Removal of Refractory Contaminants from Mature Landfill Leachate Utilizing Fungi and Evaluation of Lignocellulosic Enzymatic Activity

By

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Abstract

The aim of this research was to evaluate the refractory contaminants removal efficiency from the MLL deploying selected fungi and to investigate their lignocellulosic enzymatic activities. Among the six deployed fungal species (*Trichoderma asperellum, Cladosporium* sp., *Penicillium* sp., *Tyromyces chioneus, Bjerkandera adusta,* and *Phanerochaete chrysosporium*), *Penicillium* sp. and *T. chioneus* appeared to be most effective strains with 66% and 59% COD reduction; and associated highest laccase (Lac), lignin-peroxidase (LiP), and manganese-peroxidase (MnP) activities of 250, 8, and 11 U/L from *Penicillium* sp. and 165, 19, and 27 U/L from *T. chioneus*. *Penicillium* sp. was also utilized in a continuous pack bed bioreactor operated at 48, 72, 96, and 120 h hydraulic retention time (HRT). Bioreactor exhibited maximum 52%, 54%, 60%, 58%, and 75% of COD, soluble COD, total carbon, total organic carbon, and color removal efficiencies at HRT 120 h and maximum enzymatic activities were 149, 27, and 16 U/L of Lac, LiP, and MnP, respectively. The findings seem to be promising for further exploration to enhance refractory contaminants removal from problematic wastewater.

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Chapter 1: Introduction

1.1. Solid waste disposal and leachate generation

Municipal solid waste (MSW) comprised with those materials which are produced daily from human activities and considered as useless such as leftovers, papers, woods, packages, yard waste, clothes, glasses, equipment, electronics, batteries, chemicals etc. (Ekmekçioğlu et al., 2010; Farrell and Jones, 2009). These materials have detrimental health and environmental impacts especially when they come across the living being or environment through air, soil, water or sewage systems (Arıkan et al., 2017). The commercial, medical, and industrial hazardous or radioactive wastes are often not included in MSW and a separate treatment option is must for them (Farrell and Jones, 2009). Waste production is an inevitable consequence of today's life and the waste generation rate is in increasing trend around the world to meet the increasing demand of rapid growing population, industrialization and urbanization. Approximately 1.3 billion tons of solid waste was generated in 2012, which is expected to rise of 70% more in 2025 of about 2.2 billion tons (The World Bank, 2017) and the estimated forecast indicated that waste generation rate would not reach the peak in this century (Hoornweg and Bhada-Tata, 2012). Therefore, proper disposal of waste could be a very crucial and possible positive step to minimize the detrimental impacts.

Engineered landfilling is still considered as the most economic and environmental friendly MSW disposal method (Tengrui et al., 2007). Many of the landfilling site around the world are not engineeringly constructed especially in the under developed and developing countries without having any provision of liner, top cover, liquid (leachate) and biogas collection system (Naveen et al., 2017). Regardless of the construction process of the landfilling site, landfill leachate (LL) is the unavoidable byproduct from the landfill due to the microbial degradation of waste along with the water content of incoming waste, percolated precipitation and ground water accumulation

(Alslaibi et al., 2010; Blight and Fourie, 2005). The waste of the landfill undergoes at least four major evolutionary phase such as aerobic hydrolysis, anaerobic acetogenic, unstable methanogenic, and stable methanogenic (stabilization) phases based on the duration of the waste in the landfill cell (Bove et al., 2015). Thus, the landfill age has a significant effect on the characteristics of the LL contaminants based on the level and duration of waste stabilization (Baig et al., 1999).

1.2. Contaminants and toxicity of landfill leachate

Although, landfill age plays a vital role on LL characteristics, there are many other important factors which also affect the LL characteristics such as composition of incoming waste, level of pre-treatment of the waste, recirculation of leachate to the waste, seasonal weather variation, and regional climatic condition (Blight and Fourie, 2005; Mohammad-pajooh et al., 2017).

LL is classified into three distinct category based on the composition of leachate and age of the landfill such as young (0-5 years), intermediate (5-10 years), and mature leachate (>10 years) (Ahmed and Lan, 2012; Foo and Hameed, 2009; Renou et al., 2008). Young LL contains higher proportion of biodegradable contaminants (> 80% volatile fatty acids) having higher biochemical and chemical oxygen demand ratio (BOD_5/COD > 0.5 and COD >10,000 mg/L), lower ammonia (NH_4^+–N < 400 mg/L) concentration and pH < 6.5. Higher level of microbial stabilization occurs with time which leads to lower biodegradable portion and eventually reduces BOD_5/COD ratio in intermediate leachate and continues to further lowering in mature leachate. The contaminants ranged in intermediate leachate 4,000–10,000 mg/L of COD, 0.1–0.5 of BOD_5/COD ratio, 20%–30% of volatile fatty acid (VFA), and 6.5–7.5 of pH (Ahmed and Lan, 2012). The mature landfill leachate (MLL) become highly contaminated due to the very lower biodegradable proportion

having BOD_5/COD ratio < 0.1, COD < 4,000 mg/L, higher ammonia (NH_4^+–N < 400 mg/L), and pH (>7.5) (Ahmed and Lan, 2012).

MLL is considered as one of the highly polluted toxic wastewaters due to the higher fraction of recalcitrant compounds such as higher concentration of heavy metals, organic and inorganic contaminants, and humic and fulvic substances. The waste and LL go through a series of interrelated concurrent biological, chemical and physical processes over the time in the landfill and lead to formation of humic-like-substance which are non-biodegradable anionic macromolecules and become predominant components of the leachate (> 70% of the total organics) (Qin et al., 2017). These contaminants have serious health and environmental impact and are responsible for contamination of the surrounding surface water, ground water, soil and biota (Kjeldsen et al., 2002). Ghosh et al., (2017) assessed the toxicological risk of landfill leachate on different trophic organisms and revealed the mutagenic effects and cytogenetically damages of organism cell due to the presence of wide range of toxic xenobiotic compounds. Cheng et al., (2017) evaluated the genotoxic potential of MLL on human hepatoma cells and explored high cytotoxicity (97.1%) after 24 h of exposure period. Higher concentrations of leachate have genotoxic and mutagenic effect on plant with cytogenetic alterations of biomass cells and also have physiological effect on seed germination, thus the cytological analysis demonstrates the persistent of ecological risk of leachate (Li et al., 2017; Morozesk et al., 2017).

1.3. Methods of landfill leachate treatment

LL treatment gets significant attention around the world due to the environmental concern raised by social, political, and governmental agencies and straighten regulations have been introduced in some developed countries like Canada incorporating regular monitoring of the surface and ground water near the landfill location with leachate collection, containment, treatment and proper

disposal options (MSD, 2016). Conventional and advanced technological approaches have been employed over the years to develop cost effective safe, secure and efficient sustainable treatment options of landfill leachate. Although, some of the treatment methods have been reported as efficient enough to remediate young LL, but satisfactory treatment of leachate is not an easy task especially for mature leachate and therefore, developing sustainable leachate treatment options have been considered a hot-topic for the researchers. Schiopu and Gavrilescu, (2010) classified leachate treatment methods into three main categories as: 1) On-site treatment. 2) Disposal to sewerage systems, and 3) Off-site treatment. More than one treatment options could also be incorporated in sequential manner which is discussed as the 4) sequential treatment option along with the aforementioned methods.

1.3.1. On-site leachate treatment

The most economic and easily manageable on-site leachate treatment option is recycling back to the landfill through the tip which has been frequently used during the past decades. Leachate recirculation increases the moisture content, a vital factor for microorganisms' activity, of the landfill cell and facilitates uniform distribution of nutrients and enzymes among microbial communities, wastes and liquids. Leachate recirculation improves the leachate quality through dropping of COD from further biodegradation and reduces stabilization time and methane production (Renou et al., 2008; Rodríguez et al., 2004). Recycling of leachate is advantageous providing recirculation and distribution of methanogens, better pH buffering capacity and dilution of inhibitory compounds (Chung et al., 2015; Xu et al., 2014).

Long term and high leachate recirculation rate have some adverse effect such as occurrence of higher organic acid concentration (pH < 5) results toxicity to methanogens and inhibit methanogenesis (Ledakowicz and Kaczorek, 2004). MLL contains higher concentration of

ammonium and leachate recirculation leads to further ammonium accumulation in landfill, which is also toxic for microorganisms and inhibits the anaerobic biodegradation process (Chung et al., 2015). Reduction of COD and accumulation of ammonium of leachate causes lowering of carbon/nitrogen (C/N) ratios and toughens nitrogen removal process due to insufficient electron donor (Trabelsi et al., 2000). The removal of nitrogen from the leachate before recirculation back to landfill is advantageous for enhanced landfill stabilization process. Youcai et al., (2002) evaluated the in situ aged-refuse (8–10 years old waste) based biofilter with anaerobic and aerobic sections to remove nitrogen from the leachate before recirculation and achieved 99.5% of ammonium removal. In situ bioreactor landfill and aerobic/anoxic/anaerobic bioreactor systems have been evaluated and reported as effective methods to manage nitrogen levels in the leachate (He et al., 2007; He and Shen, 2006).

Mæhlum, (1995) evaluated the on-site LL treatment efficiency using lagoons and constructed wetlands and obtained 70-95% of nitrogen, phosphorus, and pathogen removal efficiency, and concluded the promising utilization of conventional (lagoons) and natural systems (wetland) for on-site leachate treatment.

1.3.2. Disposal to sewerage systems: Co-treatment of leachate with domestic wastewater

The most common LL treatment practice is discharging to sewerage systems to treat in the municipal wastewater treatment plants (WWTPs) because of low operation cost and easy maintenance. Co-treatment of leachate with sewer in WWTPs is widely used in many European countries such as Ireland and Poland (Brennan et al., 2016), while some other EU countries are switching their practice due to the ineffectiveness of the conventional WWTPs to remove non-biodegradable organic inhibitory contaminants and heavy metals from the leachate. For instance, about 20% LL was co-treated in WWTPs in France and Germany in 2002 (Renou et al., 2008).

Many studies reported blending of leachate to the influent of WWTPs have significant negative effect to the effluent quality, interfere with disinfection process, decrease the nitrification process, increase the aeration consumption, and occurs less or partial biodegradation of hardly biodegradable contaminants (Brennan et al., 2017; Ferraz et al., 2014; Kalka, 2012). The co-treatment of leachate with domestic wastewater is mainly associated with activated sludge process and removes 80% – 92% of influent COD with less than 10% v/v leachate dose to minimize the high ammonia and refractory contaminants effect (Ferraz et al., 2014).

There is a lack of publicly available data about the LL treatment methods used around the world. There could be two possible reasons: landfills owner keeps this commercially sensitive data confidential as many of them are privately owned, and there is dearth of regulatory requirement for landfill owner to report them.

1.3.3. Off-site leachate treatment

The off-site treatment includes various physical, chemical and biological process (could be a combination of more than one processes) where leachate is treated in a separate facility. The off-site leachate treatment processes can be further classified based on the utilization of the type of technological approach.

1.3.3.1. Physicochemical treatment

The effectiveness of leachate treatment options has been evaluated through a wide range of physicochemical treatment methods over the several decades. The technological approaches include air (or ammonia) stripping, coagulation–flocculation, absorption–adsorption, membrane filtration, precipitation, ion-exchange, and chemical and electrochemical oxidation (Kumarathilaka et al., 2017).

Air stripping is effective to remove ammonia (> 80%) from the leachate and further treatment is necessary for the organic and inorganic contaminant removal (Ferraz et al., 2013).

Coagulation-flocculation process is used in pretreatment or finial polishing option to separate suspended particles from leachate. Various substances such as alum, aluminum sulfate, ferric chloride, poly-aluminum chloride, and seed starch are used as coagulants/flocculants and significant amount of COD removal (> 50%) has been reported (Li et al., 2010; Yusoff et al., 2018).

Zeolite, activated carbon, combination of zeolite-carbon, and biochar seem to be more effective as the adsorption media to remediate LL among the variety of tested materials (i.e. illite, vermiculite, biochar, and incinerator bottom ash) (Halim et al., 2010; Shehzad et al., 2016).

Pressure-driven membrane filtration includes reverse osmosis, microfiltration, nanofiltration, and ultrafiltration process which separate the influent to cleaner solution passing through the filter (permeate) and left behind concentrated contaminant solution (retentate). High energy input, limited membrane lifetime, and fouling problem limit the process, although small footprint and automatic operation make them user friendly (Peng, 2017).

Precipitation of non-biodegradable organic compounds, ammonium and heavy metals is occurred through chemical reaction by controlling pH and molar ratio of ions, for instance, struvite (MAP: magnesium ammonium phosphate, $MgNH_4PO_4 \cdot 6H_2O$) precipitation from landfill leachate to remove ammonia (Huang et al., 2015). Though nutrients recovery occurs from struvite precipitation, addition of Mg and P is required for optimum stochiometric molar ratio.

Ion exchange treatment of landfill leachate is typically employed as the polishing post-treatment option to remove ammonia nitrogen and heavy metals from leachate (Bashir et al., 2010; Zamri et al., 2017).

Chemical oxidation is employed with oxidants such as ozone (O_3), chloride (Cl^-), hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), and permanganate (MnO_4^+) to oxidize contaminants and pathogens from the leachate (Gao et al., 2015). A combination of higher oxidative compounds is used to generate highly reactive oxidizing agents (i.e. hydroxyl radicals: ·OH) from advanced oxidation process (AOP) such as ultraviolet ray (UV)/O_3, O_3/H_2O_2, H_2O_2/UV, Fenton (H_2O_2/Fe^{2+}), titanium dioxide (TiO_2)/UV, ultrasound (US)/O_3, solar/O_3, US/H_2O_2, and persulfate ($S_2O_8^{2+}$)/H_2O_2; whereas electrochemical advanced oxidation processes (EAOPs) further utilize electrical potential such as electro-Fenton (EF), photo-electro-Fenton (PEF), and solar PEF (SPEF) (Asaithambi et al., 2017; Chemlal et al., 2014; Hilles et al., 2016; Moreira et al., 2016; Poblete et al., 2017). Most of the chemical treatment produce some byproducts which might cause secondary contamination which can be minimized by a combination of other treatment options (Del Moro et al., 2016).

1.3.3.2. Biological treatment

Biological treatment utilizes microorganisms such as bacteria and fungi, which consumes organic compounds from the leachate as their food and degrades to CO_2 and produce sludge (mainly microorganisms) or biogas. Utilization of diverse bacterial community is known as activated sludge process. Biological treatment is considered as one of the sustainable treatment options with minimal secondary contamination effect and has been reported to be very efficient process to remove contaminants from young leachate, although the results of sole biological treatment of mature landfill leachate is not satisfactory (Renou et al., 2008). Based on the provision of aeration, biological treatment process could be aerobic or anaerobic condition.

1.3.3.2.1. Aerobic treatment

Aerobic treatment occurs with the provision of aeration to the systems either by diffuse bubble or surface aerator and allows biological degradation of organic pollutants along with ammonium

nitrogen nitrification. The aerobic treatment of LL is widely investigated applying the technological approaches of aerated lagoon, conventional activated sludge, aerobic granular sludge, and sequencing batch reactor (SBR) in suspended growth, while rotating biological contactor (RBC), moving-bed biofilm reactor (MBBR), suspended carrier film reactor, trickling bed/filter reactor, biofilter reactor, and membrane bioreactor (MBR) are based on attach-growth of biomass (Gao et al., 2015; Renou et al., 2008; Schiopu and Gavrilescu, 2010). Some previous studies reported more than 90% of ammonia nitrogen removal efficiency by aerobic treatment process regardless of the type of leachate due to the higher activity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), although recalcitrant compounds removal efficiency from MLL is limited in many cases (Gao et al., 2015; Jokela et al., 2002). Excess sludge production, low sludge settle-ability, longer aeration time and high energy consumption are some of the challenges associated with aerobic treatment process.

1.3.3.2.2. Anaerobic treatment

Anaerobic treatment is considered as the microbial degradation of leachate in absence of oxygen (aeration), where leachate is commonly co-treated with the sludge in anaerobic digestion process. In contrast to aerobic process, anaerobic digestion process produces less sludge and produces methane (CH_4) in addition of CO_2, which is considered as biogas and could be utilized as the energy source to warm up the digester in the mesophilic condition (35°C). The addition of nutrient rich leachate in anaerobic digester improves the nutrient imbalance condition and enhances synergistic effects of microorganisms by diluting the toxic elements of sludge (Xu et al., 2018). Increased rate of biogas production has been reported with limited dose of young or intermediate leachate (up to 12% v/v) in anaerobic digestion process and demonstrated no significant negative effect (Guven et al., 2018; Hombach et al., 2003; Montusiewicz and Lebiocka, 2011). Another recent study, Berenjkar et al., (2018) investigated anaerobic co-digestion of sewage sludge and

MLL by and demonstrated higher biogas production and ammonia removal rate with 5% - 20% MLL dose compared to control, although percentage of COD reduction was decreased, which suggests the interference of the MLL contaminants with the functional microorganisms.

1.3.3.2.3. Phytoremediation of leachate

Phytoremediation is carried out utilizing plants to remove, accumulate, degrade, metabolize or stabilize substances from leachate. Phytoremediation of LL is widely investigated due to inexpensive, safe and naturally occurring environmental friendly process (Jerez Ch. and Romero, 2016; Yan et al., 2016). A wide range of plant species such as cattail, reed, rush, sweet flag, yellow flag, manna grass, willow, poplar, stiff goldenrod, soft rush, arrow arum, pickerel weed, lizard's tail, soft stem bulrush, and burreed are efficient enough to remediate ammonia, COD, and heavy metals of leachate (Kumarathilaka et al., 2017). Constructed wetland based phytoremediation process seem to be most effective and achieved 63% - 95% of COD removal and > 90% of heavy metals, ammonia and phosphorus removal efficiency (Chiemchaisri et al., 2009; Kadlec and Zmarthie, 2010; Lavrova and Koumanova, 2010; Yalcuk and Ugurlu, 2009).

In addition to the inexpensive treatment option, phytoremediation process can exclude leachate transportation cost through on-site remedial facility (Granley and Truong, 2012), nearly zero net discharge of leachate can be achieved through recycling of leachate back to the plantation area (Dimitriou et al., 2006), and the short-rotation coppice management practice can yield higher dry matter production which could be utilized in heat and electricity generation (Kumarathilaka et al., 2017). In spite of the positive aspects of LL phytoremediation, evidence showed that LL has detrimental human health effect and phytotoxic outcomes, which causes leaf damage and premature senescence, yields less biomass, and reduces survival rate (Dimitriou et al., 2006).

1.3.3.2.4. Fungal treatment

Myco-remediation or fungal bioremediation seems to be promising in many ways over the conventional biological treatment process (activated sludge) due to the ligninolytic enzymatic activities of certain fungal strains such as Basidiomycetes white rot fungi (WRF). WRF fungi are well known for the secretion of lignocellulosic enzymes (lignin, hemicellulose, and cellulose degrading enzymes) such as laccase (Lac), lignin peroxidase (LiP), versatile peroxidases (VP), and manganese peroxidase (MnP), which are capable to breakdown the complex structure of recalcitrant contaminants and thus enhances biodegradation (Goltapeh et al., 2013; Sardrood et al., 2013). Biological fungal treatment of LL has recently been investigated to remove the refractory recalcitrant compounds (Ren and Yuan, 2015), and several studies reported effective removal of recalcitrant compounds from the MLL, unlikely for conventional biological treatment, due to the significant fungal enzymatic activities (Bardi et al., 2017; Kalčíková et al., 2014). Fungal treatment is also effective to reduce heavy metal concentration from the leachate through biosorption or bio-accumulation process, for instance, *Pleurotus ostreatus* and *Galerina vittiformis* mushrooms can absorb a wide range of heavy metal contaminants such as Cd, Pb, Hg, Fe, Zn, Cu, Cr, and Ni (Damodaran et al., 2014; Vaverková et al., 2018).

Leachate has been treated deploying different strains of white rot fungi (*Dichomitus squalens, Phanerochaete chrysosporium, Trametes trogii, Lentinus tigrinus, Porostereum spadiceum, Ganoderma austral, Pleurotus ostreatus)* and yeast or filamentous fungi (*Aspergillus niger, Saccharomyces cerevisiae*) species and in all cased the COD reduction ranged 60% - 90% and color removal ranged 50% - 80% (Brito et al., 2012; Ellouze et al., 2008; Tigini et al., 2013). State of art technological approach has also been incorporated to obtain best performance of fungal bioremediation through submerged microfiltration module membrane bioreactor (Brito et al., 2012), suspended growth of sequencing batch bioreactor (Ghosh et al., 2014), Ecomat-

immobilized fungus packed in a glass column (Abdullah et al., 2013), fixed bed biofilm bioreactor filled with cinder (Hang et al., 2004), and immobilized fungus in polyurethane foam cubes with rotating packed bed fungal bioreactor (Bardi et al., 2017).

The extracellular enzyme secretion is enhanced in the limiting nutrient condition such as at lower concentration of carbon, nitrogen, phosphorus, and sulfur (Ren and Yuan, 2015). MLL contains higher concentration of ammonia nitrogen and a pretreatment option to reduce ammonia concentration could be beneficial to achieve better fungal bioremediation performance. Kim et al., (2003) utilized zeolite filters (natural zeolite Clinoptilolite) as the pretreatment option and obtained higher COD removal (65%) efficiency with reduced ammonia concentration from *Phanerochaete chrysosporium* strain and concluded high C/N ratio is favorable for fungal treatment.

