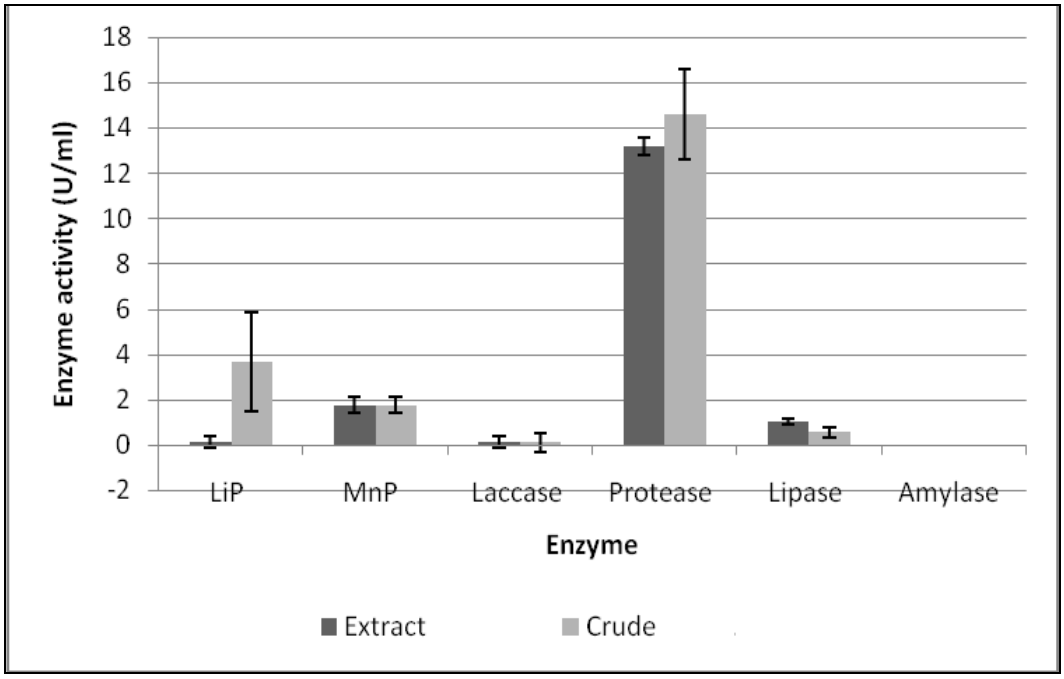


Figure 6.5 Activity of enzymes in extract and crude enzyme of *G. australe* incubated at room temperature, agitated at 150 rpm for four days. The experiment was performed in triplicates.

Comparison of productivity of enzymes between extract (extracellular enzyme) and crude enzyme (cell-free enzyme) found that the productivity of MnP, Laccase, protease, and lipase of extract and crude enzymes was not significantly different eventhough the productivity of MnP and protease were higher for crude enzyme than extracellular enzyme. Significant difference was exhibited for the productivity of LiP between extract and crude enzymes where productivity for crude enzymes was much higher (3.66 ± 2.19 U/ml) than extracellular enzyme (0.12 ± 0.24 U/ml).

Based on the result obtained, it showed that the ligninolytic enzymes (LiP, MnP and Laccase), which have been shown the ability to degrade a variety of environmental pollutant can be produced by *G. australe*. This finding is supported by Wesenbertg *et al*. (2003) who stated that white-rot fungi have the capacity to produce one or more extracellular lignin-modifying enzymes (LME) due to their lack of substrate specificity therefore, also capable of degrading a wide range of xenobiotics. Furthermore, Reddy

(1995) and Cameron *et al*. (2000) also indicated that fungi are better candidate than bacteria to be used in bioremediation since they possess a very powerful extra cellular oxidative enzymatic system: the lignin-degrading enzyme system (LDS), which has broad substrate specificity and is able to oxidize several environmental pollutants. The potential of ligninolytic enzymes to degrade several of environmental pollutant have been reported in previous study (Levin *et al*., 2005; Novotny *et al*., 2004; Mohammadi & Nasernejad, 2009; Ehlers & Rose, 2005). This suggested that the extracellular enzyme produced can be used in the leachate bioremediation. The result also indicates that the crude enzyme which possess higher enzyme activity of LiP compared to extracellular enzyme is best to be used in leachate remediation.

6.2.3 Remediation of leachate by crude enzyme at different parameters

Previous studies have focused on the lignin-degrading enzymes of *P. chrysosporium* and *T. versicolor*. However, in recent years the interest in studying the lignin-modifying enzymes of white-rot fungi has arisen (Levin *et al*., 2005) and their focus is for finding better lignin-degrading systems for use in various biotechnological applications. According to Kissi *et al*. (2001), potential applications of white-rot fungi and their enzymes in the detoxification of industrial waste waters and of a vast range of xenobiotic environmental pollutants have gaining increasing importance. Previous experiments in this study on production of extracellular enzymes by *G. australe* showed that crude enzyme has better ligninolytic activity (especially LiP) compared to extract enzyme. As a consequence, in the following study, the use of crude enzyme to remediate 100% leachate was carried out. Different concentration of enzymes did not significantly ($p>0.05$) affect the BOD_5 content in leachate (Appendix 34). Without enzyme (0 U/ml) the BOD_5 value is 3935.00 ± 0 mg/ml, whereas at enzyme concentration of 5, 10 and 20 U/ml, the BOD_5 content were 3962.50 ± 3.54 mg/ml,

3912.50 ± 67.18 mg/ml and 3985.00 ± 7.07 mg/ml respectively (Table 6.1). However, different concentration of crude enzyme showed significant ($p<0.05$) effect on the content of leachate COD (Appendix 35). In the absence of crude enzyme, the COD value obtained was 3260.00 ± 0 mg/ml; while by applying 5 U/ml, 10 U/ml and 20 U/ml obtaining 1710.00 ± 664.68 mg/ml, 840.00 ± 424.26 mg/ml and 6620.00 ± 141.42 mg/ml of COD respectively (Table 6.1). As for the content of leachate ammoniacal nitrogen (NH_3-N) it shows significantly different ($p<0.05$) at all different concentration of crude enzyme (Appendix 36). Without crude enzyme, the content of leachate NH_3-N showed the highest value (78.44 ± 0 mg/ml) compared to the value when using other concentration of crude enzyme. By using 5, 10 and 20 U/ml of crude enzyme, the content of leachate NH_3-N obtained decreased to 29.40 ± 0.28, 26.90 ± 1.27 and 36.30 ± 2.12 mg/ml respectively (Table 6.1). Different crude enzyme concentration showed no significant effect ($p<0.05$) on leachate pH (Table 6.1) (Appendix 37). The pH of leachate when 0, 5, 10 and 20 U/ml of crude enzyme were added were 6.46 ± 0, 6.47 ± 0.01, 6.45 ± 0.04 and 6.53 ± 0.06 respectively.

Based on the results obtained, percentage removal of leachate BOD_5, COD and NH_3-N were calculated (as in Section 5.1.8). Table 6.1 shows that the highest removal of leachate BOD_5 (0.57%) was obtained when 10 U/ml concentration of crude enzyme was used. The highest percentage removal of leachate COD (74.23%) was achieved when using 10 U/ml concentration of crude enzyme. Significant percentage removal of NH_3-N from leachate by crude enzyme at all concentrations was achieved. The highest removal of NH_3-N (65.71%) was exhibited when 10 U/ml of crude enzyme concentration was applied.

Remediation of leachate by crude enzyme at different concentration of enzymes revealed that the highest removal efficiency of leachate BOD_5 (0.57%), COD (74.23%) and NH_3-N (65.71%) was exhibited when 10 U/ml of crude enzyme concentration was

applied. Hammel (1989) reported that lignin peroxidase produced by *P. chrysosporium* oxidized not only lignin-related compounds, but also a wide variety of environmentally significant aromatics.

TABLE 6.1. Percentage removal of BOD_5, COD, NH_3-N and pH changes in 100% leachate after treatment with crude enzymes of *G. australe* incubated on an orbital shaker at 80 rpm with different concentrations of crude enzyme for 24 hours

Enzyme Concentration (U/ml)	Parameters	Levels in Untreated Leachate	Levels in Treated Leachate	Percentage Removal (-), Increased (+)
0	BOD_5 (mg/l)	3260.00	3264.00 ± 2.00	+0.12
	COD (mg/l)	3935.00	3933.00 ± 6.56	-0.05
	NH_3-N (mg/l)	78.44	78.42 ± 0.17	-0.02
	pH	6.46	6.46 ± 0.04	none
5	BOD_5 (mg/l)	3260.00	3282.80 ± 176.11	+0.70
	COD (mg/l)	3935.00	2063.83 ± 97.64	-47.55
	NH_3-N (mg/l)	78.44	29.40 ± 4.39	-62.52
	pH	6.46	6.47 ± 0.60	+
10	BOD_5 (mg/l)	3260.00	3241.40 ± 125.92	-0.57
	COD (mg/l)	3935.00	1014.05 ±156.44	-74.23
	NH_3-N (mg/l)	78.44	26.90 ± 2.36	-65.71
	pH	6.46	6.45 ± 0.30	-
20	BOD_5 (mg/l)	3260.00	3301.00 ± 154.78	+1.26
	COD (mg/l)	3935.00	7990.80 ± 130.71	+103.07
	NH_3-N (mg/l)	78.44	36.30 ± 2.29	-53.72
	pH	6.46	6.53 ± 0.17	+

– indicates reduced (removed); + indicates increased.

values expressed are means ± S.D. of triplicate measurements.