1.3.4. Sequential treatment

Achieving satisfactory level of contaminants removal efficiency from LL, especially form MLL using a single treatment option is very difficult due to the presence of higher proportion of refractory and hardly biodegradable contaminants in LL. Multistage sequential treatment options, a combination of several physicochemical and/or biological treatment process, has significantly enhanced the contaminants removal efficiency to a several folds such as Moreira et al., (2015) incorporated a multistage treatment strategy in combination with bio-process (activated sludge SBR) /coagulation/EAOP/bio-process and successfully eliminated the organic matter and nitrogen contaminants. A wide range of sequential treatment options has been reported to be very effective (> 90% removal of COD and ammonia) to treat MLL such as up-flow anaerobic sludge blanket (UASB)/Reverse osmosis (RO), activated sludge/RO, Fenton/activated sludge, coagulation/electro-Fenton/SBR (Gao et al., 2015), air stripping/Fenton/SBR/coagulation (Guo et al., 2010), partial nitritation sequencing batch reactor/anammox reactor/photo-Fenton (Anfruns et

al., 2013), and sequencing batch biofilter granular reactor/photo-Fenton (or ozone) (Cassano et al., 2011).

1.4. Objectives and hypothesis

This research was conducted in three experimental studies with the following overall objective: to remove organic and inorganic contaminants from the mature landfill leachate (MLL) utilizing fungal and activated sludge biological treatments. The specific objectives were as follows:

Study I examined the effect of glucose, woodchips and wheat-straws as co-substrates on contaminants (COD and sCOD) removal efficiencies by *Trichoderma asperellum, Cladosporium* sp.*, and Tyromyces chioneus* strains. The secondary objectives were to evaluate contaminants removal efficiency between the suspended fungal biomass treatments and immobilized in polyurethane foam (PUF) cubes and to study the solubilization of woodchips and wheat-straws.

Study II evaluated the effect of fungal lignocellulosic enzymatic activities (Lac: laccase, LiP: lignin-peroxidase, and MnP: manganese-peroxidase) secreted from six selected fungi: *T. asperellum, Cladosporium* sp., *Penicillium* sp., *T. chioneus, Bjerkandera adusta and Phanerochaete chrysosporium)* on contaminants removal efficiencies of MLL. Additionally, three newly isolated fungi from MLL were identified through PCR sequencing, and a relationship was established between the COD/sCOD removal efficiencies and their corresponding ligninolytic enzymatic activities.

Study III investigated the contaminated removal efficiency from MLL through the sequential treatment of aerobic activated SBR and rotating packed bed continuous fungal bioreactor utilizing the most effective fungal strain obtained from the previous study (*Penicillium* sp.). Furthermore, enzymatic activity in fungal bioreactor was examined, and the effect of initial ammonia nitrogen concentration of leachate on contaminant removal efficiencies was explored.

Hypotheses of this research are as follows:

- In study I, the hypothesis was that co-substrate addition could enhance fungal growth and thereby affect the contaminant removal efficiency from MLL.
- In study II, it was hypothesized that increased fungal lignocellulosic enzymatic activity could contribute to enhance contaminants removal efficiency.
- In study III, the hypothesis was that the sequential biological treatment of MLL through SBR and fungal bioreactor could achieve long term enhanced contaminants removal efficiency.

1.5. Organization of this thesis

This thesis was prepared in manuscript style following the guideline of the Department of Civil Engineering and Faculty of Graduate Studies, University of Manitoba. This consists of seven chapters including introduction (Chapter 1), three stand-alone specific research chapters (Chapter 2, 3 and 4), summary and conclusions (Chapter 5), engineering significance including importance of this research and implications (Chapter 6), and recommendations for future studies (Chapter 7). Chapter two discussed treatment of mature landfill leachate with three selected fungi utilizing glucose, woodchips and wheat-straws as co-substrates. Chapter three investigated the effect of fungal lignocellulosic enzymes from six selected fungi on the contaminants removal efficiency of mature landfill leachate. Chapter four examined the advanced biological sequential treatment options of mature landfill leachate using aerobic activated sludge SBR and fungal bioreactor utilizing the most effective fungal strain obtained from previous studies.

Chapter 2: Fungal Treatment of Mature Landfill Leachate Utilizing Woodchips and Wheat-straws as Co-substrates

2.1. Introduction

Pure water, clean air, and uncontaminated soils are the most important natural resources for the sustainable environment, which are being severely contaminated by the waste. Daily human activities typically generate waste, and the process is accelerated due to the global population growth, increasing affluent lifestyles, continuous urbanization, and industrial development. World Bank reported that global solid-waste generation would be almost double (more than 6 million tons per day) in 2025 as compared to 2010 (3.5 million tons per day) whilst the global waste generation forecast estimated that it will not reach its peak in this century (Hoornweg and Bhada-Tata, 2012). Generation of waste is much faster than the world's population growth that poses a major environmental, social, and economical challenge for municipal solid wastes (MSW) management (Renou et al., 2008).

The most common, environmental friendly, and economical method of MSW disposal is engineered landfill (Tengrui et al., 2007). The microbial degradation of waste causes landfill leachate (LL) production from the water content of incoming waste (Blight and Fourie, 2005) along with the percolated precipitation and ground water accumulation (Alslaibi et al., 2010). LL characteristics depend on many important factors like the age and type of landfill waste composition (Baig et al., 1999; Trebouet et al., 2001), and pre-treatment level of waste (Mohammad-pajooh et al., 2017). Recirculation of leachate to the waste, seasonal weather variation and regional climatic condition also affect LL characteristics (Blight and Fourie, 2005; Qasim and Chiang, 1994).

LL has been categorized into young (0-5 years), intermediate (5-10 years), and mature leachate (>10 years) based on the landfill age and the leachate composition (Foo and Hameed, 2009; Renou et al., 2008). Young LL is characterized by a high biodegradable fraction of total contaminants. It contains more than 80% biodegradable volatile fatty acids (VFAs), higher ratio between biochemical and chemical oxygen demand (BOD_5/COD > 0.5), low to moderate ammonia concentration (NH_3-N < 400 mg/L), and pH < 6.5 (Foo and Hameed, 2009). The biodegradable portion is decomposed with time due to the stabilization process of microbial activity while the non-biodegradable fraction mostly remains unchanged, which decreases BOD_5/COD ratio (< 0.1) and increases pH (>7.5) in the mature landfill leachate (MLL) (Ahmed and Lan, 2012). MLL is considered as one of the most problematic wastewaters due to high concentration of ammonia nitrogen (NH_4^+–N > 400 mg/L) (Schiopu and Gavrilescu, 2010) and higher fraction of recalcitrant components (Foo and Hameed, 2009). These are mostly toxic and are not easily biodegradable, like humic and falvic substances, xenobiotic compounds, i.e. pharmaceutical and personal care products (PPCPs), polycyclic aromatic hydrocarbons (PAHs), fertilizers, pesticides, etc., and heavy metals such as copper, cadmium, nickel, chromium, lead, zinc, etc. (Hilles et al., 2016; Kjeldsen et al., 2002; Moreira et al., 2015). Fungal bioremediation approaches have been widely investigated in recent decades due to their lignocellulosic enzymatic activities, such as laccase (Lac), lignin-peroxidase (LiP), versatile-peroxidases (VP), and manganese-peroxidase (MnP), to remediate the recalcitrant components of the problematic wastewaters (Bardi et al., 2017; Retes-Pruneda et al., 2014).

The treatment efficiency of MLL has been investigated in this study by utilizing *Trichoderma asperellum*, *Cladosporium* sp., and *Tyromyces chioneus* fungal strains under non-sterile conditions through flask-level batch experimental tests. The ammonium-nitrogen (NH_4^+–N) concentration of

MLL was reduced (< 350 mg/L) to minimize the toxicity (Ellouze et al., 2009) before the batch experiment through aerobic pretreatment in a sequencing batch reactor (SBR).

The objectives of this study were: (1) to compare the COD and soluble COD (sCOD) removal efficiencies between the immobilized fungal biomass in polyurethane foam (PUF) cubes and suspended treatments, (2) to study the solubilization of the slowly soluble co-substrates, woodchips (WC) and wheat-straws (WS), and (3) to investigate the effect of glucose (GL), WC, and WS as co-substrates toward COD/sCOD removal as well as to examine the extracellular enzymatic activities (Lac, LiP, and MnP).

2.2. Materials and methods

2.2.1. Analytical procedures

The pH was measured by using a pH meter. The BOD_5 were determined following the Standard Methods for Examination of Water and Wastewater (SMEW, 18th Edition) (APHA, 1998a). COD was measured from unfiltered samples by using a spectrophotometer (DR2800, HACH Ltd., London, ON, Canada) according to the standard dichromate methods (APHA, 1998a). Soluble COD (sCOD) was quantified by the COD of filtered samples using 1–5 µm plain filter paper (Qualitative Grade P2, Fisher Scientific Company, Ottawa, ON, Canada). Flow inject analyzer (FIA) (QuikChem 8500, LACHAT Instruments, Loveland, CO, USA) was used to determine the concentration of ammonia (NH_4^+–N), nitrate nitrogen (NO_3^-–N), and nitrite nitrogen (NO_2^-–N).

2.2.2. Characterization of MLL

MLL was collected from the Brady Road Resource Management Facility, Winnipeg, Canada (Well No. # 24) and stored at 4°C. The landfill has been functional since 1973 and accepts both residential and commercial wastes. MLL was always collected from the same well and physicochemical characterization was carried out before each test (Table 2.1). The raw MLL was

aerobically pretreated with an activated sludge operated SBR to minimize the NH_4^+–N content, which was used as the feed for the batch experimental tests. The MLL was also characterized after the aerobic treatment (Table 2.1).

Table 2.1. Physicochemical parameters of raw and aerobically treated MLL.

Parameters	Raw leachate	After aerobic treatment
pH	8.5 ± 0.5	7.2 ± 0.4
BOD_5 (mg/L)	155 ± 16	132 ± 14
COD (mg/L)	1827 ± 78	1495± 93
sCOD (mg/L)	1695 ± 26	1431± 39
NH_4^+–N (mg/L)	872 ± 25	0 - 350 ± 22
BOD_5/COD	0.085 ± 0.01	0.088 ± 0.01

Note: Parameters are presented as average values with standard deviations.

2.2.3. Collection and sub-culture of fungal strains

The fungal strains (*Cladosporium* sp., *T. asperellum*, and *T. chioneus*) were locally isolated from MLL. All the fungal strains were periodically cultured and inoculated in 90-mm Petri dishes on MEA (Malt Extract Agar, containing 2 g/L peptone and 20 g each of malt extract, agar, and glucose) media at 25°C for one week in the dark and stored at 4°C. Selected strains were cut into small pieces (about 0.1×0.1 cm^2) with a sterile scalpel from the outer part of the colony and blended with sterile saline (9 g/L sodium chloride solution) under aseptic conditions to homogenize fungal strains whilst maintaining the ratio of 1 cm^2 fungal colony per mL of sterile saline. For the suspended growth of fungal strains, homogenized solution was inoculated into 2 L flasks containing sterilized GLY broth (glucose and yeast extract of 5.0 and 1.9 g/L, respectively) at the rate of 90 mL homogenate per liter solution. Sterilized PUF cubes were added into the homogenized GLY solution at the rate of 1.2 mL homogenate per cube (3 pieces of PUF cubes per 40 mL of solution) for the immobilized growth of fungal strains. Flasks were incubated for one

week at 150 rpm in a shaker agitator at 23±3°C. After complete fungal growth, suspended and immobilized fungal strains were used for the batch experimental tests. The fungal strains were separated from the broth by using sterile sieve and residual GLY was removed by washing with sterile deionized water.

2.2.4. Batch experimental tests

Several batch tests were carried out following the same procedure and environmental conditions to evaluate the performance of different fungal strains in suspended and immobilized state to treat MLL in terms of COD and sCOD removal efficiencies and lignocellulosic enzymatic activities (Lac, LiP, and MnP) by using GL, WC, and WS as co-substrates. The WC was collected from municipal trimmings of forest residue containing a mixture of tops, branches, roots, and barks. The WS was wheat harvested residue and collected from the local field. The pH of MLL was adjusted to 4.5 for all trials before the experiment with 10% sulfuric acid (H_2SO_4) and co-substrates were added at the rate of 0.2 g/L of GL (approximately 213 mg/L COD load) and 5 g/L each of WC and WS. Four immobilized cubes or proportionate amount (based on wet weight) of suspended fungal strains were placed in 500 mL flasks containing 100 mL of effluent for each treatment and trials were triplicated including control (without fungal inoculum) treatment. Flasks were placed on a shaker at 150 rpm for 288 hours (h) at 23±3°C. Leachate samples were collected before the treatment preparation, immediately after the preparation of trials, and after 24, 48, 72, 96, 144, 168, 192, 240, and 288 h for analyzing enzymatic activities and COD/sCOD removal efficiencies.

2.2.5. Determination of ligninolytic enzymes

Enzymatic activities (Lac, MnP, and LiP) were determined at 25°C by using a multi-detection microplate reader spectrophotometer (Synergy 4, BioTek Instruments Inc., Winooski, VT, USA). Lac activity was measured according to the method devised by Bourbonnais and Paice, (1990).

Briefly, the readings were taken at 420 nm in sodium citrate buffer (100 mM) at pH 3 from the oxidation of ABTS (2,2'-azino-bis-(3-ethylbenozthiazoline-6-sulfonic acid) (coefficient 36000 M^{-1} cm^{-1}). MnP assay was based on the oxidation of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) and DMAB (3-dimethylaminobenzoic acid) that was initiated by adding 4 mM H_2O_2 solution (Vyas et al., 1994). The readings were taken at 590 nm (coefficient 32900 M^{-1} cm^{-1}) using the reaction mixture of 100 mM succinate lactate buffer (pH 4.5), 25 mM DMAB, 1 mM MBTH, and 4 mM $MnSO_4$. LiP activity was approximated at 310 nm according to Tien and Kirk, (1984) that is based on the oxidation of veratryl alcohol (3,4-Dimethoxybenzyl alcohol) (coefficient 9300 M^{-1} cm^{-1}) in the reaction mixture of 100 nM sodium tartrate buffer (pH 3) and 0.4 mM H_2O_2.

2.2.6. Statistical analysis

SAS software for Windows 10 (SAS 9.4, SAS Institute Inc., Toronto, ON, Canada) was used for the statistical analysis. Possible outliers for all dataset were examined prior to each analysis.

2.2.6.1. Analysis of variance (ANOVA) and repeated measures analysis

PROC GLIMMIX procedure was used for the ANOVA tests and repeated measures analysis ($p < 0.05$). Repeated measures analysis was used to evaluate the effect of suspended and PUF immobilized biomass (3-way factorial), solubilization of co-substrates (2-way), effect of co-substrates on COD/sCOD removal efficiencies (2-way), and to analyze the enzymatic activities (2-way). Inoculum time was considered as the random variable and covariance structures were examined for all repeated measures data. The minimum values of Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and Generalized Chi-Square were utilized to choose the covariance type.

Two-way ANOVA (Co-substrates: NO, GL, WC, and WS; Treatments: *Cladosporium* sp., *T. asperellum*, and *T. chioneus*) for the multiple means comparison tests were used to examine the

effect of co-substrates on sCOD removal efficiency after 24 and 288 h of inoculum time. All data sets were analyzed for homogeneity of variance. Residual normal distribution ($W > 0.9$ and $p > 0.05$) was confirmed by the Gaussian distribution of the residuals with the Shapiro-Wilk test for normality. Satterthwaite denominator degrees of freedom (DDF) method and Tukey's tests were considered for the least squares means adjustment.

2.2.6.2. Partial least square (PLS) regression

The COD/sCOD of the co-substrates were primarily plotted against the treatment duration to understand the solubilization curve pattern, which revealed a time-dependent polynomial functional curve (equation 2.1).

$$y_i = \alpha_0 + \alpha_1 x_i + \alpha_2 {x_i}^2 + \ldots\ldots + \alpha_n {x_i}^n + \varepsilon_i \qquad \text{for}, i = 1, 2, 3, \ldots\ldots, n \tag{2.1}$$

Where, y_i = dependent variable (amount of co-substrate COD/sCOD), α = regression coefficient, x_i = independent variable (inoculum time), and n=degree of polynomial term.

PLS regression study was performed to obtain the best fit curve. PROC PLS procedure was used to identify quantitative relationships between the inoculum time and contribution of co-substrate's COD/sCOD to MLL. Five degrees of polynomial terms (simple linear, quadratic, cubic, quartic, and quintic) for inoculum time were considered as the predictor variables in the preliminary model. The cross validation method (CV = testset()) was used to select the number of PLS factors. Polynomial terms of inoculum time which had significant effect on the co-substrate COD/sCOD contribution (Variable Importance in Projection: VIP > 0.8) were selected for the reduced model and the least impact to the model was observed.

2.3. Results and discussion

2.3.1. Comparison of suspended and PUF immobilized biomass

The effect of suspended and PUF immobilized fungal treatments with respect to inoculum time for two different experimental conditions (presence and absence of GL as a co-substrate) on the performance of *Cladosporium* sp., *T. asperellum*, and *T. chioneus* to remove sCOD from the MLL is shown in Figure 2.1. The removal efficiencies of COD and sCOD showed similar patterns over the experimental period. Therefore, only the percentages of sCOD removal data is presented here.

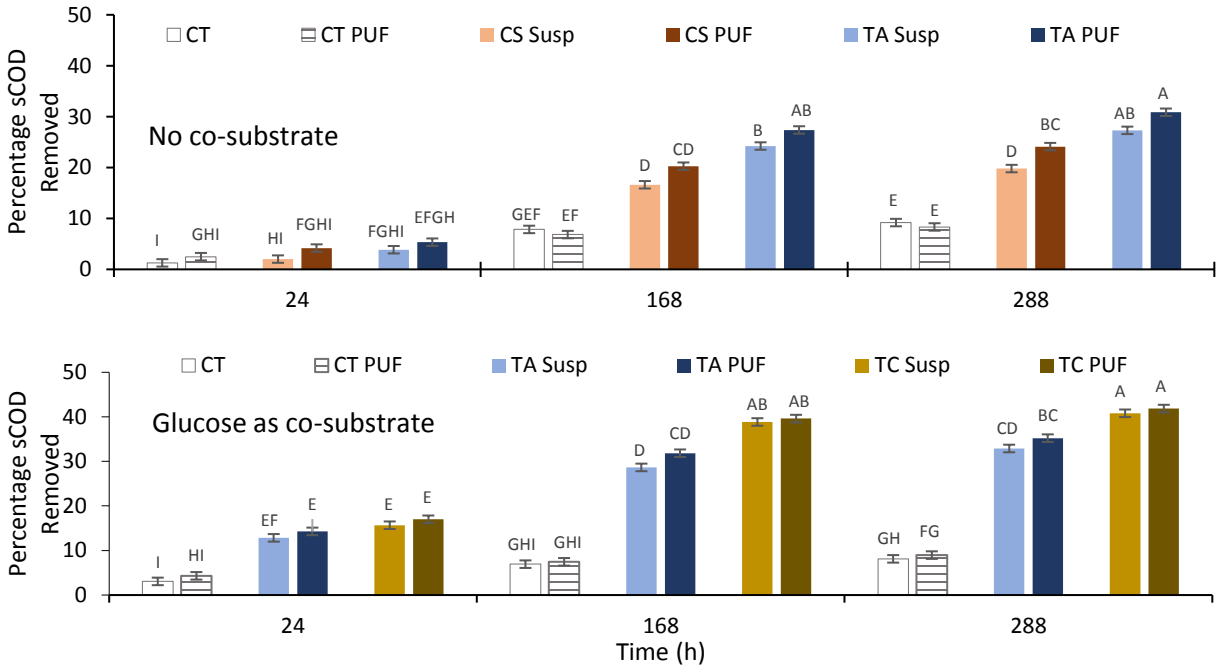

Figure 2.1. Soluble COD removal efficiencies in suspended and PUF immobilized conditions (a) without co-substrate and (b) with glucose as co-substrate.

Note: Three-way factorial design (Growth conditions: Susp and PUF; Treatments: CT, CS, and TA; Inoculum time: 24, 168, and 288 h). Sharing same letter between the bars represents no statistical significance at $p < 0.05$. The error bar represents the standard error.
CT = Control (without biomass and co-substrate), Susp = Suspended biomass, PUF = Immobilized biomass into PUF, CS = *Cladosporium* sp., TA = *Trichoderma asperellum*, TC = *Tyromyces chioneus*.

Although the PUF immobilized treatments numerically achieved higher percentage of sCOD removal efficiency as compared to the suspended treatments; however, there was no significant difference ($p < 0.05$) between them after 24, 168, and 288 h, except for the *Cladosporium* sp. at

288 h (Figure 2.1). Therefore, in terms of MLL treatment efficiency, the performance of free cell culture (suspended biomass) is equivalent to the performance of immobilized culture. In a different study, the fungal growth pattern in suspended mycelia was reported to be similar to the PUF immobilized culture (Saetang and Babel, 2012). Additionally, the immobilized fungal culture enhances the biosynthetic capacity and stability of the biomass growth (Ehlers and Rose, 2005). It also exhibits better biological activities, improves contaminant removal efficiency for a long-time operation to treat wastewaters having higher fractions of biodegradable components (Sharari et al., 2013).

With the progression of inoculum time, the fungal species (*Cladosporium* sp., *T. asperellum*, and *T. chioneus*) achieved significantly higher sCOD removal efficiencies in contrast with the control for both experimental conditions. After 288 h of treatment in the absence of co-substrates, *Cladosporium* sp. exhibited sCOD removal efficiency of 19.80% and 24.11% in suspended and immobilized cell, respectively. Under the same experimental conditions, significantly higher removal efficiencies of 27.30% and 30.85% were achieved by *T. asperellum* in suspended and immobilized cell, respectively. In the presence of GL, the removal efficiency of *T. asperellum* increased to 32.90% in suspended and 35.22% in PUF, whereas in all cases the control treatment (CT) showed only 8–9 % removal (Figure 2.1). Similarly, *T. chioneus* achieved significantly higher sCOD removal efficiencies of 40.80% and 41.86% in suspended and PUF, respectively. Therefore, this experiment demonstrates the potential of the fungal species to remove COD and sCOD from wastewater under different treatments. This finding is particularly interesting as the fungal species *T. asperellum* is known to remove contaminants through bio-sorption and biodegradation by excreting ligninolytic enzymes (Ali et al., 2010; Sen et al., 2016; Xin et al.,

2012), although no previous study has investigated this characteristic for *Cladosporium* sp. and *T. chioneus* species.

2.3.2. Evaluation of COD/sCOD contribution from the slowly soluble co-substrates

It was found that the co-substrates WC and WS were slowly soluble in MLL. Therefore, calibration curves were developed to predict the approximate amount of co-substrate's COD/sCOD into MLL from the difference of MLL + co-substrate and MLL treatments employed over the course of experimental duration. The COD solubilization showed the same pattern as the sCOD.

$$\mathrm{COD_{WC}} = 39.365 + 2.551 \times \mathrm{T} + 5.606 \times 10^{-4} \times \mathrm{T}^2 - 1.480 \times 10^{-5} \times \mathrm{T}^3 \quad (2.2)$$

$$\mathrm{sCOD_{WC}} = 49.646 + 2.693 \times \mathrm{T} + 3.487 \times 10^{-4} \times \mathrm{T}^2 - 1.638 \times 10^{-5} \times \mathrm{T}^3 \quad (2.3)$$

$$\mathrm{COD_{WS}} = 16.890 + 6.002 \times \mathrm{T} - 3.209 \times 10^{-2} \times \mathrm{T}^2 + 5.65 \times 10^{-5} \times \mathrm{T}^3 \quad (2.4)$$

$$\mathrm{sCOD_{WS}} = 13.330 + 5.154 \times \mathrm{T} - 2.485 \times 10^{-2} \times \mathrm{T}^2 + 4.081 \times 10^{-5} \times \mathrm{T}^3 \quad (2.5)$$

Where, the COD/sCOD is reported in mg/L and inoculum time (T) was recorded in hours.

The PLS regression study revealed that the three-degree polynomial term (Equations 2.2-2.5) can be used to better predict COD/sCOD. The regression model equations exhibited 98% accuracy between the time intervals of 24 and 288 h.

The solubilization study (Figure 2.2) represents that the amount of COD/sCOD contributed by the WC and WS to MLL has two distinct steps of 0–168 h and 168–288 h. During the first step of 0–168 h, the amount of co-substrate COD/sCOD and the solubilization rate were significantly higher ($p < 0.05$) at higher inoculum time. During the second step (168 – 288 h), there was no significant amount of COD/sCOD contributed by the co-substrates and solubilization rate was much lower than the previous period. The reason for the change in COD/sCOD contribution between the two periods was due to the availability of organic soluble fraction in WC and WS. The soluble fraction was higher during the first step and solubilized more rapidly while in contact with MLL. The

amount of soluble fraction in WC and WS was significantly reduced during the second step and became nearly insoluble at the end.

During the entire period (0 – 288 h), equivalent amount of COD/sCOD was contributed by WC and WS, although the contribution of WC was numerically higher than WS. The maximum COD/sCOD contribution from the WC and WS was 475.68/474.48 and 428.39/408.05 mg/L, respectively, after 288 h (Figure 2.2).

Figure 2.2. Solubilization study of slowly soluble co-substrates (woodchips and wheat-straws) to quantify the amount of (a) COD and (b) sCOD contributed from the co-substrates into the leachate with respect to the inoculum time.

Note: Two-way factorial design (Co-substrate: woodchips and wheat-straws; Inoculum time: 24, 72, 168, 216, and 288 h). Sharing same letter between the bars represents no statistical significance at $p < 0.05$. The error bar represents the standard error.

2.3.3. Effect of co-substrates on COD/sCOD removal efficiency

In the presence of co-substrates as the supplemental carbon source, sCOD removal efficiency significantly increased as compared to the control (Figure 2.3). It is known that the fungal growth cannot be fully supported by the available organic compounds in the leachate whilst the growth can be significantly enhanced/inhibited with the presence/absence of co-substrate as the carbon source (Saetang and Babel, 2012). The WC and WS favor immobilization of fungal biomass by facilitating the attached-growth process and offer slow addition of carbon to the liquid media, which makes them better co-substrates than GL. The sCOD removal efficiency of *T. chioneus* in the presence of WC was significantly higher than the other two co-substrates after 288 h of

operation (Figure 2.3c). The possible reason is that WC ascertains an immobilization support media by offering a stable surface area to enhance the attached-growth biomass process and contributes more COD/sCOD to the liquid media than the WS. Moreover, WS started disintegrating within 48 h of operation, was visibly pulpy after 168 h, and had completely disintegrated into the fungal biomass by 288 h while the visual integrity of WC remained unchanged over the entire experimental period.

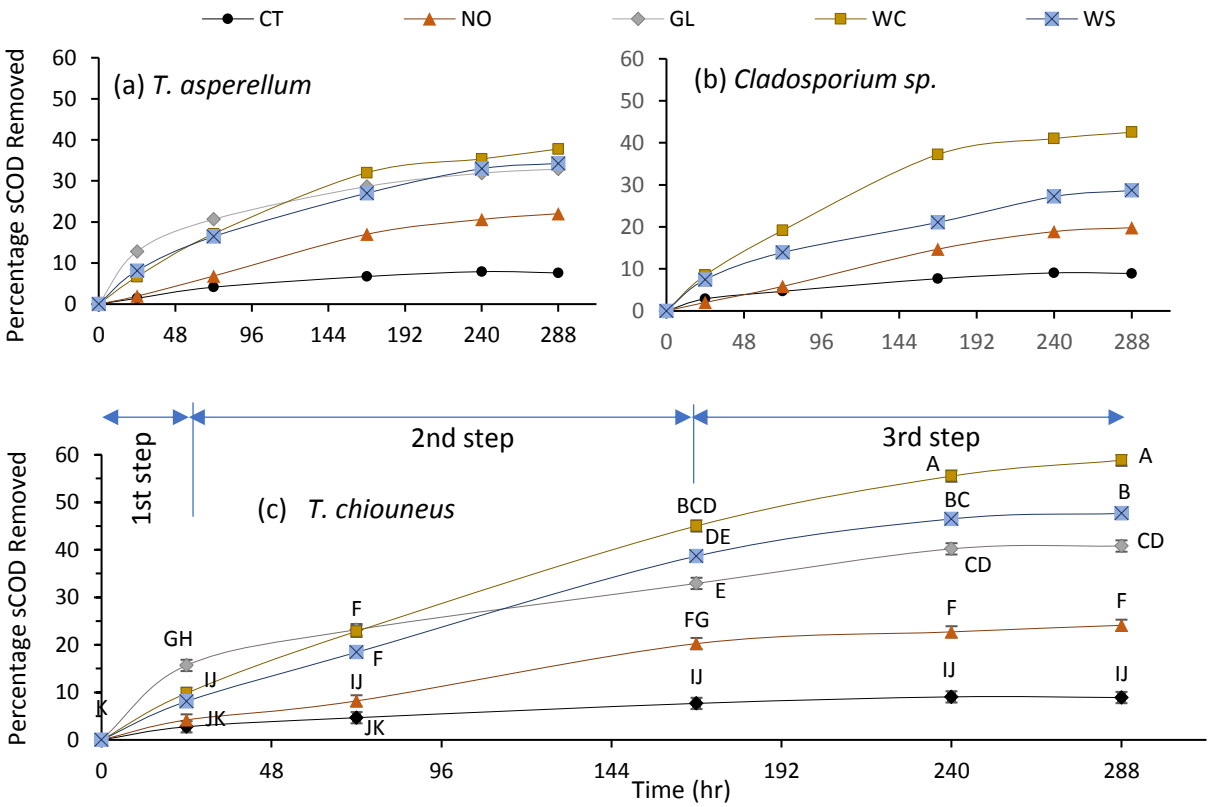

Figure 2.3. Effect of co-substrates on sCOD removal efficiencies by (a) *T. asperellum*, (b) *Cladosporium* sp., and (c) *T. chioneus*. The three steps of sCOD removal efficiencies are indicated in (c).

Note: Two-way factorial design (Co-substrates: CT, NO, GL, WC, and WS; Inoculum time: 0, 24, 72, 168, 240, and 288 h). Sharing the same letter between the treatments in (c) represents no statistical significance at $p < 0.05$. The error bar represents the standard error.
CT = Control (without biomass and co-substrate), NO = No co-substrate (only with biomass), GL = Glucose, WC = Woodchips, and WS = Wheat-straws

The WC also provides extra nutrients to the biomass to further enhance fungal growth (Forss and Welander, 2009; Yum and Peirce, 1998). However, Shin et al., (2002) reported that WS

demonstrated higher fungal growth than maple woodchips and the possible reason is the difference between the WC used in both studies. The WC used in the current study were retrieved from municipal trimmings of forest residue chips containing a mixture of tops, branches, roots, and barks. It has also been reported elsewhere that the pure woodchips exhibit less microbial activity and biodegradation of contaminants than the mixture (Forss and Welander, 2009).

In terms of the increasing pattern of sCOD removal efficiencies, three different time steps were identified during the inoculum time of 0–24 h, 24–168 h, and 168– 288 h (Figure 2.3c). Sharari et al., (2013) reported a similar three-step trend based on BOD and COD reduction during the 9-day operation and recognized the first step as the lag phase where the contaminants are digested as the nutrients. Lignin degrading enzymes started to secrete during the second step (log phase) and the amount of sCOD removed in the second step was greater than the other steps (Figure 2.3). In the third step, the sCOD removal efficiency reached a plateau and the removal rate was decreased. Similar results were obtained from (Sharari et al., 2013). During the final stages of treatment, no significance difference was found among the treatments at different inoculum time except the treatments with co-substrates at 168 h.

The pattern of sCOD removal efficiency in the first stage was completely different than the others. At the end of the first stage, the sCOD removal efficiencies with GL were significantly higher (Figure 2.4a) compared to the second and third stages while the maximum sCOD removal efficiency was observed with WC. Since GL is readily soluble into MLL and instantly accessible for the fungal biomass, microbial activity was more prominent in presence of GL during the 0–24 h period. After 24 h, the carbon source from WC and WS became accessible for the biomass and remained accessible for a longer duration due to the slow rate of solubilization and thus contributed to higher sCOD removal than the GL. The sCOD reduction with GL, WC, and WS were

significantly different from each other by the *Cladosporium* sp. and *T. chioneus* at the end of the treatment periods while significant reduction was observed only with WC by *T. asperellum* (Figure 2.4b).

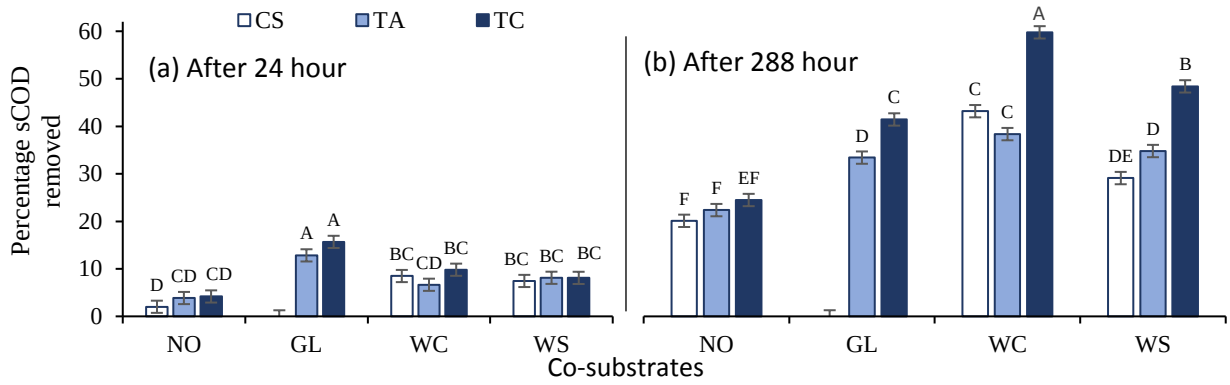

Figure 2.4. Effect of co-substrate on sCOD removal efficiency by *Cladosporium* sp., *T. asperellum* and *T. chioneus* species after (a) 24 and (b) 288 h of inoculum time.

Note: Two-way factorial design (Co-substrates: NO, GL, WC, and WS; Treatments: CS, TA, and TC). Sharing same letter between the bars represents no statistical significance at $p < 0.05$. The error bar represents the standard error.
NO = No co-substrate (only with biomass), GL = Glucose, WC = Woodchips, WS = Wheat-straws, CS = *Cladosporium* sp., TA = *Trichoderma asperellum*, and TC = *Tyromyces chioneus.*

After 288 h, the maximum sCOD removal efficiency of 58.85% was obtained by *T. chioneus* with WC, which was significantly higher than the treatment without the co-substrate (24.11% removal), and inclusion of GL (40.80%) and WS (47.66%) (Figure 2.3c and 2.4b). The sCOD removal efficiencies were about 2.5, 2, and 1.75 times greater with WC by *T. chioneus*, *Cladosporium* sp., and *T. asperellum*, respectively, as compared to the treatment without co-substrate at 288 h. During the third step, *T. asperellum* had similar sCOD removal patterns for GL and WS with the removal percentages of 32.90% and 34.26% at 288 h, respectively, while the removal pattern was completely different by *Cladosporium* sp. and *T. chioneus* during that time (Figure 3). The possible reason is the production of certain enzymes (Lac, LiP, MnP) by *T. asperellum*, *Cladosporium* sp., and *T. chioneus*, which is further explained in the next section. Furthermore, fungal growth and contaminant removal efficiency is affected by other factors such as the type and amount of carbon

sources (co-substrates), level of ammonia concentration, heavy metal content, and organic matter that are not easily degraded by the secreted enzymes (Saetang and Babel, 2012).

2.3.4. Enzymatic activities

All three fungal species exhibited maximum enzymatic activities (LiP, MnP and Lac) with WC co-substrates as compared to GL and WS. Due to the similar trend of enzymatic activities with all three co-substrates, only the data for WC is presented here.

The repeated measures study of enzymatic activities for the fungal treatments revealed that *T. chioneus* produced significant amount of all three enzymes at 168 and 288 h while *Cladosporium* sp. produced a high amount of LiP and MnP and *T. asperellum* mostly produced Lac (Figure 2.5). Overall, the amount of Lac production was much higher than the LiP and MnP. Even with a low production of LiP and MnP, these enzymes can degrade higher content of xenobiotic compounds as compared to Lac due to their higher redox potential (Li et al., 1999; Ricotta et al., 1996). Significant amount of LiP and MnP (5.98 and 6.41 U/L) were detected at 288 h by the *Cladosporium* sp. but no Lac activity was observed. *T. asperellum* produced significant amount of Lac at 168 and 288 h (82.48 and 119.62 U/L), but no LiP and MnP activities were observed. The maximum amount of these enzymes (LiP, MnP, and Lac) were detected by *T. chioneus* (14.08, 19.72, and 164.58 U/L, respectively) after 288 h (Figure 2.5).

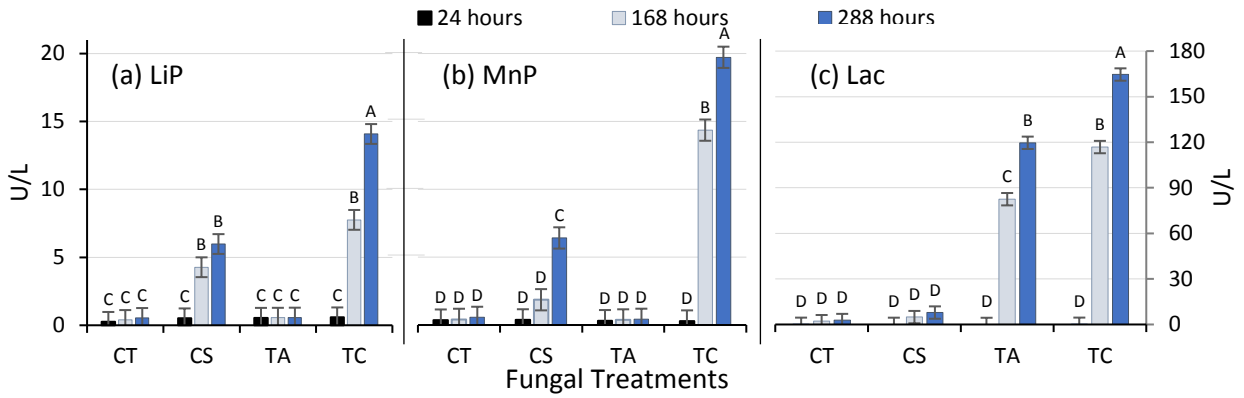

Figure 2.5. Repeated measures study of enzymatic activities (a) LiP, (b) MnP and (b) Lac of fungal treatments after three different inoculum times of 24, 168 and 288 h with WC co-substrate.

Note: Two-way factorial design (Treatments: CT, CS, TA, TC; Inoculum time: 24, 168, and 288 h). Sharing same letter between the bars represents no statistical significance at $p < 0.05$. The error bar represents the standard error.
CT = control (without biomass and co-substrate), CS = *Cladosporium* sp., TA = *Trichoderma asperellum*, TC = *Tyromyces chioneus*, LiP = Lignin-peroxidase, MnP = Manganese-peroxidase, Lac = Laccase

The potential of producing Lac by the filamentous fungi (*Cladosporium* sp. and *T. asperellum*, phylum Ascomycota) and white root fungus (*T. chioneus*, phylum Basidiomycota) is widely reported in the literature (Kubicek, 2012; Polizeli and Rai, 2013). The most investigated lignocellulosic enzymes in white root fungi are LiP and MnP due to their potential capabilities to break down the complex chemical structure (Reddy, 1995). The active ligninolytic enzymatic activities in the current study confirmed that they play a vital role for the removal of COD/sCOD from MLL.

2.4. Conclusion

Both suspended and PUF immobilized fungal biomass performed analogously to remove recalcitrant fractions from the mature landfill leachate (MLL). Woodchips (WC) and wheat-straws (WS) were slowly soluble in MLL and acted as the carbon sources for the fungal growth to enhance the COD/sCOD removal efficiency. WC proved to be a better co-substrate to treat MLL by facilitating immobilized growth of fungus on its stable and integrated surface. *Cladosporium* sp., *Trichoderma asperellum*, and *Tyromyces chioneus* secreted lignocellulosic enzymes and thus

contributed to removal of COD/sCOD from MLL. Collectively, Lac secretion was significantly higher than the LiP and MnP. *T. chioneus* was the most effective species, and the current study is the first ever reporting of lignocellulosic enzymatic activity by the *T. chioneus* species. This finding is promising, and further investigation could explore the potential of the *T. chioneus* species to treat problematic wastewater. It is also recommended to investigate the feasibility of using different wood matrices as the immobilized media in the fungi operated bioreactor. It is concluded that fungal treatment is as an advanced biological treatment option for treatment of MLL to remove refractory components and further research is recommended to optimize this sustainable technological approach.

Chapter 3: Effect of Fungal Lignocellulosic Enzymes on the Treatment of Mature Landfill Leachate

3.1. Introduction

Engineered landfill is considered as the most common economical and environmentally acceptable solid waste disposal method (Tengrui et al., 2007). The landfill waste undergoes four evolution stages of aerobic, anaerobic acetogenic, unstable methanogenic, and stabilization phases (Bove et al., 2015; Kjeldsen et al., 2002). The leachate is generated in the landfill due to the microbial degradation of wastes in combination with the water content of incoming waste (Blight and Fourie, 2005), percolated precipitation (rainfall and snowfall) and ground water accumulation (Alslaibi et al., 2010). It goes through a series of interrelated concurrent biological, chemical and physical processes (An et al., 2008; Kreith and Tchobanoglous, 2002; Renou et al., 2008). Although, many important factors affect the landfill leachate (LL) characteristics, the composition of LL predominantly depends on the landfill age (Baig et al., 1999).

Based on the landfill age and the compositional changes of leachate, LL has been categorized into young, intermediate, and mature leachate when the landfill age range is 0-5 years, 5-10 years and more than 10 years, respectively (Ahmed and Lan, 2012; Chian and DeWalle, 1976). Due to the longer duration of stabilization process of microbial activity in the mature landfill, the biodegradable portion remains comparably less in the mature landfill leachate (MLL) and the non-biodegradable refractory fraction becomes prominent. MLL is characterized by high concentration of ammonia (NH_4^+-N > 400 mg/L) with higher content of toxic recalcitrant compounds, mainly four basal groups of contaminants, such as organic pollutants, inorganic salts, heavy metals and xenobiotic compounds in addition to the wide range of pathogens (Cheng et al., 2017; Foo and Hameed, 2009; Schiopu and Gavrilescu, 2010).

Generally, LL is treated together with municipal sewage in the wastewater treatment plant. Due to the toxic nature and very low biodegradability of the MLL, the conventional biological wastewater treatment processes are inefficient to remove the refractory fractions from the MLL (Li et al., 2010). Thus, the potential adverse effect of toxic recalcitrant constituents of the MLL has become a growing concern. To meet the stringent effluent discharge regulations and mitigate the concern of toxic refractory fractions of MLL, a separate alternative sustainable treatment technology for the efficient removal of recalcitrant fractions from the MLL is very essential.

In recent decades, advanced and innovative biological approaches, for instance, fungal bioremediation or myco-remediation, have been extensively investigated by using different fungal strains to remediate the refractory and recalcitrant components of the problematic wastewaters (Bardi et al., 2017; Kim et al., 2003; Retes-Pruneda et al., 2014). Fungi produce and secrete different lignocellulosic enzymes (that degrade lignin, hemicellulose, and cellulose) such as laccase (Lac), lignin peroxidase (LiP), versatile peroxidases (VP), and manganese peroxidase (MnP) (Goltapeh et al., 2013; Wesenberg, 2003). These enzymes catalyze and break down the complex structure of selective refractory toxic components which are simultaneously present in MLL such as oxidation of xenobiotic compounds (Barr and Aust, 1994) lignin (Goltapeh et al., 2013), tannins (Gamble et al., 1996), humic substances (Hofrichter and Fritsche, 1997; Liu et al., 2015), PPCPs (Rodarte-Morales et al., 2011), and PAHs (Farnet et al., 2009; Kotterman et al., 1998). Additionally, fungi have the ability to detoxify polluted wastewaters through adsorption of heavy metals (Kapoor et al., 1999; Tobin et al., 1984; Yang et al., 2017).