The result on effect of time exposure on leachate remediation is as shown in Table 6.2. The content of leachate BOD_5 is not significantly different ($P>0.05$) at all four different exposure time. At 0 hour of exposure time, the leachate BOD_5 content was 3935.00 ± 0 mg/ml. Meanwhile at 4 hours, 8 hours and 24 hours of exposure of leachate to crude enzyme shows 3965.00 ± 7.07 mg/ml, 3970.00 ± 7.07 mg/ml and 3912.00 ± 67.18 mg/ml of leachate BOD_5 respectively. In addition, Table 6.2 also

showed that the removal of BOD_5 in leachate almost did not occured at all different time exposure to enzymes. On the other hand, Table 6.2 shows significant difference ($p<0.05$) on leachate COD content. The leachate COD content decreased drastically after four hours exposure to crude enzyme. The leachate COD was 3260.00 ± 0 mg/ml at 0 hour of time exposure but then it dropped to 600.00 ± 424.26 mg/ml after four hours of exposure, 300.00 ± 141.42 mg/ml after eight hours of exposure and 840.00 ± 424.26 mg/ml after 24 hours of exposure. The removal of leachate COD at 4 hours of time exposure to crude enzyme was 81.60% and was constant at 8 hours of exposure (90.80%) and at 24 hours of exposure (74.23%) (Table 6.2).

Similar patterns of leachate ammoniacal nitrogen degradation was shown by crude enzyme (Table 6.2). At 0 hour exposure time to crude enzyme, the content of leachate ammoniacal nitrogen was 78.44 ± 0 mg/ml, and it dropped drastically to 30.30 ± 0.71 mg/ml after 4 hours of exposure to crude enzyme; 29.90 ± 0.71 mg/ml at 8 hours, and maintained at 28.80 ± 0.57 mg/ml after 24 hours of time exposure to crude enzyme. The percentage removal of leachate ammoniacal nitrogen at different exposure time to crude enzyme was shown in Table 6.2. The result shows that 61.37% of leachate ammoniacal nitrogen was removed at 4 hours of time exposure and the percentage of ammoniacal nitrogen removal was maintained with 62.14% at 8 hours, and 63.28% after 24 hours of time exposure to crude enzyme. Meanwhile, the value of leachate pH quite similar at all different time exposure of leachate to crude enzyme. The pH values showed no significant difference ($p>0.05$) with pH ranged from 6.46 to 6.54 (Table 6.2) (Appendix 39).

Meanwhile, remediation of leachate using 10 U/ml crude enzymes at four different time exposures demonstrated that the removal of leachate COD after four hours of time exposure to crude enzyme was 81.60% and quite constant after 8 hours of exposure (90.80%) and reduce to 74.23% after 24 hours exposure. The removal of

TABLE 6.2. Percentage removal of BOD_5, COD, NH_3-N and pH changes in 100% leachate after treatment with 10 U/ml of crude enzyme of *G. australe* incubated on an orbital shaker at 80 rpm at different time exposure.

Time (hrs)	Parameters	Levels in Untreated Leachate	Levels in Treated Leachate	Percentage Removal (-), Increased (+)
0	BOD_5 (mg/l)	3260.00	3260.00	nc
	COD (mg/l)	3935.00	3935.00	nc
	NH_3-N (mg/l)	78.44	78.44	nc
	pH	6.46	6.46	
4	BOD_5 (mg/l)	3260.00	3284.77 ± 105.64	+ 0.76
	COD (mg/l)	3935.00	724.04 ± 54.78	-81.60
	NH_3-N (mg/l)	78.44	30.30 ± 3.24	-61.37
	pH	6.46	6.46 ± 0.14	nc
8	BOD_5 (mg/l)	3260.00	3289.01 ± 186.59	+0.89
	COD (mg/l)	3935.00	362.02 ± 24.56	-90.80
	NH_3-N (mg/l)	78.44	29.70 ± 2.29	-62.14
	pH	6.46	6.54 ± 0.26	+
24	BOD_5 (mg/l)	3260.00	3241.09 ± 148.00	-0.58
	COD (mg/l)	3935.00	1014.05 ± 96.81	-74.23
	NH_3-N (mg/l)	78.44	28.80 ± 3.21	-63.28
	pH	6.46	6.45 ± 0.25	-

– indicates reduced (removed); + indicates increased; nc indicates no changes.

values expressed are means ± S.D. of triplicate measurements.

leachate ammoniacal nitrogen showed similar patterns as the removal of leachate COD. The removal of leachate ammoniacal nitrogen after 4 hours of exposure was 61.37% and was maintained with 62.14% after 8 hours, and 63.28% after 24 hours of exposure to crude enzyme. These observations were coherent with Ehlers and Rose (2005) who noted that maximum LiP activity was detected after 5-6 h being introduced to the phenol and trichlorophenol (TCP).

Singh, (2008) stated that the use of cell-free enzyme for bioremediation has been gaining popularity as compared to the fungal treatments. This is due to the potential advantages that include: application to biorefractory compounds, operation at high and low contaminant concentrations, operation over a wide range of pH, temperature and

salinity, absence of shock loading effects, absence of delays associated with the acclimatization of biomass, reduction in sludge volume (no biomass generated) and the ease and simplicity of controlling the process (Karam & Nicell, 1997). In addition, Gianfreda and Rao (2004) elaborated that the advantage of applying cell-free enzymes over the use of microbial cells in wastewater treatment is due to their unique substrate-specificity and catalytic power; their capability to act in the presence of many toxic, even recalcitrant, substances, and/or under a wide range of environmental conditions, often unfavourable to active microbial cells (i.e. relatively wide temperature, pH and salinity ranges, high and low concentrations of contaminants); and their low sensitivity or susceptibility to the presence of predators, inhibitors of microbial metabolism, and drastic changes in contaminant concentrations.

Previous study done by Novotny *et al*. (2004) showed that in a majority of experiments using white rot fungi, the degradation rates could be correlated with the levels of extracellular ligninolytic enzymes known to be involved. Besides that, Wesenberg *et al*. (2003) also reported that ligninolytic enzymes are involved in degradation of various xenobiotic compounds.

The capability of ligninolytic enzymes in bioremediation was reaffirmed by Novotny *et al*. (2004). They described that enzyme LiP, MnP, and Laccase have been shown to take part in vitro transformation of nonpolymeric, recalcitrant pollutants such as nitrotoluens, PAHs, organic and synthetic dyes and pentachlorophenol. All these pollutants were reported to be contained in leachate (Baun *et al*., 2004; Chu *et al*., 1994; Jemec *et al*., 2012; Kjeldsen *et al*., 2002; Lakshmi, & Joseph, 2010; Park *et al*., 2001; Zakaria *et al*., 2005). LiP was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds (Figure 6.6) (Karam & Nicell, 1997).

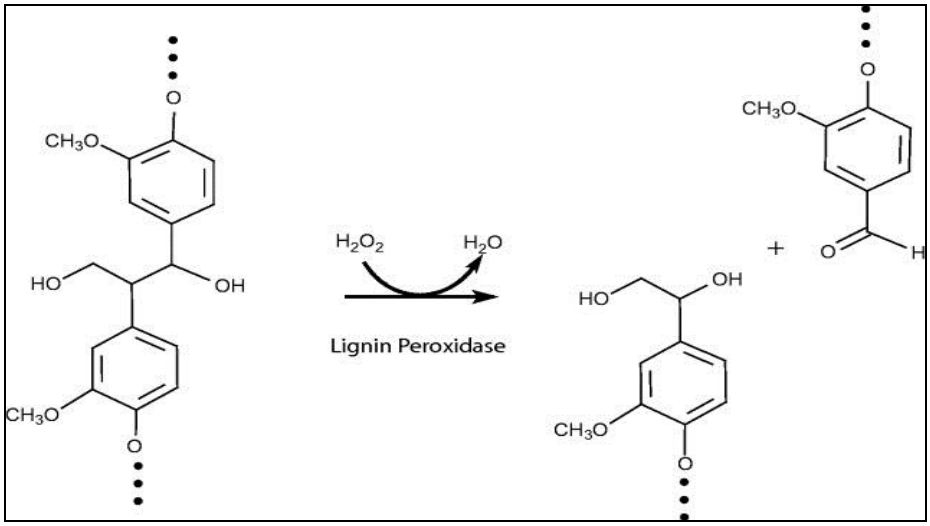


Figure 6.6 The oxidative reaction of lignin peroxidase.

Source: Sigma Aldrich (2013).

Laccase is a bleu copper phenoloxidase that contains four copper atoms per polypeptide chain and is capable of catalyzing the four-electron transfer reaction necessary to fully reduce oxygen to water (Lema *et al*., 2000) (Figure 6.7). Therefore, these enzymes have a broad substrate specificity including polyphenols, methoxy-substituted monophenols, aromatic amines and a considerable variety of other natural and synthetic substrates (Lema *et al*., 2000). In a great number of white-rot fungi, MnP expression requires the Mn (II) ion which involved in the catalytic cycle of MnP and then, oxidized to Mn (III) (Figure 6.7). Complexed Mn (III) with an organic acid present in wastewater able to attack a great number of substrates with a phenolic structure similar to that of lignin (Lema *et al*., 2000).

6.2.4 Remediation of concentrated (100%) leachate by immobilized *G. australe* followed by treatment with crude enzyme.

The results obtained from the previous experiments demonstrated that treatment of leachate with immobilized *G. australe* on Ecomat only achieved significant BOD_5 removal, while treatment of leachate with crude enzymes attained notable effect on