This study investigates the treatment efficiency of MLL by utilizing six selected fungal strains. Three of them are newly isolated from MLL, one strain was previously isolated from the same MLL and none of them has previously been reported to treat MLL. The rest two strains were

collected, whilst both of them have previously been reported to be efficient for specific contaminants removal and one of them has also been reported to treat MLL treatment.

The objectives of this study were: (1) to identify the newly isolated fungal strains from MLL, (2) to investigate the COD/sCOD removal efficiencies from the MLL by the six selected fungi with woodchips as co-substrate, (3) to examine the effect of produced lignocellulosic enzymatic activities (LiP, MnP and Lac) on the COD/sCOD removal efficiencies, and (4) to establish a relationship between the COD/sCOD removal efficiencies and their corresponding ligninolytic enzymatic activities.

3.2. Materials and methods

3.2.1. Leachate characterization

The source of MLL was the landfill (Well No. # 24) located at the Brady Road Resource Management Facility, Winnipeg, MB, Canada, which is in operation since 1973 and receives both commercial and residential wastes. The MLL was periodically collected from the same well, stored at 4°C and physicochemical characterization was carried at the laboratory. Before the experimental tests, the ammonia nitrogen concentration (NH_4^+–N) of the raw MLL was reduced to minimize the ammonia toxicity (Ellouze et al., 2009) by using an aerobic (2 L/min air flow rate) sequencing batch reactor (SBR) operated with activated sludge (VSS: volatile suspended solids ranged 6,000 – 8,000 mg/L). A 5 L glass cylinder with 3 L working volume was used for the SBR and operated as: 8 h per cycle, 7.5 h aeration, and 5 – 10 min settlement before discharge of 1.5 L.

3.2.2. Analytical procedures

The protocols described in the Standard Methods for Examination of Water and Wastewater (SMEW, 18th Edition) (APHA, 1998a) were followed to determine BOD_5, COD and soluble COD (sCOD). COD and sCOD were measured using a spectrophotometer (DR2800, HACH Canada

Ltd., London, ON, Canada); while unfiltered samples represent COD and filtered samples using 1–5µm plain filter paper (Qualitative Grade P2, Fisher Scientific Company, Ottawa, ON, Canada) represent sCOD. The difference between COD and sCOD was considered as the particulate COD (pCOD). The pH was measured with a pH meter/probe (Oakton pH meter, Eutech Instruments, Singapore). Ammonium (NH_4^+–N), nitrates (NO_3^-–N), and nitrites (NO_2^-–N) concentrations were determined using a flow inject analyzer (FIA) (QuikChem 8500, LACHAT Instruments, Loveland, CO, USA).

3.2.3. Selection of fungal species

This study was carried out utilizing three filamentous Ascomycetes fungi *Trichoderma asperellum* (*T. asperellum*), *Cladosporium sp., and Penicillium sp*., and three Basidiomycetes which are commonly referred to as white-rot fungi (WRF), *Tyromyces chioneus* (*T. chioneus*), *Bjerkandera adusta* (*B. adusta*) and *Phanerochaete chrysosporium* (*P. chrysosporium*). The *T. asperellum*, *Cladosporium sp.,* and *Penicillium sp.* strains have been isolated form the MLL. The *T. chioneus* strain was obtained from the environmental engineering lab, University of Manitoba, which was also previously isolated and identified from the same MLL. The *B. adusta* strain MUT 2295 was collected from the collection center of Mycotheca Universitatis Taurinensis (MUT), University of Turin, which was previously used to treat MLL (Bardi et al., 2017), PAHs (Schützendübel et al., 1999), PPCPs (Rodarte-Morales et al., 2011), and textile and pharmaceutical wastewaters (Bardone et al., 2012; Robinson and Nigam, 2008; Sodaneath et al., 2017a). The *P. chrysosporium* strain DSMZ 6909 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. *P. chrysosporium* was previously evaluated to remove humic acid (Liu et al., 2015), cadmium and 2, 4-dichlorophenol (Chen et al., 2011), DDT (Zheng et al.,

2012), PPCPs from aqueous solutions (Rodarte-Morales et al., 2011), and to treat landfill leachate (Hu et al., 2016).

3.2.4. Isolation and cultivation of fungi

Three fungal strains were isolated from the mature landfill leachate (MLL) and these were named as MD3, UB, and UG, before the identification. Two different techniques were followed for isolating fungi. For the first method, 150 µl of 100-fold diluted MLL with sterile saline buffer (per 1 L: 9 g NaCl) was spread onto malt extract agar (MEA) plates (per 1 L: 2 g peptone and 20 g each of malt extract, agar, and glucose) and incubated for one week. In the second method, 15 mL of MLL was used to inoculate 135 mL of sterile basal media (per 1 L: 2.2 g Na_2HPO_4, 0.8 g KH_2PO_4, and 3 g NH_4NO_3); this culture as agitated on a shaker at 150rpm for one week and then 150 µl of this culture was dispersed onto MEA plates and incubated for one week. The individual fungal colonies were excised from these plates and transferred onto new MEA plates using sterile scalpel and sub-culturing was continued until morphologically pure strains were obtained. All the incubations were carried out at 25°C in darkness and stored at 4°C.

After one week of fungal growth on MEA plates, the fungal colony were cut into small pieces (nearly 0.1×0.1 cm^2) using sterile scalpel and cultivated for one week into sterilized GLY broth (per l L: 5g glucose and 1.9 g yeast extract) on a shaker at 150 rpm. After one week of growth at 25°C, the mycelium was harvested for DNA extraction and experimental batch tests. Harvested mycelium was washed with sterile deionized water in order to remove residual GLY media components.

3.2.5. DNA extraction, PCR amplification and identification of fungi

For the DNA extraction, the mycelium was harvested (roughly 10 g of wet weight) by suction filtering through 1-5µm plain filter paper (Qualitative Grade P2, Fisher Scientific, Ottawa, ON, Canada) and mycelium was washed with sterile water. A variation of Hausner and co-workers (Hausner et al., 1992a, 1992b) protocol was followed to extract DNA. Pestle and mortar were used to grind the mycelium (1 g wet weight) with 1.5 g acid-washed sand (Fisher Scientific) and 2mL extraction buffer (per 200 mL: 4 g cetyl-trimethyl-ammonium bromide (CTAB), 20 mL of 1 M Tris-HCl (pH 8), 16 mL of 0.25 M ethylene-diamine-tetraacetic acid (EDTA) (pH 8), 16.4 g NaCl). The ground mixture was incubated in a centrifuge tube at 65°C for 1 h with gentle mixing every 10 min and then cooled down at 15°C. Equal volume of chloroform was added and thoroughly mixed. The sample was centrifuged at 3500 rpm using a general-purpose benchtop centrifuge (IEC Centra CL2, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min and the aqueous layer was transferred into new tube. The chloroform extraction steps were repeated until a clear aqueous layer was obtained. The aqueous solution was treated with 5 µl RNase A (100 mg/mL) and incubated at 65°C for 1 h. Then 2.5 volumes of 95% ethanol were added and placed at -20°C for 1 h after gently mixing through inverting the tube. The sample was centrifuged at 3500 rpm for 15 min and the supernatant was decanted. The nucleic acid pellet was washed with 1 mL of 70% ethanol followed by centrifugation at 3500 rpm for 5min. Finally, ethanol was completely removed, and the DNA pellet was re-suspended in 300 mL of sterile water (ddH_2O, Fisher Scientific) and stored at -20°C.

The internal transcribed spacer (ITS) region was amplified by the polymerase chain reaction (PCR) using the primer pairs, ITS1/ITS4 (White et al., 1990) and V9G (de Hoog and Ende, 1998) / LS266 (Masclaux et al., 1995). Segments of the small subunit rRNA (SSrRNA) genes, β-tubulin genes

(BT) and the Calmodulin (CMD) genes were amplified using the primer pairs, SSZ/LS4 (Hausner et al., 1993), Bt_2a/Bt_2b (Glass and Donaldson, 1995) and CMD5/CMD6 (Hong, 2006), respectively. The Hot Start *Taq* DNA Polymerase (New England Biolabs, Ipswich, MA, USA) protocol was followed as recommended by the manufacturer for assembling the PCR reactions (per 50 µL: 5 µL 10x PCR buffer, 0.5 µL *Taq* DNA polymerase, 40.5 µL sterile distilled water and 1 µL each of 10 mM deoxyribonucleotide triphosphates (dNTPs), 10 µM forward primer, 10 µM reverse primer, and template DNA). Typical thermal cycling conditions for the amplification process were as follows: initial denaturation at 94°C for 300 s; 35 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 45 s, and primer extension at 72°C for 60 s; and final extension at 72°C for 420 s. PCR products were purified with the Wizard SV Gel and PCR Clean-Up system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The DNA content was evaluated using a spectrophotometer (NanoDrop 2000c UV-Vis spectrophotometer, Thermo Fisher Scientific). The quality of PCR products was assessed by resolving on 1% agarose gels through gel-electrophoresis (Prim Midicel Horizontal Submarine Gel System, E-C Apparatus Corporation, Waltham, MA, USA). Sanger DNA Sequence analysis of the amplified PCR products were performed at the Manitoba Institute of Cell Biology, Winnipeg, MB, Canada. Sequence analysis was carried out with the nucleotide Basic Local Alignment Search Tool (BLASTn) program of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) to identify the fungal isolates.

3.2.6. Batch tests

The mycelium was harvested by using a sterile sieve. Diluted sulfuric acid solution (10% H_2SO_4) was used to adjust the pH to 4.5 of MLL (pH of raw MLL was about 7.8 – 8.5) for all trials before transferring the mycelium into the leachate. Woodchips co-substrate was added at a rate of 5 g/L

as a supplemental carbon source and to support attached growth of fungi on the woodchip surfaces. The source of WC was the municipal trimmings of forest residue containing a mixture of tops, branches, roots, and barks. Equivalent amount of mycelium (approximately 10 g wet weight) was added to the 100 mL MLL in 500 mL flasks for the fungal treatments and all trials were triplicated. The control treatments were prepared without fungal mycelium and woodchips. The flasks were placed on a shaker at 150 rpm to facilitate mixing at 23±3°C for 12 days. Samples were collected before adding mycelium, immediately after treatment preparation, after 24 h and then every second day during the experimental duration to determine the percentage of COD/sCOD removal efficiency and to evaluate the corresponding enzymatic activities.

3.2.7. Lignocellulosic enzymes determination

A multi-detection microplate reader spectrophotometer (Synergy 4, BioTek Instruments Inc., Winooski, VT, USA) was used to determine enzymatic activities at 25°C. Lac activity was evaluated from the oxidation of 2, 2'-azino-bis-(3-ethylbenozthiazoline-6-sulfonic acid) (ABTS, ε: 36000 M^{-1} cm^{-1}) at 420 nm in 100 mM sodium citrate buffer at pH 3 (Bourbonnais and Paice, 1990). MnP activity was determined from the oxidation of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH: 1 mM) and 3-dimethylaminobenzoic acid (DMAB: 25 mM) (ε: 32900 M^{-1} cm^{-1}) initiated by adding 4 mM H_2O_2 solution at 590 nm in the mixture of 100 mM succinate lactate and 4 mM $MnSO_4$ buffer at pH 4.5 (Vyas et al., 1994). LiP activity was examined from the oxidation of 3, 4-dimethoxybenzyl alcohol (veratryl alcohol, ε: 9300 M^{-1} cm^{-1}) initiated by adding 0.4 mM H_2O_2 solution at 310 nm in the 100 mM sodium tartrate buffer at pH 3 (Tien and Kirk, 1984).

3.2.8. Statistical analysis

SAS software (SAS 9.4, SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis. Possible outliers were carefully observed for all dataset.

3.2.8.1. Analysis of variance (ANOVA)

PROC TTEST procedure was used to compare the leachate characterization parameters between the raw and pre-treated leachate. Two-way repeated measure analysis ($p < 0.05$) was carried out using PROC GLIMMIX procedure to evaluate the interaction effect of fungal species and inoculum time on the particulate COD, COD/sCOD removal efficiency and enzymatic activities for Lac, LiP and MnP enzymes. Inoculum time was considered as the random variable. Covariance structures were chosen based on the minimum value of Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC) and Generalized Chi-Square. Correlation study was done between the enzymatic activity and COD/sCOD removal efficiency using PROC CORR procedure. Homogeneity of variance and residual normal distribution was confirmed ($W > 0.9$ and $p > 0.05$) for each dataset. Tukey's adjustment and denominator degrees of freedom were considered for the least squares multiple means comparisons.

3.2.8.2. Partial least square (PLS) regression

The percentage of COD/sCOD removal efficiency was predicted from the multivariate regression of lignocellulosic enzymatic activities following the PLS method. The predictor variables were considered as the enzymatic activities (Lac, LiP and MnP) and their corresponding polynomial terms of up to five degrees. The predictor terms which have significant effect on the COD/sCOD removal efficiencies (variable importance in projection, VIP > 0.8 and p > 0.1) were selected for the reduced model. The basic form of multivariate regression is (Equation 3.1):

$$y = \beta_0 + \sum \beta_{i1} X_i + \sum \beta_{i2} {X_i}^2 + \sum \beta_{i3} {X_i}^3 + \ldots\ldots + \sum \beta_{in} {X_i}^n + \varepsilon_{ij} \quad (3.1)$$

Where, y = dependent variable (percentage of COD/sCOD removed), X_i = predictor variables (enzymatic activity of LiP, MnP, and Lac), i = number of predictor variables, j = degree of polynomial terms, and β_{ij} = regression coefficients.

3.3. Results and Discussion

3.3.1. Identification of fungal isolates

The concentration of purified PCR amplified DNA products ranged from 90 to 150 ng/µL based on NanoDrop spectrophotometer readings. The fungal isolate designated as MD3 yielded PCR products as follows 580 bp, 980 bp, 1.3 kb, 550 bp, 480 bp, and 500 bp with ITS1/ITS4, V9G/LS266, SSZ/LS4, SS3/LS2, Bt2a/Bt2b, and CMD5/CMD6 primers, respectively (Figure 3.1). Similarly, the approximate PCR product sizes obtained for fungal isolate UB were 550 bp, 950 bp, 1.3 kb, 480 bp, and 400 bp with ITS1/ITS4, V9G/LS266, SSZ/LS4, SS3/LS2, and Bt2a/Bt2b, primers, respectively, whilst the PCR products size of the fungal isolate UB with CMD5/CMD6 primers could not be resolved. The approximate PCR product size obtained for fungal isolate UG was 1.3 kb with the SSZ/LS4 primers. The initial PCR products showed significant amount of non-specific products and double bands with SSZ/LS4, SS3/LS2, Bt_2a/Bt_2b, and CMD5/CMD6 primers (lanes from 1 to 8), this required optimization of the PCR conditions in order to remove the non-specific products (lanes from 9 to 19).

Schoch et al. (2012) suggested that the internal transcribed spacer (ITS) region should be adopted as the universal and primary fungal DNA barcode marker due to its high success for identification for a wide range of fungi. Additional targets, namely the SSrRNA, BT, and CMD genes, were also used to support the identification of fungi. These loci have been applied towards a variety of fungi with regards to identification and taxonomic investigations (Glass and Donaldson, 1995; Hong, 2006; Selouane et al., 2009; Visagie et al., 2014).

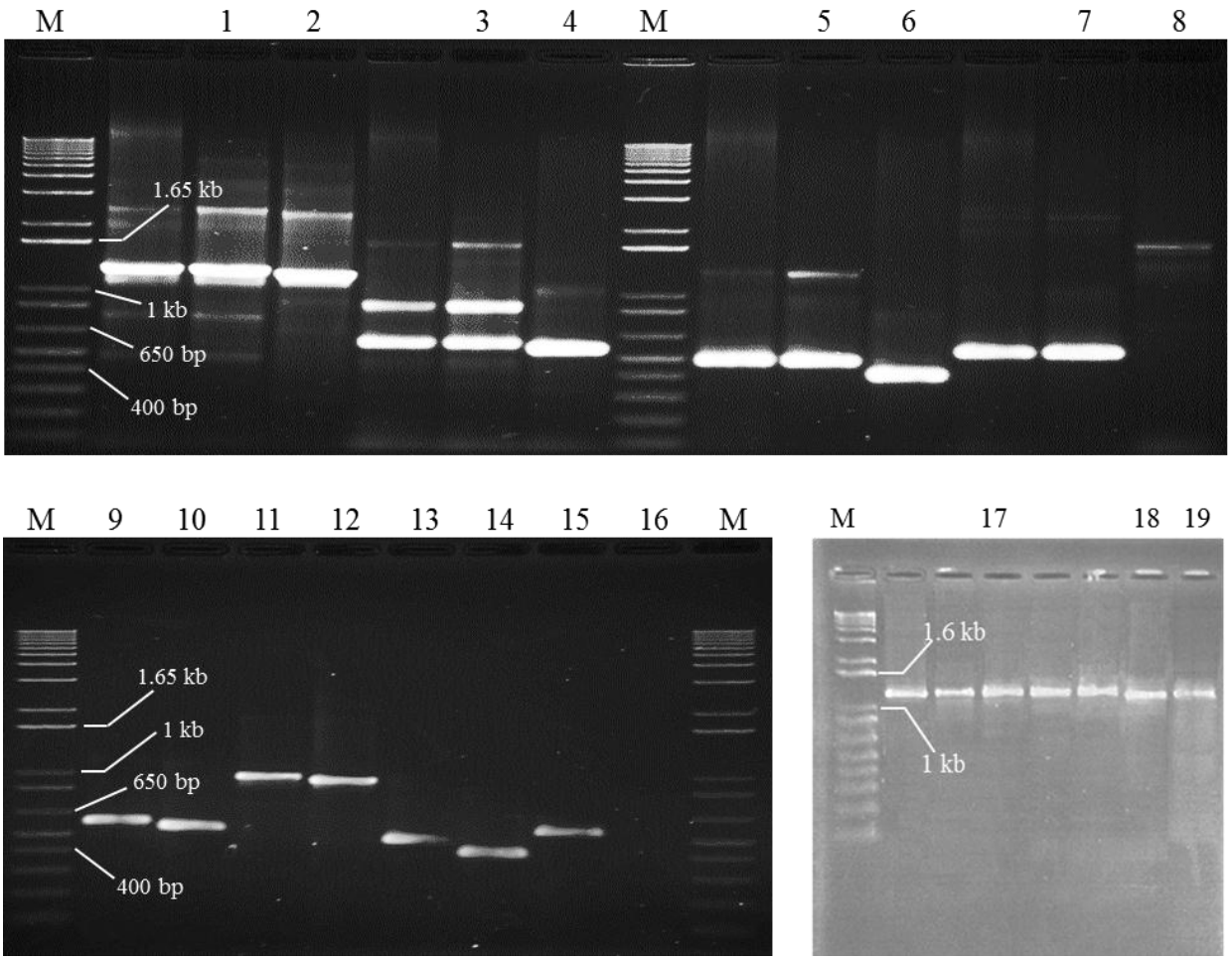

Figure 3.1. Gel image with the approximate PCR products size from the gel-electrophoresis.

Note: Ran 3 µL PCR product through 1% agarose gel stained with ethidium bromide (0.5 µg/mL). Lane denoted "M" for 0.1 µg 1 Kb plus DNA ladder. The lane number and corresponding species and primers used as:

1 = MD3 (SSZ/LS4)	2 = UB (SSZ/LS4)	3 = MD3 (SS3/LS2)	4 = UB (SS3/LS2)	5 = MD3 (Bt_2a/Bt_2b)
6 = UB (Bt_2a/Bt_2b)	7 = MD3 (CMD5/CMD6)	8 = UB (CMD5/CMD6)	9 = MD3 (ITS1/ITS4)	10 = UB (ITS1/ITS4)
11 = MD3 (V9G/LS266)	12 = UB (V9G/LS266)	13 = MD3 (Bt_2a/Bt_2b)	14 = UB (Bt_2a/Bt_2b)	15 = MD3 (CMD5/CMD6)
16 = UB (CMD5/CMD6)	17 = MD3 (SSZ/LS4)	18 = UB (SSZ/LS4)	19 = UG (SSZ/LS4)	

Utilizing ITS, SSrRNA, BT, and CMD sequences as queries in BLASTn searches against the NCBI nucleotide database showed that isolate MD3 can be aligned with a strain of *Penicillium palitans*, although the MD3 sequence also shared similarity with other *Penicillium* species. Similarly, the BLAST search suggested that isolated UB belongs to the Genus *Cladosporium* and isolate UG can be assigned to *Trichoderma asperellum*. The sequences obtained for the isolate UB did not allow for species designation as *Cladosporium* is a species-rich (> 500) genus and for many members' sequences are not yet available within NCBI; also currently available loci may not

provide enough resolution (variability) to resolve all the species assigned to this Genus (Dugan et al., 2004). Sequence similarities of the fungal isolates obtained in this work (queries) with respect to the species retrieved (subjects) from the NCBI database ranged from 100 to 98%. Thus, the PCR of three loci and their sequences (ITS, SSrRNA, BT, and CMD) permitted the identification of three fungal isolates isolated from leachate as *Cladosporium* sp., *Trichoderma asperellum,* and *Penicillium* sp.