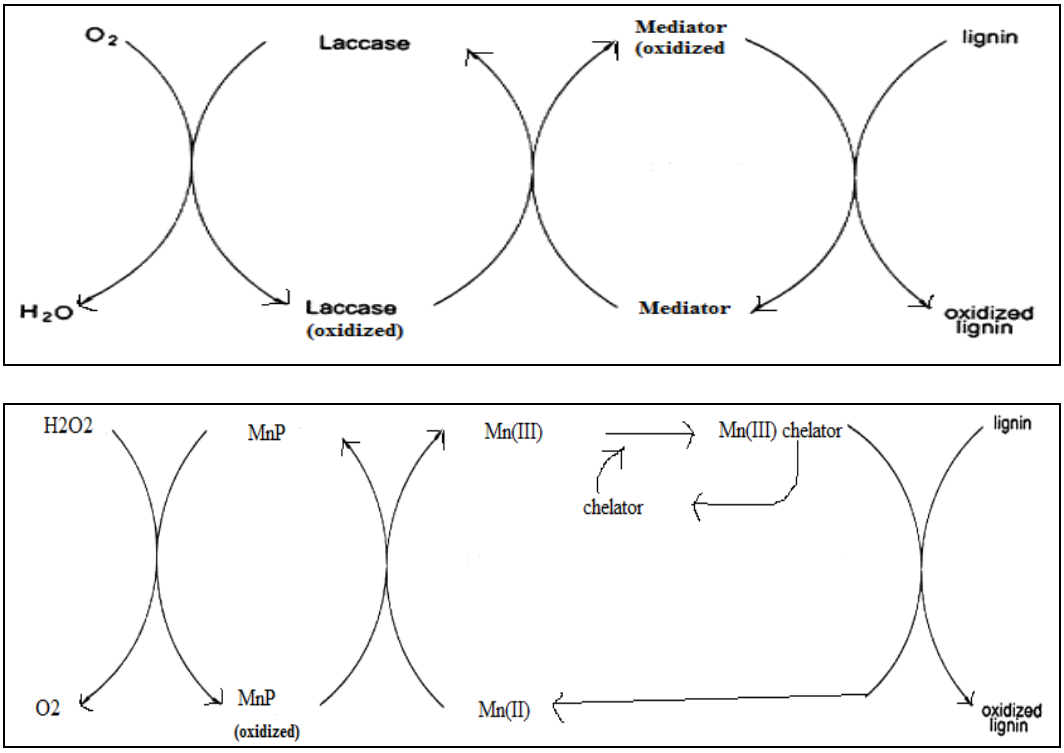


Figure 6.7 The oxidative pathway for catalytic action of laccase and manganese peroxidase on lignin.

Source: Lema *et al*. (2000)

NH_3-N removal. For this reason, final experiment for remediation of leachate using fungi was carried-out by combining immobilized *G. australe* on Ecomat and crude enzymes. It consists of two phases (as in Section 6.1.8) where in the first phase concentrated leachate was treated with immobilized *G. australe* on Ecomat. Result ofleachate remediation after the first phase was as shown in Table 6.3. It demonstrated that the percentage removal of leachate components obtained was 50.36% for BOD_5, COD (31.86%) and -39.67% for NH_3-N. At the second phase, the leachate was collected and treated with 10 U/ml of crude enzyme at 4 hours time exposure on an orbital shaker at 80 rpm. Table 6.3 also revealed that after the second phase of experiments, 16.34%, 36.93%, and 72.92% of BOD_5, COD and NH_3-N removal were achieved, respectively. Finally, by the end of the experiment, the percentage removal of leachate components was enhanced (Table 6.3). Overall, after concurrent treatment with

immobilized *G. australe* and crude enzymes, 58.47%, 57.02% and 62.17% percentage removal of leachate BOD_5, COD and NH_3-N were achieved, respectively. Meanwhile, the pH at both phases of the experiment was not much different with the value of 9.11 after the first phase of experiment and 8.87 at the end of second phase of the experiment (Table 6.3). Eventhough these concurrent methods demonstrated promising percentage removal of leachate organics and NH_3-N, but the value of BOD_5, COD and NH_3-N are still not achieved the standard limits of regulation (EQA, 2009).

TABLE 6.3 Percentage removal of BOD_5, COD, NH_3-N and pH changes after incubated with immobilized *G. australe* on Ecomat incubated at room temperature for 7 days, shaking at 150 rpm and with 10 U/ml of crude enzyme at 4 hours time exposure on an orbital shaker at 80 rpm.

Treatments	Parameters	Levels in Untreated Leachate	Levels in Treated Leachate	Percentage Removal (-), Increased (+)	Total Percentage Removal (-), Increased (+)
Immobilized *G. australe*	BOD_5 (mg/l)	4166.00	2068.00 ± 424.79	-50.36	
	COD (mg/l)	5980.00	4075.00 ± 207.75	-31.86	
	NH_3-N (mg/l)	30.93	43.20 ± 5.24	+	
	pH	8.14	9.11 ± 0.72	+	
Crude enzyme	BOD_5 (mg/l)		1730.00 ± 131.15	-16.34	-58.47
	COD (mg/l)		2570.00 ± 126.30	-36.93	-57.02
	NH_3-N (mg/l)		11.70 ± 3.61	-72.92	-62.17
	pH		8.87 ± 0.74	-	+

– indicates reduced (removed); + indicates increased.

values expressed are means ± S.D. of triplicate measurements.

Remediation of concentrated leachate by immobilized *G. australe* followed by treatment with crude enzyme revealed that the first phase of treatment which is by immobilized *G. australe* on Ecomat only exhibited 50.36% and 31.86% of BOD_5 and COD removal. Result obtained showed the ability of white-rot fungi *G. australe* to

remove BOD_5 and COD but not NH_3-N. 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. Besides that, Coulibaly *et al*. (2003) noted 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 because of their capacities to adapt to severe environmental constraints. Cell immobilization that involved the entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate. According to Ramakrishna, and Prakasham (2010), cell immobilization technology which involved the use of whole microbial cells and/or organelles is pertinently suited to produce extracellular enzymes especially by microorganisms. This is for the reason that immobilization eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes. It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation.

However, the continuous experiment with 10 U/ml of crude enzyme at 4 hours time exposure on an orbital shaker at 80 rpm not only could remove BOD_5 and COD but also ammoniacal nitrogen. The percentage removal achieved were 58.47%, 57.02% and 62.17% for leachate BOD_5, COD and NH_3-N, respectively. These result illustrated that enzyme can be applied to remove leachate NH_3-N significantly. They also showed the ability to remove BOD_5 and COD. Previous result shows that *G. australe* was able to produce ligninolytic enzymes such as LiP, MnP and Laccase. These enzymes were known for their capability in degrading a variety of environmentally pollutants (Yateem *et al*., 1998) that include leachate. Ikehata *et al*. (2004) described the use of enzymes for the treatment or the removal of environmental and industrial pollutants has attracted

increasing attention because of their high efficiency, high selectivity, and environmentally benign reactions.

Torres *et al*. (2003) illustrated peroxidases and laccases which have broad substrate specificities can catalyze the oxidation of a wide range of toxic organic compounds. Gianfreda and Rao (2004) elaborated that enzymes were carry-out when no efficient chemical transformations could have been devised. They stated the degradative efficiency of biological processes depends on the biodegradability of the contaminants. The contaminants must interact with enzymatic systems in the degrading organisms. Certain contaminants are soluble and can easily enter cells, thus able to degrade by extracellular enzymes of fungi but not for insoluble substances, including xenobiotics. So, in order to be biodegraded, these contaminants must be transformed into soluble or easily cell-available products by interacting with enzymatic systems in the degrading organisms. Besides applying extracellular enzymes, other alternative for using enzyme in bioremediation is utilizing cell-free enzymes isolated from their originating cells (Gianfreda *et al*., 1999) such as using crude enzyme.

In addition, one of the most important advantages in the use of enzymes over microbes where the disposal of the sludge due to biomass produced in bioremediation processes using living cells, mainly aerobic treatments, could be avoided. Besides that, since conservation of natural resources and the protection of the environment become increasingly important issues, enzymes have been suggested as the only solution to remediate certain sites, such as those polluted with deep and dispersed subsurface halogenated hydrocarbons. Trombly (1995) stated that this is based on the claimed made by some companies that enzyme-enhanced bioremediation processes can be cost-competitive with ex-situ approaches.

Consequently, the findings in this study are consistent with the statement by Davis *et al*. (1993) who said that degradation of xenobiotics under aqueous conditions,

both by fungal cultures and by enzymes preparations *in vitro*, suggests that bioremediation using lignin-degrading fungi has potential hence, can be applied in wastewater such as leachate treatment.

Table 6.4 summaries the use of *G. australe* and *T. menziesii* in the treatment of 100% leachate using various methods. Treatment of leachate by free-cell mycelia of *G. australe* and *T. menziesii* in this study were capable in removing BOD_5 by all study treatments with the highest (89.33%) was shown by using *G. australe* in 50% unsterilised leachate. Meanwhile, COD removal showed variations among the treatments for both *G. australe* and *T. menziesii*. Overall, the highest leachate COD removal (56.49%) has shown by *T. menziesii* in unsterilised medium of 50% leachate. Sterility of the medium (sterilised or unsterilised) demonstrated significantly different ($p<0.05$) in BOD_5 removal. Treatment of 100% leachate by *G. australe* revealed that using unsterilised leachate medium resulted in better BOD_5 removal where in unsterilised leachate medium 85.24% of BOD_5 was removed compared to 48.48% in sterilised medium. Moreover, dilution of leachate (50% or 100%) did not significantly increase the removal efficiency of leachate BOD_5. Besides that, after 28 days 100% leachate being treated by immobilized *G. australe*, *T. menziesii* and coculture of *G. australe* and *T. menziesii* in flask, 93.09% and 17.84%; 93.48% and - 7.12%; and 67.66% and -2.35% of BOD_5 and COD removal was obtained respectively. Based on these results, we can conclude that immobilized *G. australe* give the best result in the removal of BOD_5 and COD of concentrated (100%) leachate.