3.3.2. MLL characterization

The characterization parameters of raw MLL were evaluated immediately after arrival of the leachate to the laboratory facility. The NH_4^+–N concentration was reduced from the raw leachate with a pre-treatment option using an aerobic sequencing batch reactor (SBR) to minimize the ammonium toxicity and the MLL was again characterized before the experimental tests. The BOD_5/COD ratio was less than 0.1 (0.082 ± 0.01) of the pre-treated leachate, which confirmed the leachate category as the mature landfill leachate. The pH of the raw and pre-treated leachate was 8.3 and 6.5, respectively. The t-test between the samples of raw leachate and pre-treated leachate showed that the NH_4^+–N concentration was significantly reduced to 345.58 mg/L form 861.89 mg/L, after the pre-treatment, although pH, COD, and sCOD reduction were also shown to be significant (Figure 3.2). After the pre-treatment, no significant reduction of BOD_5 concentration and BOD_5/COD ratio was observed.

Among the diverse toxic compounds of MLL as discussed earlier, the concentration of ammonia is more prominent in MLL which is problematic to microorganisms. On the other hand, the expression of lignocellulosic enzymes is occurred during secondary metabolism when nutrients availability is limited such as lower concentration of carbon and nitrogen (Reddy, 1995). Lower initial ammonia nitrogen concentration significantly influences the ligninolytic enzymes secretion

and fungal biodegradation activity (Swamy and Ramsay, 1999). Ellouze et al., (2009) reported that 360 mg/L NH_4^+–N concentration (20 mM nitrogen) as the optimum condition for fungal growth and enzymatic activities, and almost similar NH_4^+–N concentration (345.58 mg/L) of MLL was achieved from the utilization of SBR pretreatment process.

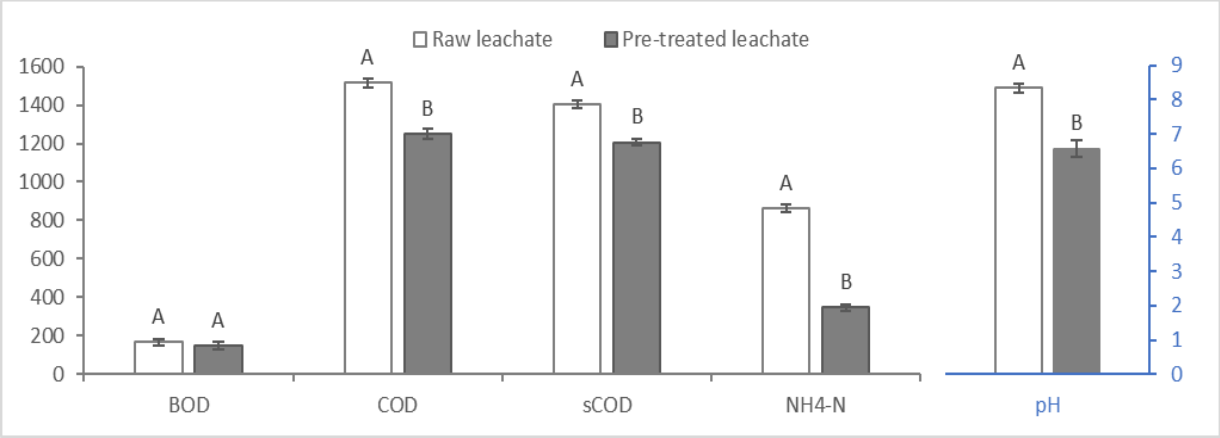

Figure 3.2. Physicochemical parameters of raw and aerobically pre-treated MLL.
Note: The left side vertical axis represents the concentration of BOD_5, COD, sCOD and NH4-N in mg/L and the right side vertical axis represents the pH. Sharing same letter between two bars represents no significant difference at $p < 0.05$ from the t-test of the samples of raw and pre-treated leachate.

3.3.3. Effect of fungal species on COD/sCOD removal efficiencies

The fungal treatments did not exhibit significant increasing pattern of the particulate COD (pCOD) when compared to the control except for the *Penicillium sp.*, for which it was significant over the treatment duration (Table 3.1). Since the high rate of fungal growth and addition of woodchips co-substrate can possibly lead to cause pCOD in the solution, the steady pattern of pCOD suggests that fungal biomass was stable except for the *Penicillium sp*. Similarly, the significant increase of pCOD from *Penicillium sp.* suggests that this strain grows faster than the other fungi used in this study. The average pCOD of studied fungal strains ranged from 20 – 65 mg/L, while the maximum observed value was 77 mg/L at 168 h for *Penicillium sp*.

Table 3.1. Particulate COD (pCOD) of fungal treatments over the experimental duration.

Time → Treatments ↓	24 h	168 h	288 h
Control	21 F	38 CD	30 DEF
T. asperellum	29 DEF	28 DEF	30 DEF
Cladosporium sp.	28 DEF	30 DEF	22 EF
Penicillium sp.	48 B	64 A	48 BC
T. chioneus	24 EF	22 F	32 DE
P. chrysosporium	24 EF	30 DEF	30 DEF
B. adusta	30 DEF	25 EF	38 CD

Note: Two-way ANOVA (factor 1: treatments, factor 2: time or duration of treatment) was done with repeated measure analysis. All values represent the average. Sharing the same letter between treatments represent no significance deference at $p < 0.05$.

The two-way ANOVA statistical analysis (factor 1: fungal treatments, factor 2: duration of treatments) was carried out to identify interaction effect between treatments (fungal strains) and duration of treatments on COD/sCOD removal efficiency (Table 3.2). There was significant interaction which means that the effect of one independent variable (fungal strains) is influenced by another independent variable (treatment duration). For example, no significance difference was observed among the treatments at 24 h with without co-substrates, but with increases of treatment duration, treatments showed significant difference with one another.

The statistical difference between sampling times for each fungal strain (row-wise lettering in Table 3.2) illustrated that COD/sCOD removal efficiency significantly increased with increases of treatment duration for all fungal strains except the control and maximum removal achieved at 288 h. It was noticeable that there was no further significant COD/sCOD reduction occurred after 240 h for all species, as no significant difference was observed between 240 h and 288 h. For the *T. asperellum* and *Cladosporium sp.* strain, no further significant COD/sCOD reduction occurred after 168 h without co-substrates.

Table 3.2. Percentage of COD/sCOD removal efficiencies of various fungal strains with woodchips co-substrate.

Co-substrate	Treatments	24 hr	72 hr	168 hr	240 hr	288 hr	24 hr	72 hr	168 hr	240 hr	288 hr
		% COD removed					% sCOD removed				
No co-substrate	Control	2 h	5 gh	6 gh	7 g	8 fg	2 k	4 jk	8 hij	9 hi	9 hi
	Cladosporium sp.	3 h	8 fg	16 de	18 d	18 d	2 jk	6 ijk	17 ef	19 ef	20 de
	T. asperellum	4 gh	12 ef	23 c	26 bc	27 bc	4 jk	11 gh	24 cd	26 c	27 c
	Penicillium sp.	6 gh	14 e	30 b	35 a	37 a	6 ijk	14 fg	32 b	36 ab	38 a
Woodchips	Control	3 o	5 o	6 o	8 o	9 no	3 r	5 r	7 r	9 pqr	9 pqr
	T. asperellum	6 o	18 m	27 j	34 ij	36 ghi	7 r	17 no	27 kl	35 jk	38 hij
	Cladosporium sp.	8 no	22 kl	30 ij	40 gh	42 efg	8 pqr	19 no	30 ijk	41 fgh	43 efg
	Penicillium sp.	***10 mno***	***27 jk***	***41 cd***	***60 ab***	***64 a***	***9 pq***	***29 lm***	***46 bcd***	***63 a***	***66 a***
	T. chioneus	***11 mn***	***24 kl***	***34 de***	***54 bc***	***57 bc***	***10 opq***	***23 lm***	***38 ef***	***56 bc***	***59 b***
	P. chrysosporium	8 o	21 lm	31 gh	40 fg	43 ef	7 r	17 o	27 k	40 fgh	44 efg
	B. adusta	8 o	23 kl	32 f	46 ef	47 de	8 qr	20 mn	29 fgh	47 de	49 cde

Note: Two-way ANOVA (factor 1: treatments, factor 2: duration of treatment) was done with repeated measure analysis. All values represent the average. Sharing the same letter between treatments represent no significance deference at $p < 0.05$.

The statistical difference among fungal strains for each treatment duration (column-wise lettering in Table 3.2) revealed that at 24 h, COD reduction by *Penicillium sp.* and sCOD reduction by both *Penicillium sp.* and *T. chioneus* was significantly higher than the control and also significantly higher than any other treatments with woodchips co-substrates. At 72 h and onward, the performance of all fungal strains was significantly higher than the control except the *Cladosporium sp.* at 72 h without co-substrate. During the period of 168 h – 288 h, *Penicillium sp.* and *T. chioneus* showed to be most effective fungal strains exhibiting significantly higher removal efficiency than any other strains. At 288 h with woodchips co-substrate, *Penicillium sp.* exceeded by 7% more removal than *T. chioneus* (64% and 57% of COD removal, and 66% and 59% of sCOD removal from *Penicillium sp.* and *T. chioneus* respectively).

The COD/sCOD of control with woodchips treatment (data not shown) was higher than the control alone at all treatment duration, which illustrated that the amount of organic contaminants adsorbed on woodchips was negligible. The highest COD/sCOD removal efficiencies were observed for the tested strains of *Penicillium sp.* and *T. chioneus* (Table 3.2). The higher growth rate of *Penicillium sp.* enhanced higher rate of enzymatic activity and thereby exhibited higher percentage of contaminant removal efficiency. In terms of increasing COD/sCOD removal efficiencies, the fungal strains can be arranged in the order: *Penicillium sp.* > *T. chioneus* > *B. adusta* > *P. chrysosporium* > *T. asperellum* $\geq$ *Cladosporium sp*. However, this ranking does not quite reflect what was observed for the *Cladosporium sp.* and *T. asperellum* as they showed opposite performance behavior with or without co-substrate, for instance, *T. asperellum* was more efficient than *Cladosporium sp.* without co-substrate, and vice versa with woodchips co-substrate (Table 3.2).

Although white root fungi (WRF) are well-known for their ligninolytic enzymatic activities and widely investigated for their utilization as advanced biological treatment options (Levin et al., 2004), one of the locally isolated filamentous fungi, *Penicillium sp.*, appeared to be more efficient than the tested WRF, *T. chioneus*, *P. chrysosporium* and *B. adusta* strains. This suggests that some of the natively grown filamentous fungal strains could have more biodegradation capability, and sometimes even better performance can be obtained than the WRF.

3.3.4. Enzymatic activities of fungi

The secretion of Lac, LiP, and MnP enzymes was measured during the treatment of MLL. Among the six selected fungal stains, there were three filamentous fungi (*Cladosporium sp.*, *T. asperellum*, and *Penicillium sp.*) from the Ascomycota and three WRF (*T. chioneus*, *P. chrysosporium*, and *B. adusta*) from the Basidiomycota phylum. Lac enzymatic activity is widely reported in the fungal species that belong to either of these phylum (Kubicek, 2013). LiP and MnP are the most investigated ligninolytic enzymes in WRF, due to their efficient ligninolytic enzymatic systems and activities (Reddy, 1995). The amount of Lac production was much higher than the LiP and MnP for all species with woodchips co-substrate except for the *Cladosporium sp.* strain. The enzymatic activities were insignificant during the first 24 h of operation and no significant enzymatic activities were detected by the control treatment over the entire treatment period. During the treatment period of 168 h and 288 h, significant amounts of Lac, LiP, and MnP were detected for the *Penicillium sp.*, *T. chioneus*, *P. chrysosporium*, and *B. adusta* strains.

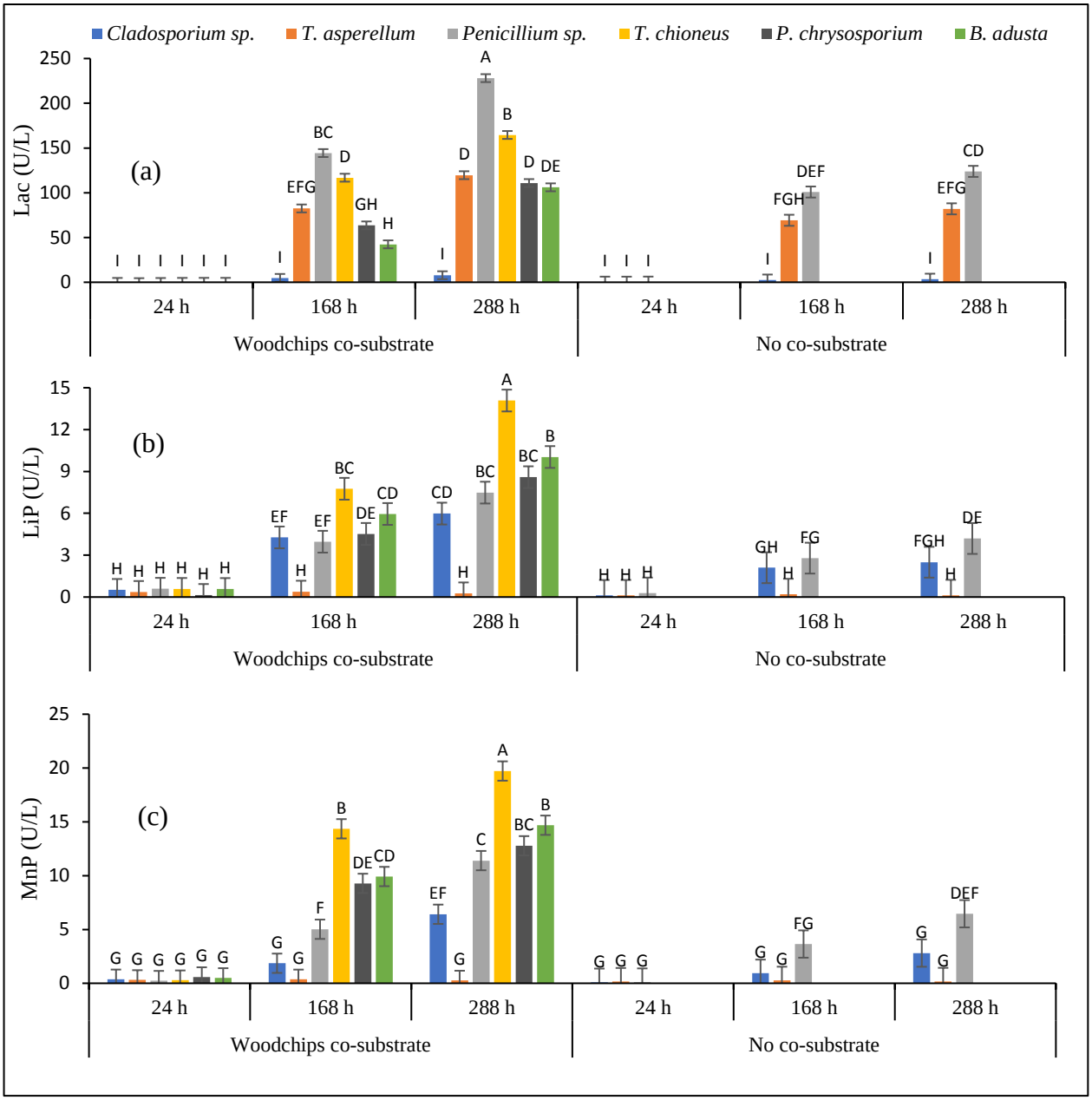

Figure 3.3. Repeated measure study of enzymatic activities (a) LiP, (b) Lac, and (c) MnP, of selected fungal treatments after three different inoculum times of 24, 168 and 288h with woodchips co-substrate.

Note: Three-way ANOVA (factor 1: co-substrates, factor 2: treatments, factor 3: duration of treatment) was done with repeated measure analysis. Sharing the same letter between treatments represent no statistical significance deference at $P < 0.05$. The error bar represents the standard error. Lac = laccase, LiP = lignin peroxidase, MnP = manganese peroxidase.

Among the filamentous fungi, *Cladosporium sp.*, produced 5.98 and 6.41 U/L of LiP and MnP, respectively, at 288 h, and Lac activity was undetected (Figure 3.3). Bonugli-Santos et al. (2010)

conveyed similar contrasting results, while Ji et al. (2014) reported the secretion of all three enzymes, Lac, MnP and LiP by the *Cladosporium sp*. The second filamentous fungi, *T. asperellum*, exhibited 119.62 U/L of Lac activity at the end of the experimental period, but LiP and MnP remained undetected. Lisboa et al. (2017) reported much higher concentrations for all three enzymes, Lac, LiP, and MnP from *Trichoderma asperellum* than evaluated in this study. The third filamentous fungus, *Penicillium sp.*, exhibited the highest amount of Lac with the activity level of 228.09 U/L (maximum detected value 249.8 U/L) at 288 h, which was significantly higher than other treatments. The LiP and MnP activities, 7.48 and 11.40 U/L, were also significant during that period. The ligninolytic enzymatic activities of *Penicillium sp*. have been widely reported in the literature such as LiP and MnP activities (Govarthanan et al., 2017; Nayanashree and Thippeswamy, 2015; Yang et al., 2005). The prevalence of Lac activity is more conspicuous from the *Penicillium sp*. (X. Li et al., 2017; Zeng et al., 2006), while a recent study revealed the highest of 1466 U/L Lac activity of this strain (Yan et al., 2017).

WRF exhibit extracellular ligninolytic enzymes, Lac, LiP and MnP for effective fungal treatments (Razarinah et al., 2015; Wesenberg, 2003), which has also been confirmed from the current study. The maximum amount of LiP and MnP were obtained by *T. chioneus* among the six selected fungal strains, with the activities of 14.08 (max. detected value 19.3 U/L) and 19.72 U/L (max. detected value 26.9 U/L), respectively, after 288 hours. *T. chioneus* also showed the maximum Lac activity (164.58 U/L) among the selected WRF followed by *Penicillium sp*. These findings are completely new, as no previous study has reported on the enzymatic activities of *T. chioneus*. Since, *T. chioneus* is reported herein to be a producer of significant ligninolytic enzymes, therefore, further investigation is recommended to explore the potential of this fungus toward contaminant remediation.

The last two WRF (*P. chrysosporium* and *B. adusta*) performed similar in terms of Lac, LiP, and MnP enzyme production as there was no significant difference between these two species at 24 h, 168 h, and 288 h (Figure 3.3). The amount of Lac, LiP, and MnP enzymatic activities were 110.82, 8.59 and 12.78 U/L for the *P. chrysosporium* and 106.08, 10.03 and 14.69 U/L for the *B. adusta*, respectively, after 288 h. Guo et al. (2014) reported similar results for a *Phanerochaete chrysosporium* strain which showed all three enzymes. *Bjerkandera adusta* has been rigorously investigated due to higher frequency of ligninolytic enzymes and comparably higher content of all three enzymes (Bouacem et al., 2018; Heinfling, 1998; Nakamura et al., 1999; Tripathi et al., 2012). The MLL used in the current study was investigated in a previous study by using *B. adusta*, which reported the maximum MnP activity of 24 U/L after 192 hours (Bardi et al., 2017).

Table 3.2 and Figure 3.3 showed that the overall COD/sCOD removal efficiency and enzymatic activity was significantly higher with the inclusion of woodchips co-substrate comparted to treatments without co-substrate at 288 h of treatment duration except for the LiP and MnP activity by the *T. asperellum* and Lac activity by *Cladosporium sp*. At 168 h, all three enzymatic activity by *Penicillium sp*. and LiP activity by *Cladosporium sp*. was also significantly higher with woodchips co-substrate than without co-substrates. The significant amount of lignocellulolytic enzyme production shown in the current study is possibly due to the active role of individual fungal species and due to the presence of the woodchips matrix, which might act as the carbon source and provided a stable surface to facilitate attached growth and thereby benefitted the fungal biomass production, that can be enhanced by immobilized growth. The expression of enzymes encoded by genes mainly depends on the fungal species which carry certain combination of genes for lignocellulosic enzymes (Allison et al., 2009). Enzymes production is typically regulated by the availability of substrates (Hanif et al., 2004; Waldrop and Zak, 2006), type of substrate (Hamilton,

2006), co-substrates (Bardi et al., 2017), pH (DeForest et al., 2012), temperature (Wallenstein et al., 2012), and moisture content (Fioretto et al., 2000).

On the other hand, the native fungal strains were isolated from the MLL, which were in long contact with toxic components and eventually developed an enzymatic system to cope up with the stressed condition. Therefore, the native fungal strains are capable to produce one or more ligninolytic enzymes and thus contribute to degrade contaminants.. Khan et al. (2015) made similar observations for fungal strains from hydrocarbon contaminated native soil, *Penicillium decumbens* and *Penicillium janthinellum*, which exhibited significant amount of Lac and MnP activity and hydrocarbon biodegradation capability.

3.3.5. Effect of lignocellulosic enzymatic activity on COD/sCOD removal efficiency

The correlation analysis was carried out between the COD/sCOD removal efficiency and the corresponding enzymatic activity to investigate the effect of lignocellulosic enzymatic activity on the COD/sCOD removal efficiency. Significant positive correlation ($p < 0.05$) was observed between the enzymatic activity and COD/sCOD removal efficiency, which indicates that the COD/sCOD removal efficiency significantly increases with the higher amount of enzymatic production. Overall, significant correlation was persistent for all treatments of fungal species except for the control as no enzymatic activity was observed in control. Similarly, LiP and MnP production form *T. asperellum* and Lac production from *Cladosporium sp.* were not significant, where the corresponding correlation were also insignificant. Although *Cladosporium sp.* showed insignificant correlation ($p > 0.05$) between the MnP production and COD/sCOD removed, the correlation coefficient (0.64/0.65) was considerably higher (> 0.5), which indicated approximately 65% of COD/sCOD removal efficiency data are correlated with MnP production. (Table 3.3).

Table 3.3. Correlation study between the COD/sCOD removal efficiency (%) and enzymatic activity for various treatments in the presence of wood chips as a co-substrate.