The treatment of leachate in column using immobilized *G. australe* mycelia on Ecomat demonstrated low percentage of BOD_5 removal (9.02% and 1.43%) in 100% leachate but none in 50% leachate. COD removal is higher than BOD removal in most cases. COD removal occurred at most of the cycles and 50% leachate demonstrated higher COD removal at all the cycles compared to 100% leachate. The degradation of

leachate ammoniacal nitrogen (NH_3-N) also achieved in this study. The highest percentage of NH_3- N removal for both leachates occurred at cycle 6 with 43.61% and 38.34% removals for 50% and 100% leachates, respectively.

Meanwhile, the application of crude enzyme for leachate treatment obtained the highest removal efficiency of leachate COD and NH_3-N when 10 U/ml concentration of crude enzyme was used. The highest percentage removal of leachate COD was 74.23% and 65.71% for leachate NH_3-N.

Remediation of concentrated (100%) leachate by immobilized *G. australe* in combination with crude enzymes demonstrated the most promising ability in removing leachate BOD_5, COD and NH_3-N. The percentage removal achieved were 58.47%, 57.02% and 62.17% for leachate BOD_5, COD and NH_3-N, respectively.

Table 6.4. Summarize results of leachate treatment based on percentage removal of BOD_5, COD, NH_3-N and pH changes using concentrated (100%) leachate at various conditions.

Treatment Methods	Percentage removal (-)/ increased (+)			
	BOD_5	**COD**	**NH_3-N**	**pH**
1) Free-cell mycelia				
- *G. australe*	-85.28	+6.72	+3.76	-
- *T. menziesii*	-81.39	-11.23	+20.60	-
2) Immobilization				
i) In flask				
- *G. australe*	-93.09	-17.84	+45.61	+
- *T. menziesii*	-93.48	+7.12	+116.85	+
ii) In column				
- *G. australe*	-1.43	-19.23	-11.28	+
3) Crude enzymes from *G. australe*	-0.57	-74.23	-65.71	
4) Immobilized *G. australe* followed by crude enzyme	-58.47	-57.02	-62.17	+

– indicates reduced (removed); + indicates increased.

These findings suggested that in order to achieve optimum removal of leachate BOD_5, COD and NH_3-N, combination treatment of immobilized *G. australe* and cell-free enzymes must be applied.

6.3 CONCLUSION

As a conclusion, the optimum result for leachate treatment was obtained with crude enzymes concentrations of 10 (U/ml) with 0.57% BOD_5 removal, 74.23% COD removal and 65.71% NH_3-N removal. Meanwhile, optimum exposure time of crude enzymes to leachate was achieved after 4 hours exposure with 81.60% COD removal and 61.37% NH_3-N removal were obtained. Consequently, these revealed that removal of leachate NH_3-N only occurred when leachate was treated with cell-free enzyme (=crude enzymes) which may be due to the very slow transformation of NH_3-N into soluble or easily cell-available products. On the contrary, removal of leachate BOD_5 was significantly obtained when enzyme extract of *G. australe* was applied while, COD removal was achieved with both by enzyme extract of *G. australe* and cell-free enzymes (=crude enzymes).

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

7.0 OVERALL CONCLUSION

Pollution problem that has arisen by leachate landfill has created the need to characterize leachate quality in order to ensure an effective leachate treatment. As discussed in Chapter 3, the characterization of leachate from different landfills revealed large differences between the landfills in terms of studied parameters. This may due to the different type of landfilling (closed or active landfills), landfill method (dumpsite or sanitary landfills), types of disposal waste or local environment.

The landfill's age has a significant effect on the leachate characteristics. From this study, leachate from active landfills has demonstrated higher content of BOD_5 and COD. However, pH and ammoniacal nitrogen contents of leachate from closed landfill shows increasing trend compared to the content in leachate from active landfill. Characterization of leachate studies showed varied characteristics among the landfills and the principal pollutants in the leachate samples were organic matter and ammonia loads (Objective I). Hence, the biological method specifically fungal remediation was applied for leachate treatment.

The characterization studies indicates of the difficulty to treat leachate due to its' varied characteristics. Therefore, we investigated on the fungal utilization for leachate degradation. Studies on local fungal strain as candidate for leachate bioremediation is still lacking. For that reason, in this study twelve local fungal strains were screened for their ability to grow and hence remediate the leachate content. Out of twelve different fungi tested, two white-rot fungi species namely *G. australe*, and *T. menziesii* showed the most prominent growth on MEA dissolved with 50% and 100% leachate without adjustment of the pH due to leachate. Both fungi demonstrated the ability to grow well

on the medium supplemented with leachate and were not affected by the pH of the medium. (Objective II).

The degradation of some leachates content is effective by certain white rot fungi. From this study, it was found that the fungal *G. australe* and *T. menziesii* displayed a good degradative capability against the complex leachate content. Treatment of leachate with free mycelia of *G. australe* and *T. menziesii* exhibited promising BOD_5 removal only. The classical fermentations using batch-mode operations suffer from various constrains such as low cell density, nutritional limitations, and high down times.