Treatment	Name	LiP	Lac	MnP
Control	COD	0.43 0.2459	0.73 0.0265	-0.01 0.9745
Control	sCOD	0.55 0.1258	0.65 0.0576	-0.02 0.9522
Cladosporium sp.	COD	0.92 **0.0005**	0.40 0.292	0.64 0.063
Cladosporium sp.	sCOD	0.85 **0.0036**	0.51 0.1629	0.65 0.0569
T. asperellum	COD	0.04 0.9278	0.93 **0.0003**	0.53 0.1383
T. asperellum	sCOD	0.21 0.5921	0.92 **0.0005**	0.60 0.0845
Penicillium sp.	COD	0.84 **0.0046**	0.98 **<.0001**	0.80 **0.0094**
Penicillium sp.	sCOD	0.88 **0.0019**	0.97 **<.0001**	0.79 **0.0109**
T. chioneus	COD	0.79 **0.0106**	0.94 **0.0002**	0.95 **<.0001**
T. chioneus	sCOD	0.86 **0.003**	0.96 **<.0001**	0.90 **0.001**
P. chrysosporium	COD	0.77 **0.0148**	0.95 **0.0001**	0.96 **<.0001**
P. chrysosporium	sCOD	0.79 **0.0112**	0.95 **<.0001**	0.92 **0.0004**
B. adusta	COD	0.85 **0.0038**	0.83 **0.0055**	0.89 **0.0014**
B. adusta	sCOD	0.88 **0.0019**	0.84 **0.0046**	0.91 **0.0007**

Note: The data of 24, 168, 288 hours were considered for the correlation study. The first value represents the correlation coefficient and the second value represents the corresponding probability at a 95% confidence level. The significant correlations have been highlighted with bold underline fonts.

In terms of correlation coefficient between the sCOD removal efficiency and enzymatic activity of treatments, the relationship could be organized in the following order:

Penicillium sp. vs Lac (0.97) > *T. chioneus* vs Lac (0.96) > *P. chrysosporium* vs Lac (0.95) > *T. asperellum* vs Lac (0.92) ≥ *P. chrysosporium* vs MnP (0.92) > *B. adusta* vs MnP (0.91) > *T. chioneus* vs MnP (0.90) > *Cladosporium sp.* vs LiP (0.85)

Table 3.4. The percentage of sCOD removed in relation to the corresponding enzymatic activities at the end of 288 h of treatment duration.

Treatments	% sCOD removed	Enzymatic activity (U/L)		
		Lac	LiP	MnP
Control	9 D	3 D	1 C	1 D
Cladosporium sp.	43 BC	8 D	6 B	6 C
T. asperellum	38 C	120 C	0 C	0 D
Penicillium sp.	66 A	228 A	7 B	11 BC
T. chiouneus	59 A	165 B	14 A	20 A
P. chrysosporium	44 BC	111 C	9 B	13 B
B. adusta	49 B	106 C	10 AB	15 AB

Note: One-way ANOVA analysis of sCOD reduction and enzymatic activities (Lac, LiP, and MnP) for fungal treatments. All values represent the average. Sharing the same letter between treatments represent no significance deference at $p < 0.05$.

The contaminants degradation capability of enzymes is regulated by their redox potential and the order of relative strength of these three enzymes is: LiP > MnP > Lac, with the corresponding redox potential at 1.2, 0.8, and 0.7 V, respectively (Kubicek, 2013)which illustrates that same amount of LiP and MnP have higher contaminant degrading ability than Lac. Table 3.4 showed that higher percentage of sCOD reduction occurred when the enzymatic activity was also significantly higher, for instance, 66%, 59% and 49% of sCOD reduction exhibited by the *Penicillium sp., T. chiouneus*, and *B. adusta* species after 288 h and the enzymatic activities were: 228, 165, and 106 U/L of Lac; 7, 14, and 10 U/L of LiP; and 11, 20, and 15 U/L of MnP, respectively. Table 2 also illustrated that when the enzymatic activity was higher, the correlation between COD/sCOD and enzymatic activity was significant, and the corresponding correlation

coefficient was also higher. *Cladosporium sp.* showed somehow an exceptional behavior, which achieved 43% of sCOD reduction although the enzymatic activities were comparably lower (8, 6, and 6 U/L of Lac, LiP, and MnP respectively) (Table 3.4). Whilst *P. chrysosporium* achieved almost similar percentage of sCOD (44%) reduction with higher enzymatic activities (111, 6, and 13 U/L of Lac, LiP, and MnP). The possible reason could be due to some unknown enzymes secretion by *Cladosporium sp.* which has not been investigated in this study or due to active role of autochthonous microorganisms in presence of this species. Overall, the higher amount of produced Lac, LiP and MnP played vital role toward biodegradation of contaminants and enhanced the COD/sCOD removal efficiencies from the MLL.

The LiP, MnP and Lac enzymatic activity is due to the secretion of extracellular oxidoreductase enzymes from the fungal mycelium. The exact mechanism of pollutants degradation by these enzymes is a multi-steps complex phenomenon which is still poorly understood (Karigar and Rao, 2011; Khan et al., 2015). Apparently, microbes are energized from the exothermic biochemical reactions facilitated by the enzymes to break down the chemical bonds and thereby mediate to transfer electron from electro donor (reduced organic substrate) to electro acceptor (another compound) (Karigar and Rao, 2011) and thus enhance the biodegradation of pollutants. The finding of the current study is promising and explored the feasibility of utilizing the ligninolytic enzymes secreted from the selected fungi, especially *Penicillium sp.* and *T. chiouneus*, to degrade toxic recalcitrant contaminants from the MLL.

3.3.6. Estimation of COD/sCOD removal efficiency due to enzymatic activities

Collinearity Diagnostics test using up to five polynomial terms of LiP, MnP, and Lac with respect to the corresponding COD/sCOD removal efficiencies revealed moderate multicollinearity of the dataset (Condition Index $\geq$ 30, and Proportion of Variation $\geq$ 0.5), which lower the accuracy of

anticipated values due to unstable estimates with high standard errors (Belsley et al., 2004). Partial least square (PLS) multilinear regression procedure was adapted to avoid the multicollinearity problem and to get a better estimation of COD/sCOD removal efficiencies from the predictor variables of LiP, MnP, and Lac.

Table 3.5. Summary of split-sample validation for number of factors

Properties	**COD**	**sCOD**
Minimum root mean PRESS	0.2423	0.2234
Minimizing number of factors	9	11
Smallest number of factors with $p > 0.1$	6	6

Note: PRESS = Predicted residual sum of squares.

Although, the absolute minimum predicted residual sum of squares (PRESS) was obtained with 9 and 11 extracted factors for COD and sCOD (Table 3.5), respectively, from the total of 15 variables, the SPLIT cross-validation test (CVTEST) option (statistical model comparison test) revealed that there was no significance difference of these two models (containing 9 and 11 factors) from the models with 6 factors. Since, the model with fewer factors is always preferred, the models with six factors have been considered from this analysis.

Table 3.6. Percent variation accounted for by partial least squares factors

Number of Extracted Factors	COD		sCOD	
	Model Effects	Dependent Variables	Model Effects	Dependent Variables
1	64.34	69.44	64.33	69.82
2	88.71	81.69	88.84	81.87
3	95.42	92.35	95.36	92.58
4	97.29	95.92	97.29	96.38
5	99.07	96.46	99.38	96.49
6	99.88	97.42	99.88	96.84

The cross-variation summary (Table 3.6) exhibited that over 99% of the predictor variation (model effects) and over 96% of the response variations (dependent variables) have been accounted for by the six factors models, both for COD and sCOD. The scores of VIP values exhibited that the six factors regression models were consisting with Lac, LiP, MnP, Lac^2, Lac^3, and Lac^5 terms both for the COD and sCOD models (Table 3.7).

Table 3.7. Scores of variable importance for projection (VIP)

Factors	COD		sCOD	
	Label	**VIP**	**Label**	**VIP**
1	Lac	1.51	Lac	1.53
2	MnP	1.17	Lac^2	1.18
3	Lac^2	1.17	MnP	1.14
4	LiP	1.12	LiP	1.13
5	Lac^3	1.04	Lac^3	1.04
6	Lac^5	1.02	Lac^5	1.03
7	Lac^4	1.01	Lac^4	1.02
8	MnP^2	0.96	MnP^2	0.95
9	LiP^2	0.94	LiP^2	0.93
10	MnP^3	0.83	MnP^3	0.82
11	LiP^3	0.83	LiP^3	0.82
12	LiP^5	0.80	LiP^5	0.79
13	LiP^4	0.79	LiP^4	0.79
14	MnP^4	0.77	MnP^4	0.77
15	MnP^5	0.76	MnP^5	0.75

Table 3.8 showed that the corresponding multivariate linear regression models equations to estimate the percentage of COD and sCOD removal efficiencies as:

$$\mathrm{COD} = 8.031 + 0.211 \times \mathrm{Lac} + 0.439 \times \mathrm{LiP} + 0.454 \times \mathrm{MnP} + 4.2 \times 10^{-4} \times \mathrm{Lac}^2 - 1.2 \times 10^{-7} \times \mathrm{Lac}^3 - 1.1 \times 10^{-10} \times \mathrm{Lac}^{-10} \quad (3.2)$$

$$\mathrm{sCOD} = 7.644 + 0.227 \times \mathrm{Lac} + 0.402 \times \mathrm{LiP} + 0.448 \times \mathrm{MnP} + 4.5 \times 10^{-4} \times \mathrm{Lac}^2 - 1.4 \times 10^{-7} \times \mathrm{Lac}^3 - 1.2 \times 10^{-10} \times \mathrm{Lac}^5 \quad (3.3)$$

Table 3.8. Parameter estimates of the PLS regression models

Parameters	COD	sCOD
Intercept	8.031	7.644
Lac	0.211	0.227
LiP	0.439	0.402
MnP	0.454	0.448
Lac^2	4.2×10^{-4}	4.5×10^{-4}
Lac^3	-1.2×10^{-7}	-1.4×10^{-7}
Lac^5	-1.1×10^{-10}	-1.2×10^{-10}

Factors that have a significant (VIP > 0.8 and p > 0.1) effect are only considered in the model equations. In the multivariate regression equations, the first value represents the regression coefficient (β_0), which is independent of the enzymatic activities and cannot be exemplified by the enzymes. This explains that under the condition of no enzymatic activities, the COD/sCOD removal efficiency will reach to β_0 percentage, i.e. 8.03% and 7.64% of COD and sCOD, respectively, which validated the removal efficiency achieved by the control treatment (8–9%) when there was no enzyme production. The coefficient of the higher degree terms (Lac^2, Lac^3, and Lac^5) in the model equations (equation 3.2 and 3.3) is very less in compared to the linear term (Lac) suggesting that at lower concentration of lignocellulosic enzymes, the higher degree terms have less effect on COD/sCOD reduction efficiency and follows approximately a linear increasing or decreasing pattern. The coefficient of Lac, LiP and MnP indicates (i.e. 0.211, 0.439, and 0.454 for COD) the approximate percentage of COD/sCOD reduction per unit of these enzymatic activities. As mentioned earlier, Lac has less redox potential thus less pollutants degrading ability than the same amount of LiP and MnP enzymes (Kubicek, 2013). The lower coefficient value of Lac than LiP and MnP in the model equations also confirms that per unit of LiP and MnP cause higher percentage of COD/sCOD reduction than Lac.

3.4. Conclusion

This study investigated the recalcitrant contaminants removal efficiency utilizing six selected fungi, where three strains were newly isolated. The PCR with primers specific for ITS, β–tubulin and CMD genes was able to identify the three newly isolates of fungal species as *Cladosporium sp., Trichoderma asperellum,* and *Penicillium sp.* Both filamentous and white rot fungi were capable to produce ligninolytic enzymes to remove COD/sCOD from the OLL. Two of the locally isolated native fungal strains, *Penicillium sp. and Tyromyces chioneus,* were more efficient in removing COD/sCOD (66% and 59% of COD, and 64% and 57% of sCOD removal respectively). They also produced a higher amount of ligninolytic enzymes such as max. 249.8 U/L of Lac from *Penicillium sp.* and 19.3 and 19.72 U/L of LiP and MnP from *Tyromyces chioneus*. Overall, Lac activity was significantly higher than LiP and MnP activity. The lignocellulosic extracellular enzyme production was strongly correlated with the percentage of COD/sCOD removal efficiency. Higher content of contaminants removal efficiencies from the MLL were obtained due to the vital role of produced enzymes. The enzymatic activities acted as the predictors to estimate the percentage of COD/sCOD removal efficiency. In conclusion, lignocellulosic biomass could be a sustainable and advanced biological treatment option to remove refractory components from the problematic wastewater. *Tyromyces chioneus* is the first ever reported species in this study with significant ligninolytic enzymes and further investigation is recommended to explore its' potential toward contaminants remediation.

Chapter 4: Advanced Biological Sequential Treatment of Mature Landfill Leachate Using Aerobic Activated Sludge SBR and Fungal Bioreactor

4.1. Introduction

Leachate is defined as mature landfill leachate (MLL) when landfill age is higher than 10 years. It is considered as one of the highly polluted wastewater due to the existence of various inherent toxic contaminants such as humic and fulvic substances, xenobiotic endocrine disrupting compounds, trace organic contaminants, heavy metals, higher concentration of ammonium, and lower biodegradable portion (Foo and Hameed, 2009; Mandal et al., 2017). Although, biological processes have effectively been used to remediate young leachate with higher ratio between biochemical oxygen (BOD) and chemical oxygen demand (COD) (BOD/COD > 0.5), MLL treatment still remains challenging (Peyravi et al., 2016; Saleem et al., 2018). Conversely, physico-chemical process such as advanced oxidation, chemical precipitation, absorption-adsorption, coagulation-flocculation, and ion-exchange have been investigated to treat MLL (Ren, 2017), but high operating cost and limited versatility (Torretta et al., 2016) have restricted the applicability and use of these processes. Consequently, treatment of leachate together with municipal sewage in the wastewater treatment plant (WWTP) is still considered as the most economical option. The inadequacy of conventional WWTP to remove recalcitrant components necessitate innovation of sustainable and advanced biological treatment option to remediate MLL (Ghosh et al., 2014). Deployment of lignocellulosic biomass such as fungi is an environmental-friendly, cost effective, and sustainable technological approach. Fungi can survive under relatively higher toxic and environmentally stressed conditions (i.e. low pH and nutrient deficiency). Additionally, the lignocellulosic fungi release diverse extracellular enzymes such as lignin-peroxidase (LiP),

manganese-peroxidase (MnP), versatile peroxidase (VP), and laccase (Lac) to degrade a broad array of recalcitrant fractions (Sardrood et al., 2013).

Fungal bioreactors such as stirred tank, airlift, bubble column, and fluidized bed reactors are designed with the provision of adequate oxygen transfer capability, an essential nutrient for efficient fungal growth, to treat dyes and wastewaters (Ochoa and Gomez, 2008; Sodaneath et al., 2017b). Bioreactor performance is limited by several factors which make them inconvenient in many cases such as continuous agitation of stirred tank reactor that causes high shear stress and leads to the mycelium eruption (Papagianni, 2004; Tang et al., 2011), insufficient mixing in airlift reactor with high biomass densities (Sodaneath et al., 2017b), and non-uniform mixing in bubble column reactor due to scale-up and high pressure drop with packed column reactor (Nair et al., 2016).

Although fungal treatment of landfill leachate is a promising approach, most of the reported studies of efficient removal of contaminants from leachate was carried out in batch experiments with either young (BOD/COD > 0.5) or intermediate leachate (BOD/COD: 0.1 – 0.5), while studies with mature leachate (BOD/COD < 0.1) alone are very limited (Kalčíková et al., 2014). Likewise, limited number of continuous fungal bioreactor studies have been reported (Ghosh et al., 2014; Saetang and Babel, 2009); most studies herein have remediated young or intermediate leachate and only a single study has reportedly used MLL (Bardi et al., 2017). From the exhaustive literature review, neither full-scale studies nor successful fungal treatment of mature leachate has been investigated. On the other hand, most of the fungal biodegradation studies have been conducted under controlled aseptic condition and the influence of autochthonous microorganisms during the treatment of MLL under not-sterile condition is not well documented.

This study investigated the treatment efficiency of *Penicillium* sp. to remove recalcitrant fractions from MLL under the non-sterile condition in a sequential treatment option by using aerobic activated sludge sequencing batch reactor (SBR) and rotating packed bed continuous fungal bioreactor. The utilization of SBR was considered to be pre-treatment process to reduce the ammonium concentration. In our previous study, *Penicillium* sp. exhibited as the most efficient species among the six selected fungi (three Ascomycetes filamentous fungi and three Basidiomycetes white-rot fungi) to treat MLL and therefore it was utilized in the current study. The lignocellulosic enzymatic activities (Lac, LiP, and MnP) of *Penicillium* sp. has also previously been reported in literature (Chen and Yien Ting, 2015; Govarthanan et al., 2017; X. Li et al., 2017). White-rot-fungi got great attention from the researchers for fungal bioremediation due to their enzymatic activity (Svobodová and Novotný, 2018). However, only a single study has reportedly utilized filamentous fungi (*Aspergillus oryzae*) to treat distillery spent wash effluent through bioreactor (Chavan et al., 2013). From the authors' best knowledge, this study appears to be the first study to report the utilization of *Penicillium* sp. (Ascomycetes filamentous fungus) in a continuous bioreactor to remediate refractory contaminants from the MLL under non-sterile conditions. Thus, the novelty of the present study is obvious for bioremediation of toxic refractory contaminants from MLL.

The batch experimental test was carried out utilizing *Penicillium* sp. to evaluate the effect of NH_4^+–N concentration on the fungal enzymatic activities (Lac, LiP, and MnP) as well as on the removal efficiency of COD and soluble COD (sCOD) from MLL. The bioreactor was operated with different hydraulic retention time (HRT) to investigate corresponding response of contaminants removal efficiencies i.e. percentage of COD, soluble COD (sCOD), total carbon (TC), total organic

carbon (TOC), and color removal efficiencies in accordance with the Lac, LiP, and MnP. enzymatic activities.

4.2. Materials and methods

4.2.1. Leachate characterization

Landfill leachate was periodically collected from the Brady Road Resource Management Facility (BRRMF), Winnipeg, Canada (Well No. # 24, location: 49°46'0"N, 97°11'57.5"W) during the spring, summer, and fall of 2017. The landfill is in operation since 1973 and landfilling is done with both residential and commercial wastes. The physicochemical properties of leachate were evaluated immediately after arrival to the laboratory and before each test. The samples were stored at 4°C before conducting further tests. The historic physicochemical characterization data for MLL of the same well (Well No. # 24) was collected from BRRMF to understand the nature of the contaminants over time.

4.2.2. Pretreatment of MLL

The raw MLL was aerobically pretreated to reduce NH_4^+–N concentration in an SBR containing activated sludge, which was obtained from the local wastewater treatment plant (South End Water Pollution Control Centre, Winnipeg, MB, Canada). The feed of the SBR contained 70-80% MLL dose (v/v) with the primary effluent, which was also collected from the aforementioned wastewater treatment plant. The SBR was made up of a 5 L glass cylinder with a 3 L working volume and contained 6,000 – 8,000 mg/L volatile suspended solids (VSS) (Figure 4.1). The reactor worked for 8 h per cycle with the provision of 7.5 h aeration period per cycle at the airflow rate of 2 L/min. Half of the working volume (1.5 L) was discharged following 5 min of settlement at the end of each cycle and same amount of raw MLL was pumped into the reactor for the next cycle. The effluent having different NH_4^+–N concentration was collected from the SBR depending on

reactor's operating condition and considered as the pretreated MLL for the following fungal treatment process.

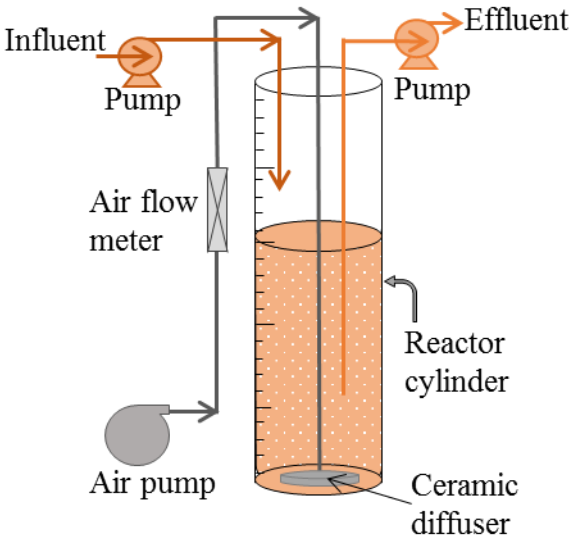

Figure 4.1. Schematic diagram of the aerobic

4.2.3. Cultivation of fungi and batch experimental tests

Penicillium sp. was previously isolated from the MLL and cultured onto malt extract agar plates (MEA) at 25°C in darkness containing 2 g peptone and 20 g each of malt extract, agar, and glucose in 1 L solution and subsequently stored at 4°C. The fungal colony of MEA plates was cut into small pieces, about 0.1×0.1 cm^2, using a sterile scalpel and cultivated into sterilized GLY broth containing 5 g glucose and 1.9 g yeast extract in 1 L solution. The mycelium was harvested and washed with sterile deionized water to remove residual GLY media components, which was used for batch experimental tests. The pH of the pretreated MLL ranged from 7.6 to 8.1 and pH was adjusted to 5 using 10% diluted H_2SO_4 solution before transferring the harvested mycelium into the resulting solution. Approximately 10 g of harvested mycelium (wet weight) was added to 100 mL pretreated MLL in 500 mL flasks for the fungal treatments except the controls and all trials were triplicated. The flasks were placed on a shaker at 150 rpm to facilitate uniform mixing at

25°C for 2 weeks. Samples were collected before adding mycelium, immediately after treatment preparation, after 24 h, and every second day for the entire experimental duration.