Therefore, in this study the biological treatment of leachate with Ecomat-immobilized *G. australe* mycelia on 50% (diluted) and 100% (raw) leachate was carried-out in packed column. The result revealed that BOD_5 was not removed from both leachate concentrations. However, COD removal occurred at most of the cycles and diluted leachate demonstrated higher COD removal at all the cycles compared to concentrated leachate. In addition, from this study also demonstrated that the degradation of leachate by immobilized *G. australe* in column showed promising removal of ammoniacal nitrogen occurred in both diluted and raw leachates but, unfortunately the percentage was not constant through-out the cycle.

Study on the effectiveness of applying enzymes (bioaugmentation) for enhancement biological treatability of leachate was quite rare. Therefore, in this study two selected white-rot fungi *Ganoderma australe* and *Trametes menziesii* were used in order to examine their ability to produce ligninolytic enzymes, hence potential to degrade leachate important parameters such as BOD_5, COD and NH_3-N. Profiling of enzymes by *G. australe* and *T. menziesii* in this study demonstrated that both fungi were able to produce ligninolytic enzymes but at different productivities. Furthermore, the

production of ligninolytic enzymes was higher by *G. australe* than *T. menziesii.* It is due to the difference in strains capability.

The production of enzymes is influence by the medium formulation such as inducers. As a consequence, in this study, production of ligninolytic enzymes was compared using different inducers. Production of enzymes by *G. australe* was studied by using two set of mediums which contain different enzymes inducer. The medium which contains skim milk, olive oil, veratryl alcohol, $MnCl_2$, $CuSO_4$ and starch demonstrated the best medium in producing LiP, MnP and Laccase since it produced higher activity of most of studied enzymes especially ligninolytic enzymes. Meanwhile, the comparison of *G. australe* and *T. menziesii* in producing ligninolytic enzymes showing *G. australe* was much promising with the highest activity of LiP was 1.39 ± 0.60 U/ml; MnP (45.83 ± 1.81 U/ml) and Laccase (21.93 ± 0.79 U/ml). Evaluation of enzyme activity of extract and crude illustrated significant difference ($p<0.05$) only for LiP but not for other studied enzymes. As a result, better production of LiP, MnP and Laccase was obtained in medium containing veratryl alcohol, $MnCl_2$ and $CuSO_4$.

This present study also investigated leachate remediation using two different approaches: different enzyme concentrations and different time of leachate exposure to enzyme. The highest removal efficiency of leachate BOD_5, COD and NH_3-N was obtained when 10 U/ml concentration of crude enzyme was used. The highest percentage removal of leachate BOD_5 was 0.57%, while 74.23% and 65.71% for leachate COD and NH_3-N respectively. Furthermore, the removal of leachate COD at 4 hours of time exposure to crude enzyme was 81.60%, quite constant at 8 hours of exposure (90.80%) before reduced to 74.23%) at 24 hours exposure. Removal of leachate ammoniacal nitrogen achieved 61.37% at 4 hours of time exposure and maintained 62.14% at 8 hours, and 63.28% after 24 hours of time exposure to crude

enzyme. However, the removal of BOD_5 in leachate was almost not occurred at all different time exposure to enzymes.

In summary, application of immobilized local white-rot fungal – *G. australe* in leachate treatment demonstrated a successful removal of BOD_5 and COD from leachate. It is due to the facts that white-rot fungi have been shown to degrade a wide variety of environmental pollutants. Meanwhile, in this present study showed that application of crude enzyme on leachate treatment after being treated with immobilized fungal revealed very noteworthy results where BOD_5, COD and ammoniacal nitrogen could be removed significantly (Objective III). The finding from this study will contribute to a new "Green Technology" for wastewater treatment especially leachate from Malaysian native fungi species.

7.1 RECOMMENDATIONS FOR FUTURE WORK

The characteristic of leachate has shown that leachate contains abundant organic matter. The identification of organic matter using UV-VS, GC-MS and/or HPLC should be done in order to elucidate the mechanism of leachate degradation.

The present study highlights that *G. australe* and *T. menziesii* are an excellent source of lignocellulolytic enzymes particularly LiP, MnP and laacase. The potential of immobilized *G. australe* in column (continuous) could be improved by reducing the flow-rate of the cycle and longer the time of treatment so that it can increased the exposure time of mycelia enzymes to leachate.

The crude enzyme extract has shown the potential to remove ammoniacal nitrogen (NH_3-N) from leachate. The identification of degradation products using GC-MS and/or HPLC should be done in order to elucidate the mechanism of leachate degradation.

The purification of enzyme is necessary in order to understand the catalytic properties as well as the difference in the properties of the enzyme. Therefore, the purification of LiP, MnP and laccase should be carried out and the catalytic properties of the enzymes should be compared to the enzymes reported by other white-rot fungi.

The potential of enzymes of *G. australe* should also be evaluated in the bioremediation of other organopollutants such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs).

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