4.2.4. Rotating packed bed fungal bioreactor

A packed bed bench-scale bioreactor was constructed using a 5 L cylinder having a working volume of 4.5 L (Figure 4.2). *Penicillium* sp. was cultivated and immobilized onto polyethylene foam (PUF) cubes following the aforementioned fungal cultivation procedure for the fungal bioreactor. A perforated polyethylene cage coupled with the shaft of the motor rotating at 5 rpm was used to contain fungal colonized PUF cubes (nearly 60 cubes). The pH controller was set to 5 to regularly adjust the reactor pH with 10% diluted H_2SO_4 solution. Continuous aeration (2 L/min) was maintained through a diffuser at the bottom connected with an air pump. The pretreated MLL was used as the feed and two pumps were used for the inlet and outlet. The reactor was operated at 6 h cycles at the room temperature (approximately 25°C) with 1 h discharge, 1 h feeding, and 4 h of lag time between the feeding and discharge. The inlet and outlet pumps' flowrate were adjusted to 9.4, 6.3, 4.7, and 3.8 mL/min for 48, 72, 96, and 120 h of HRT, respectively. The inlet and outlet samples were collected daily for analysis.

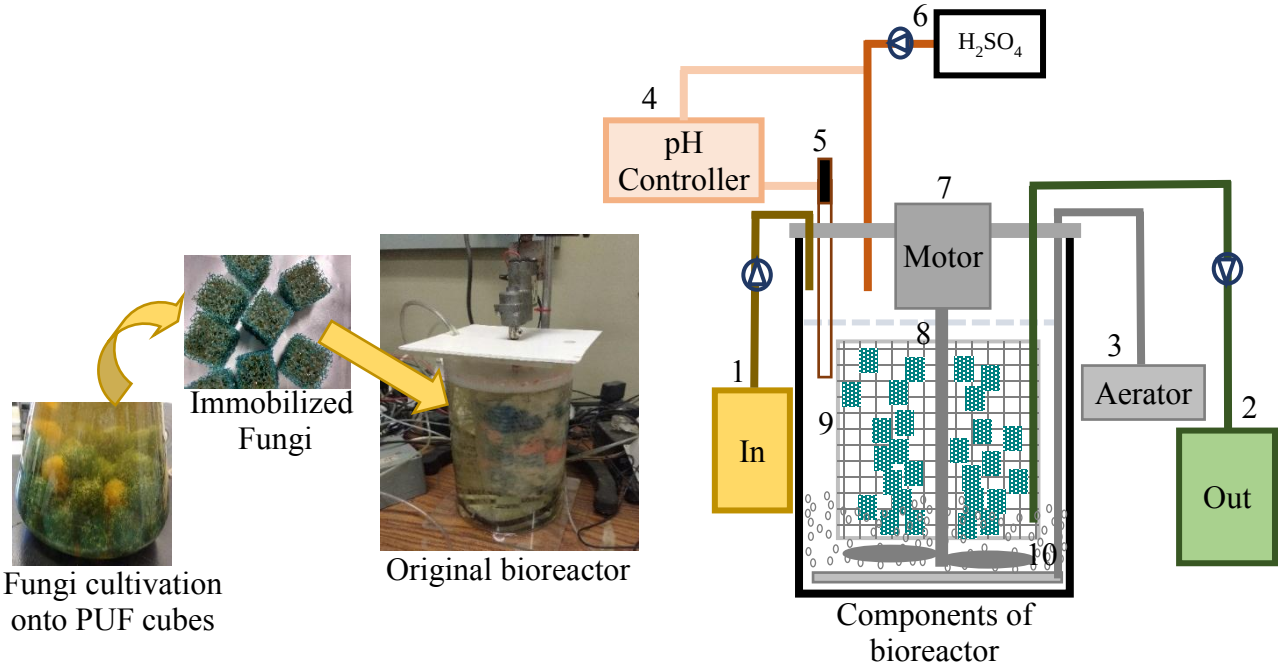

Figure 4.2. Schematic of the packed bed bioreactor. 1. Inlet feed tank, 2. Outlet discharge tank, 3. Aerator, 4. pH Controller, 5. pH probe, 6. acid solution container, 7. Mixer motor, 8. Motor shaft, 9. cage with immobilized fungi in PUF cubes, 10. Air diffuser

4.2.5. Analytical procedures

The amount of BOD_5, COD and sCOD in the samples was determined using the Standard Methods for Examination of Water and Wastewater (SMEW, 18th Edition) (APHA, 1998b). COD represents unfiltered samples and sCOD represents the COD of filtered samples that was measured using 1–5 µm plain filter paper. A Hach kit TNT 827 protocol was followed to determine total nitrogen (TN). A spectrophotometer (DR2800, HACH Canada Ltd., London, ON, Canada) was used to measure COD and TN. The particulate COD (pCOD) was obtained from the difference of COD and sCOD. The pH was measured using a pH meter (Oakton, Eutech Instruments, Singapore). A flow inject analyzer (QuikChem 8500, LACHAT Instruments, Loveland, CO, USA) was used to measure the concentrations of ammonia nitrogen (NH_4^+–N), nitrate nitrogen (NO_3^-–N), nitrite nitrogen (NO_2^-–N), and orthophosphate (PO_4^+–P). The TC and TOC contents were measured using a high-temperature TOC analyzer (FormacsHT TOC Analyzer, Skalar, Tinstraat,

Breda, The Netherlands) coupled with an autosampler (LAS-160) and nitrogen detector from the same supplier.

A microplate reader spectrophotometer (Synergy 4, BioTek Instruments Inc., Winooski, VT, USA) was used to detect enzymatic activities at 25°C. Lac activity was determined as described in Bourbonnais and Paice, (1990) from the oxidation of ABTS (2,2'-azino-bis-(3-ethylbenozthiazoline-6-sulfonic acid)) (specific activity of 36000 $M^{-1}cm^{-1}$) in 100 mM sodium citrate buffer at pH 3 and 420 nm. MnP activity was determined by the method described by Vyas et al., (1994) from the oxidation of 25 mM DMAB (3-methyl-2-benzothiazolinone hydrazone hydrochloride) and 1 mM MBTH (3-dimethylaminobenzoic acid) (specific activity of 32900 $M^{-1}cm^{-1}$) by adding 4 mM H_2O_2 in 100 mM succinate lactate and 4 mM $MnSO_4$ buffer at pH 4.5 and 590 nm. LiP activity was determined following Tien and Kirk, (1984) from the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) (specific activity of 9300 $M^{-1}cm^{-1}$) by adding 0.4 mM H_2O_2 in 100 mM sodium tartrate buffer at pH 3 and 310 nm. The color removal or decolorization percentage was determined from the differences in spectrum absorbance of initial and final values during the treatment using the above-mentioned microplate reader spectrophotometer in the visible range of 380–740 nm.

4.2.6. Statistical analysis

The statistical analysis was carried out using SAS software (SAS 9.4, SAS Institute Inc., Cary, NC, USA). Two-way analysis of variance (ANOVA) with PROC GLIMMIX procedure and repeated measure analysis was used to evaluate the interaction effect between the inoculum time and ammonia nitrogen concentration of the pretreated MLL on the COD/sCOD removal efficiency and enzymatic activity in the batch experimental tests. The minimum value of Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC) and Generalized Chi-Square considered to

choose the covariance structure. One-way ANOVA analysis was carried out to evaluate the effect of HRT on contaminants (COD, sCOD, TC, TOC and color) removal efficiencies and enzymatic activities (Lac, LiP, MnP) from the fungal bioreactor. Possible outliers and homogeneity of variance was carefully observed, and normality of residual distribution was confirmed ($W > 0.9$ and $p > 0.05$) for each dataset. For the least squares multiple means comparisons, Tukey's adjustment and denominator degrees of freedom were considered.

4.3. Results and discussion

4.3.1 MLL characterization and criteria selection for enhanced fungal enzymes secretion

The physicochemical parameters of MLL from the laboratory analysis (Table 4.1) and four years historic data (Supporting document Table 4.4) exhibited the nature and specificity of a wide range of toxic contaminants in the existing samples, and the environmental fate, effects, exposure route, and toxicity still remain unknown for many of them (Koshy et al., 2007; G. Li et al., 2017). The age of buried waste cell (> 10 years) of the landfill and the BOD_5/COD ratio (< 0.1) validated the leachate category as fully mature (Ahmed and Lan, 2012; Zhang et al., 2015).

Table 4.1. Physicochemical parameters of MLL characterized in the laboratory.

Parameters	Units	Raw leachate	Wastewater
pH		8.1 ± 0.7	7 ± 0.5
BOD_5	mg/L	141 ± 19	195 ± 15
COD	mg/L	1458 ± 239	410 ± 34
sCOD	mg/L	1385 ± 196	NA
Ammonia nitrogen (NH_4^+–N)	mg/L	914 ± 91	45 ± 11
NOx (NO_2^-–N and NO_3^-–N)	mg/L	0.60 ± 0.54	< 0.1
PO_4^+–P	mg/L	4.7 ± 1.49	3.69 ± 0.6
Total nitrogen (TN)	mg/L	938 ± 62	NA

Total carbon (TC)	mg/L	695 ± 75	NA
Total organic carbon (TOC)	mg/L	675 ± 65	NA
BOD_5/COD		0.095 ± 0.007	0.46 ± 0.05

Note: Parameters are presented as average values with corresponding standard deviations. NA = Not analyzed.

The MLL contains many of the contaminants in excess amount and many of them exceeded the *Manitoba Water Quality Standards, Objectives, and Guidelines* (Manitoba Water Stewardship, 2011) (Supporting document Table 4), which emphasize the urgent need to adapt effective treatment options for the remediation of these diverse refractory contaminants.

The equivalent amount of ammonia nitrogen (914±91 mg/L) and total nitrogen (938±62 mg/L) in MLL suggest that nitrogen is present mostly in ionized form (NH_4^+-N). Moreover, restricted nitrogen levels in the environment enhance lignocellulosic enzyme production. For instance, Ellouze et al. (2009) and Ürek and Pazarlioğlu (2005) reported that the optimum value for lignocellulosic enzyme production as 20 – 22 mM nitrogen, which is equivalent to 360 – 396 mg/L NH_4^+–N. In this study, the aerobic activated sludge pretreatment process was successfully utilized to reduce the NH_4^+–N concentration from 914 to 352 mg/L to enhance the subsequent fungal treatment process which is discussed in the next section.

It was assumed that no additional nutrients were required for the biological fungal treatment process except oxygen and some easily accessible carbon source since some minerals and essential nutrients are already present in MLL with high concentration such as Na (>1500 mg/L), K (>590 mg/L), Ca (>190 mg/L), Mg (≥500 mg/L), Mn (>0.25 mg/L), Fe (>1.95 mg/L), Chloride (≥1700 mg/L), N (>900 mg/L), and P (>5 mg/L) (Supporting document Table 4.4). The addition of easily accessible carbon source in the form of wastewater (20% - 25% v/v) could be beneficial for the biodegradation process. These findings are in agreement with Saetang and Babel, (2009) who

reported that fungal growth cannot be fully supported by the available organic compounds in MLL and therefore, additional carbon source significantly enhances fungal growth.

4.3.2 Effect of NH_4^+–N concentration of pretreated MLL on fungal treatments

The effect of the ammonia nitrogen concentrations in the pretreated MLL on the percentage of COD/sCOD removal efficiency in accordance with enzymatic activities were studied via batch experimental tests. The NH_4^+–N concentration of raw MLL was 914 mg/L, and the collected pretreated MLL concentrations were 0, 105, and 352 mg/L. The experiments were carried out with MLL containing initial NH_4^+–N concentrations of 0, 105, 352, and 914 mg/L (which have denoted as L1, L2, L3, and L4 respectively) and inoculated with *Penicillium* sp. The control included raw MLL without fungal inoculation.

Table 4.2. Effect of NH_4^+–N concentration of pretreated MLL on the percentage of COD/sCOD removal efficiencies by *Penicillium* sp.

Time (hr)	24	96	168	216	264	24	96	168	216	264
Treatments	% COD					% sCOD				
CT(914)	3 m	4 lm	5 lm	5 lm	6 lk	1 n	3 lmn	3 lmn	6 klm	6 jklm
F(0)	6 l	16 ji	21 hg	25 fg	26 fe	7 jkl	17 gh	24 ef	26 de	27 de
F(105)	7 lk	18 hi	27 fe	31 dc	32 c	8 jk	21 fg	28 dc	32 bc	33 b
F(352)	2 m	17 ji	29 de	37 b	41 a	2 mn	18 gh	32 bc	39 a	42 a
F(914)	4 lm	9 k	14 j	17 ji	17 i	3 lmn	10 ij	14 hi	16 h	17 gh

Note: Sharing same letter among treatments indicates no significance difference. CT = control (without pretreatment and fungi), F = fungal *Penicillium* sp. The number of parenthesis bracket represents NH_4^+–N concentration (mg/L) in the corresponding treatments.

No enzymatic activity was observed from the control (with L4 leachate) treatment and therefore, the data was not presented. The enzymatic activity was insignificant at 24 h for all treatments

(Figure 4.3). It is noticeable that at 24 h, L1 and L2 showed significantly higher COD/sCOD reduction as compared to the control suggesting that better acclimatization occurred with lower NH_4^+–N concentration (Table 4.2). At 96 h, no significance difference was observed among L1, L2, and L3 and after the treatment duration of 168 h, L3 expressed significantly higher COD/sCOD reduction than any other treatments. The study revealed that the fungal treatment containing 352 mg/L of ammonia nitrogen concentration (L3) exhibited higher removal efficiencies of COD (41%) and sCOD (42%) at the end of 264 hours of treatment duration. The enzymatic activity was also highest with L3 leachate at the end of the treatment duration such as 193, 37, and 25 U/L of Lac, LiP and MnP, respectively (Figure 4.3). The MLL with lower NH_4^+–N concentration (L2: 105 mg/L) reduced the COD and sCOD removal to 32% and 33%, respectively. At this same concentration (L2), the Lac, LiP, and MnP secretion was significantly reduced in contrast with L3 and inhibited by 20%, 27%, and 21%, respectively. Similarly, L1 (0 mg/L NH_4^+–N) treatment caused further reduction in COD and sCOD to 26% and 27% as compared to L3 (352 mg/L NH_4^+–N) and inhibited the secretion of all three enzymes by approximately 40%. Conversely, L4 treatment (higher concentration of NH_4^+–N:914 mg/L) also caused a drop in COD/sCOD removal to 17% and inhibited all three enzymes secretion by approximately 70% (Figure 4.3).

Ellouze et al. (2009) reported similar observations in terms of fungal growth and enzymatic activities. They reported 360 mg/L NH_4^+–N concentration (20 mM nitrogen) as the optimum condition for fungal growth and exhibited reduction in LiP and MnP activities by 50% and 60% for *P. chrysosporium,* and reduction in Lac activity by 30% for *T. trogii* with 673 mg/L NH_4^+–N (2 g/L of NH_4Cl) concentration as compared to the optimum condition. Ürek and Pazarlioğlu, (2005) also reported highest MnP activity at 396 mg/L NH_4^+–N (22 mM nitrogen) concentration. These observations are also in agreement with many white-rot-fungi behavior suggesting that the

secretion of lignocellulosic enzyme enhances at limited nitrogen levels (Singh and Chen, 2008). However, similar observations of enzymatic inhibition at very lower ammonia nitrogen concentration (0 – 396 mg/L NH_4^+–N) were not reported by Ellouze et al. (2009) and Ürek and Pazarlioğlu, (2005). The possible reason could be due to the difference of fungal culture media. The previous studies were conducted with the addition of NH_4Cl in solid-state culture media whereas the present study was conducted with MLL by reducing ammonia nitrogen concentration through aerobic activated sludge pretreatment process.

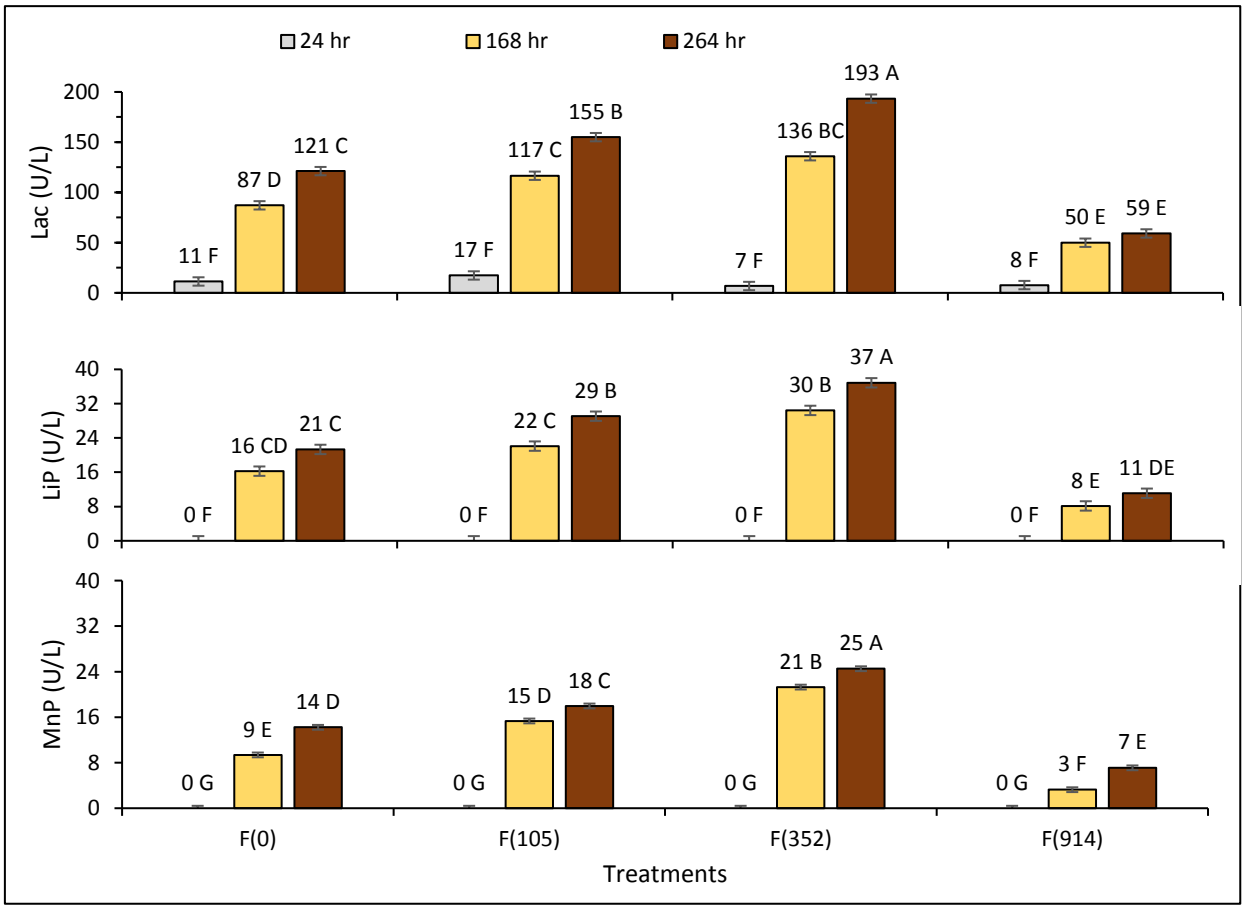

Figure 4.3. Effect of ammonia nitrogen concentration of pretreated MLL on fungal enzymatic activities. F = fungal *Penicillium* sp. The number of parenthesis bracket represents NH_4^+–N concentration (mg/L) in the corresponding treatments. The number of above bar represents mean value and Sharing same letter among treatments indicates no significance difference.

4.3.3 Effect of aerobic activated sludge pretreatment on MLL

The initial volume in the aerobic activated sludge SBR was 20% (v/v) MLL, which was successively increased by 5-10% over the next 2-3 months to acclimatize with 100% MLL. With the 100% MLL in feed, the SBR achieved 11 – 13 g/L mix liquor total suspended solids (MLTSS), 6 – 8 g/L mix liquor volatile suspended solid (MLVSS), and SVI < 100, which suggests good settleability (Tchobanoglous et al., 2013). At stable operating condition, 99% – 100% NH_4^+–N removal was achieved irrespective of the MLL dose in the feed (Figure 4.4). High NH_4^+–N removal efficiency from leachate has also been reported in other studies. For instance, Xu et al. (2010) reported 96.7% NH_4^+–N removal efficiency from leachate (BOD_5/COD ratio of 0.15 with an initial NH_4^+–N of 1451 mg/L) with relatively higher effluent NH_4^+–N (48 mg/L). In another study, Wang et al. (2011) also achieved 99% NH_4^+–N removal rate by introducing 30 min intermittent aeration followed by 24 h SBR cycle.

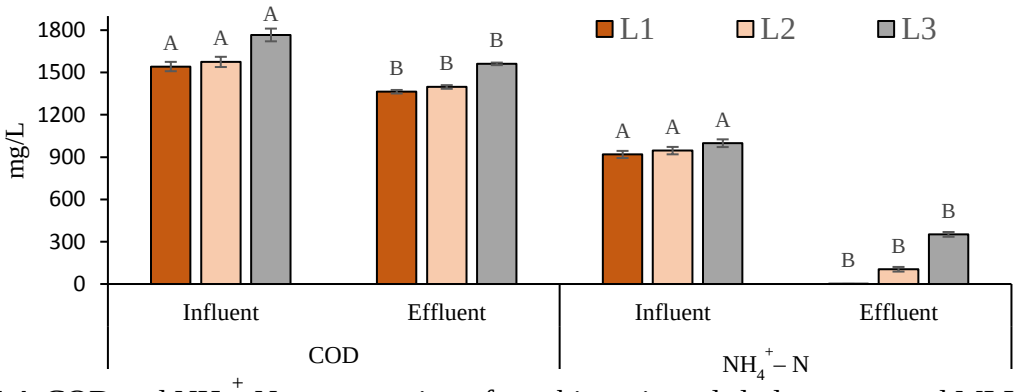

Figure 4.4. COD and NH_4^+-N concentration of aerobic activated sludge pretreated MLL.

Note: Paired t-test was done between influent and effluent for COD and NH4+-N reduction. Sharing same letter between influent and effluent indicates no significant difference ($p < 0.05$). Error bar represents standard error of the mean.

In order to obtain the pretreated MLL for the following fungal treatment process, effluent with three levels of NH_4^+–N concentration (0, 105, and 352 mg/L) was collected. The influent and

effluent characteristics of the SBR for these three categories of pretreated MLL (L1, L2 and L3) illustrate that the sole aerobic activated sludge process is not efficient enough to remove COD (effluent COD: 1363, 1397, 1561 mg/L for L1, L2, and L3 respectively), although the pared t-test between influent and effluent shows significantly different (Figure 4.4). Marañón et al. (2010) also demonstrated that sole activated sludge process could not adequately remove COD from old leachate. The possible reason could be due to the low biodegradability fraction and diverse toxicity of the recalcitrant components in MLL (Müller et al., 2015; Peyravi et al., 2016). For all three categories of SBR effluents, approximately 10% - 12% of COD removal rate was exhibited with 80% MLL feed (Figure 4.4). This observation agreed with the previous findings (Ren, 2017) that used the same MLL and reported a 14% COD reduction via aerobic activated sludge SBR fed with 40% - 65% MLL.

4.3.4 Contaminants removal and enzymatic activities in fungal bioreactor

The flask level sequential treatment revealed that the maximum COD (41%) and sCOD (42%) reduction occurred when MLL was first treated with aerobic activated sludge (at 352 mg/L NH_4^+–N concentration) following the fungal (*Penicillium* sp.) treatment. The final scale-up studies were carried out following this sequence and the performance of fungal bioreactor was evaluated based on the percent removal efficiencies of COD, sCOD, TC, TOC, and color from the pretreated MLL. In the fungal bioreactor, COD and sCOD removal efficiencies were further enhanced to 48% and 50% (max. 52% and 54% respectively) on day 87 with HRT 120 h as compared to the batch tests of 41% and 42%, respectively (Figure 4.5). Moreover, an additional 12% COD removal was achieved by the aerobic activated sludge SBR during the pretreatment process. This observation is analogous with the finding of Ghosh et al. (2014), where a 71% COD reduction in flask test was further increased to 79% in a sequential bioreactor. The sequential treatment with two or more

micro-organisms is beneficial as the first treatment initiates the catabolic reactions while the subsequent treatment activates the remaining metabolic pathways for complete mineralization (Kaushik et al., 2010). On the other hand, the feed for the sequential treatment process was prepared with 80% raw MLL and 20% municipal sewer wastewater, where wastewater contributed approximately 80 – 90 mg/L of COD. The higher BOD/COD ratio of wastewater (≈ 0.5) as compared to MLL (< 0.1) indicated that wastewater added little amount of easily accessible carbon source for the microorganisms which might further enhance the microbial activity.

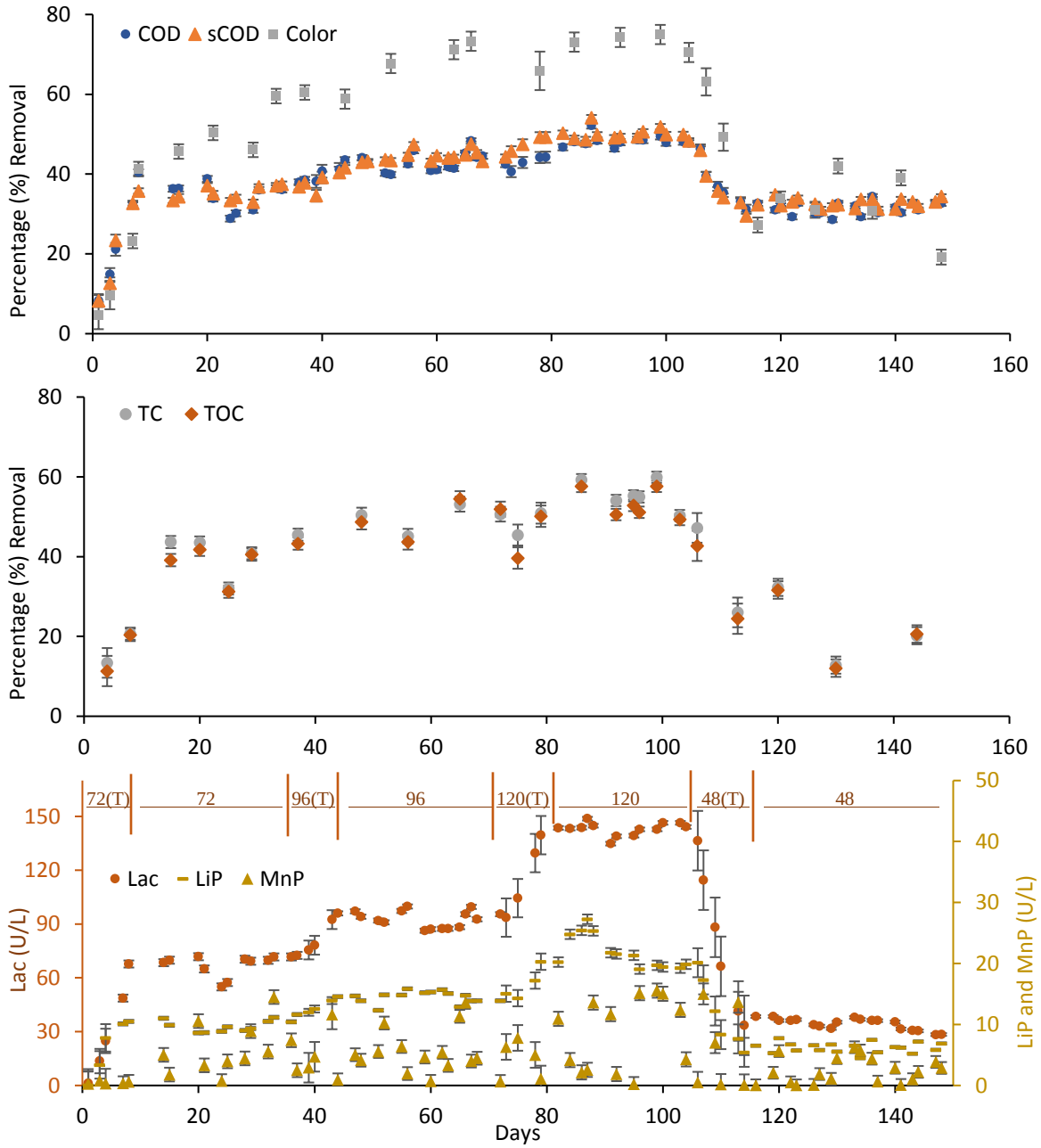

Figure 4.5. Percentage of COD, sCOD, Color, TC, and TOC removal efficiencies from the fungal bioreactor and corresponding Lac, LiP and MnP enzymatic activities.

The fungal bioreactor demonstrated stable removal efficiencies and enzymatic activities since the startup of the bioreactor and operation was continued for 150 days with varying HRT (Figure 4.5). The startup HRT (72 h) was subsequently increased to 96 h and 120 h, and finally reduced to 48 h to observe the effect of HRT on contaminants' removal efficiencies and enzymatic activities. The

system took several days to adjust to the change in HRT and regain stable removal efficiencies and enzymatic activities and these transition phases were indicated as 72(T), 96(T), 120(T), and 48(T) for the corresponding HRT of 72, 96, 120, and 48 h, respectively. The transition phase was longer, approximately 8 days, during the change in HRT from 120 h to 48 h, while it was about 5 days in all other cases. With the increase in HRT, the COD and sCOD reduction was significantly increased due to the longer contact time between MLL and fungus. Specifically, the average COD reduction of 31%, 35%, 43%, and 48% and sCOD reduction of 33%, 35%, 44%, and 50% were recorded at HRT of 48, 72, 96, and 120 h, respectively. The Lac and LiP enzymatic activities were also significantly higher in most cases, while MnP activities were statistically insignificant and low values were recorded over the entire operational period (Figure 4.5 and Table 4.3). In terms of TC, TOC, and color removal efficiencies and Lac and MnP enzymatic activities, there was no significant difference between the HRT 96 and 120 h suggesting that 96 h could be considered as optimal contact time between MLL and fungus in the bioreactor. The maximum TC, TOC and color removals were 60%, 58%, and 75% on day 99 while the maximum Lac, and LiP activities were 149 and 27 U/L on day 87 and MnP activity of 16 U/L was recorded on day 99 at HRT 120 h (Figure 4.5).

Table 4.3. Effect of HRT on contaminants removal efficiency and enzymatic activity of fungal bioreactor.

Operating	HRT	Percentage removal					Enzymatic activities (U/L)		
days	(h)	COD	sCOD	TC	TOC	Color	Lac	LiP	MnP
1 – 4	72(T)	15 E	15 E	13 C	11 D	7 E	13 C	4 C	0 A
5 – 37	72	35 C	35 C	38 B	36 CB	47 C	66 BC	10 C	5 A
38 – 43	96(T)	40 BC	38 C	NA	NA	NA	82 AB	13 BC	6 A

44 – 72	96	43 B	44 B	50 A	50 A	68 AB	93 A	14 B	5 A
73 – 79	120(T)	43 B	48 AB	48 A	45 AB	66 AB	117 A	17 AB	5 A
80 – 105	120	48 A	50 A	54 A	52 A	73 A	143 A	22 A	8 A
106 – 114	48(T)	37 C	36 C	26 BC	24 CD	56 CB	80 ABC	12 BC	6 A
115 – 148	48	31 D	33 D	22 C	21 D	32 D	34 C	6 C	2 A

Note: One-way ANOVA was carried out to evaluate the effect of HRT on the removal efficiency of COD, sCOD, color, TC, and TOC; and also the effect of HRT on enzymatic activities. The parenthesis letter (T) of operating days indicates transition period. Sharing same letter among treatments indicates no significant difference. NA = Not Analyzed.

The COD, sCOD, TC, and TOC removal efficiencies followed a similar trend during all phases of HRT except at 48 h, when the COD/sCOD reduction was approximately 10% higher than the TC/TOC due to the higher variation in TC/TOC (standard deviation for COD/sCOD < 2, while for TC/TOC ≈ 10). The color removal efficiency was much higher in contrast to the COD, sCOD, TC, and TOC at HRT of 72, 96, and 120 h. For instance, color removal was almost 1.5-fold higher than COD during those periods. Similarly, Lu et al. (2010) reported higher percentage of color removal (85.5%) than COD reduction (54.6%) in a continuous bioreactor.

A previous continuous bioreactor study with *Bjerkandera adusta* to treat MLL from the same landfill (and same well # 24) (Bardi et al., 2017) resulted in average sCOD reduction of 48% during the first stage with 0.5 g/L of glucose addition at the startup and HRT of 72 h whereas 16% sCOD reduction was recorded from MLL alone. The sCOD reduction decreased to 14% when startup glucose was depleted, and no additional glucose was added during the second stage; which increased the sCOD reduction to 24% while 0.5 g/L glucose was again added at the third stage. During the entire study period, no lignocellulosic enzymatic activity was reported and COD/sCOD reduction trend was not stable. In the current study, higher and steady sCOD removal efficiency

and lignocellulosic enzymatic activities suggest better acclimatization for *Penicillium* sp., which could lead to higher performance. In a separate study, Ren (2017) reported 43% and 31% COD reduction from the same MLL by using granular activated sludge SBR fed with 45% and 90% MLL dosage, respectively, which suggests that sequential treatment of aerobic granular sludge and lignocellulosic fungi is a feasible approach to enhance COD removal efficiency from MLL. The result of the current study is promising considering the nature of the MLL with low biodegradability and presence of toxic refractory contaminants. Moreover, a maximum COD reduction of 64% was achieved from the raw MLL considering the 12% COD reduction from pretreatment process. Further exploratory study to understand the chemical characteristics of the degraded components could be beneficial to enhance the performance of the current process.

4.4. Conclusion

This study investigated the lignocellulosic enzymatic activities and contaminants' removal efficiencies (COD, sCOD, TC, TOC, and color) from MLL through batch experimental tests and sequential treatment utilizing an aerobic activated sludge SBR (pretreatment) and a continuous backed bed fungal (*Penicillium* sp.) bioreactor. The maximum COD reduction (41%) was observed from the pretreated MLL with 352 mg/L NH_4^+–N concentration through batch tests and COD reduction efficiency was decreased regardless of the change in NH_4^+–N concentration. The optimal treatment condition (352 mg/L NH_4^+–N) exhibited 193, 37, and 25 U/L of Lac, LiP and MnP enzymatic activity, respectively. The maximum COD reduction efficiency was further increased to 52% in the continuous bioreactor fed with pretreated MLL at HRT 120 h. The total COD reduction of 64% was obtained from raw MLL including the 12% COD reduction from pretreatment process. The fungal bioreactor exhibited 60%, 58%, and 75% of TC, TOC, and color removals efficiency in accordance with the 149, 27, and 16 U/L of Lac, LiP, and MnP enzymatic

activities. The findings of this treatment process seem to be promising and the integration of advanced and sustainable technological approaches could be evaluated to enhance the removal of refractory contaminants from the MLL.

4.5. Supplementary documents

Table 4.4. Contaminants level in MLL from the monitoring data of Well # 24 from 2014 to 2017.

Sampling Date	Units	Criteria	Sep. 2017	Sep. 2016	Sep. 2015	Sep. 2014
Inorganic Parameters						
pH		6.5 – 9.0 [c]	7.27	7.2	7.51	7.66
Total Alkalinity	mg/L		5,840	6,310	4,410	6610
Hardness (as $CaCO_3$)	mg/L		2,190	2,430	2,090	2440
Specific Conductivity	uS/cm	1000 [b]	***15,500***	***15,600***	***12,000***	***15,400***
Turbidity	NTU		35.8	151	146	68.6
Total Dissolved Solids	mg/L	700 [b]	***7,170***	***7,660***	***7,850***	***8,470***
Total Suspended Solids	mg/L	25 [b]	***380***	***310***	***840***	20
Chloride (dissolved)	mg/L	2300	1,700	1,500	1,200	1,600
Sulphate (dissolved)	mg/L		82	40	198	<5
Cyanide (CN)	mg/L	0.066	0.0091	0.0118	0.0136	0.0118
Nutrients						
Dissolved Ammonia	mg/L		983	1,000	590	472
Nitrate-Nitrite Nitrogen	mg/L	10 [b]	< 0.003	< 0.003	< 0.003	< 0.003
Total Kjeldhal Nitrogen	mg/L	15 [a]	***1,050***	***660***	***700.1***	***1,008***
Total Phosphorus	mg/L	1 [a]	***5.32***	***3.62***	***3.62***	***6.3***
Organic Indicators						
Biological Oxygen Demand	mg/L	25 [a]	***87***	***189***	***120***	***<93***
Chemical Oxygen Demand	mg/L		1,460	1,430	1,270	1,470
Metals (Total)						
Arsenic (As)	mg/L	1.9	0.0167	0.0165	0.0227	0.0155
Barium (Ba)	mg/L	29	0.453	0.409	0.495	0.454
Beryllium (Be)	mg/L	0.067	< 0.00020	< 0.0005	< 0.1	< 0.0001
Cadmium (Cd)	mg/L	0.0027	0.00012	< 0.0003	0.000216	0.000099
Calcium (Ca)	mg/L		193	176	197	181
Chromium (Cr)	mg/L	0.81	0.0566	0.0488	0.0472	0.0539
Chromium (Hexavalent)	mg/L	0.14	< 0.01	< 0.01	< 0.001	< 0.002
Copper (Cu)	mg/L	0.087	0.0027	< 0.005	0.0157	< 0.0005
Iron (Fe)	mg/L		1.97	4.74	6.4	3.07
Lead (Pb)	mg/L	0.025	0.00428	< 0.0010	0.00802	0.00031
Magnesium (Mg)	mg/L		500	450	392	483

Manganese (Mn)	mg/L		0.263	0.503	0.741	0.245
Mercury (Hg)	mg/L	0.0028	< 0.00002	< 0.000002	< 0.000002	< 0.000002
Nickel (Ni)	mg/L	0.49	0.201	0.196	0.195	0.21
Potassium (K)	mg/L		597	540	523	640
Dissolved Selenium (Se)	mg/L	0.063	0.00134	< 0.0020	0.00186	0.0013
Silver (Ag)	mg/L	0.0015	< 0.0002	< 0.0005	0.000139	0.000063
Sodium (Na)	mg/L	2,300	1,530	1,270	1,070	1,400
Zinc (Zn)	mg/L	1.1	0.047	< 0.050	0.051	0.0143
Extractables						
Benzo (a) Pyrene (PAH)	ug/L	0.81 (0.15 [c])	***0.9***	***< 2***	< 0.05	< 0.1
Anthracene	ug/L	2.4	< 0.2	< 2	< 0.05	< 0.5
Benzo (a) anthracene (PAH)	ug/L	4.7 (0.018 [c])	***0.7***	***< 2***	***< 0.05***	***< 0.5***
Benzo (g,h,i) Perylene (PAH)	ug/L	0.2	***1.2***	***< 2***	< 0.05	***< 0.5***
Hexachlorobenzene	ug/L	3.1	< 0.05	< 0.005	< 0.05	< 0.05
Phenanthrene	mg/L	0.58 (0.4 [c])	< 0.0002	0.002	0.00089	NA
Phenol	ug/L	12,000 (4 [c])	***33***	***257***	0.183	0.312
Petroleum Hydrocarbons						
F1 (C6-C10 Hydrocarbons)	ug/L	750	29	280	80	NA
F2 (C10-C16)	ug/L	150	***450***	***190***	< 100	***790***
F3 (C16-C34)	ug/L	500	***1,800***	< 200	< 200	***2,400***
F4 (C34-C50)	ug/L	500	< 200	< 200	< 200	430
Volatile Organic Carbons						
BTEX	ug/L		< 25	32	47	NA
Vinyl Chloride	ug/L	1.7	***< 5.0***	***< 4.0***	***< 2.0***	***< 5***
1,4 Dichlorobenzene	ug/L	67	5.1	4.7	2.8	< 5
Chloroform	ug/L	22	< 2.5	< 2	< 1	< 2.5
Trichloroethylene	ug/L	17	< 2.5	< 2	< 1	< 2.5
Tetrachloroethylene	ug/L	17	< 2.5	< 2	< 1	< 2.5
Polychlorinated Biphenyls	mg/L	0.015	< 0.0005	< 0.00005	< 0.0005	0.0006
Pesticides and Herbicides						
2, 4-D	ug/L		< 10	< 20	< 20	< 8
Aldrin	ug/L	8.5 (1 [c])	< 0.05	< 0.005	< 0.05	< 0.05
Chlordane	ug/L	28	< 0.07	< 0.005	< 0.05	< 0.05
Hexachlorocyclohexane	ug/L	1.2	< 0.03	< 0.005	< 0.03	< 0.03
MCPA	ug/L	2.6 [c]	***< 20***	***< 40***	***< 40***	***< 16***

Methoxychlor	ug/L	6.5	< 0.1	< 0.01	< 0.1	< 0.1
DDT	ug/L	2.8	< 0.05	< 0.005	< 0.05	< 0.05
Bacteria						
Total Coliforms	MPN / 100 mL		11,000	11,000	>1,100,000	430
Fecal Coliforms	MPN / 100 mL	200 [a,b]	***1,200***	43	***>1,100,000***	23
E. coli	MPN / 100 mL	200 [a,b]	***1,200***	43	***1,100,000***	4

- Criteria from the Ontario Ministry of the Environment (2011) guideline report on Soil, Ground Water and Sediment Standards for Use Under Part XV.1 of the Environmental Protection Act. Table 3: Full Depth Generic Site Condition Standards in a Non-Potable Ground Water Condition, except for those marked as a, b, and c which are based on the *Tier I, Tier II, and Tier III of Manitoba Water Quality Standards, Objectives, and Guidelines* for wastewater effluents discharged to a water body and water use for aquatic life and wildlife, respectively (Manitoba Water Stewardship, 2011).
- The highlighted values represent exceeding the criteria.
- Data is adapted from the Annual Reports of Brady Road Resource Management Facility (BRRMF, 2017, 2016, 2015, 2014).

Chapter 5: Summary and Conclusions

Fungal lignocellulosic enzymatic activities such as laccase (Lac), lignin-peroxidase (LiP), and manganese-peroxidase (MnP) are frequently detected in most of the species of Basidiomycetes white rot fungi (WRF) and from very few species of Ascomycetes filamentous fungi. Several fungal strains were isolated from mature landfill leachate (MLL) and four of them (newly isolated filamentous fungi: *Trichoderma asperellum, Cladosporium* sp., and *Penicillium* sp.; previously isolated WRF: *Tyromyces chioneus*) were selected from a preliminary screening test to treat the same MLL assuming that the fungi survived in MLL have better adaptability and pollutants degrading capability from the MLL. Another two WRF (*Bjerkandera adusta* and *Phanerochaete chrysosporium*) were also included in the batch experimental tests, which were previously reported as very effective to degrade diverse toxic compounds.

Three newly isolated fungal species were identified as *T. asperellum*, *Cladosporium* sp., and *Penicillium* sp. from the polymerase chain reaction (PCR) with primers specific for internal transcribed spacer (ITS), β–tubulin and calmodulin (CMD) genes (chapter 3). The performance of suspended and polyurethane foam (PUF) immobilized fungal biomass was quite comparable in terms of COD and sCOD removal efficiency. Woodchips (WC) and wheat-straws (WS) acted as the carbon sources for the fungal growth by slowly solubilization and significantly enhanced COD and sCOD removal efficiencies and enzymatic activities. WC seemed to be a better co-substrate among the three tested co-substrates (glucose, WC, and WS) facilitating immobilized growth of fungi on its stable and integrated surface. Further investigation using different wood matrices as the immobilized fungal media could explore the feasibility of advanced bioprocess application.

All tested species, both filamentous and white rot fungi, secreted one or more lignocellulosic enzymes, while the overall Lac secretion was significantly much higher than LiP and MnP. Strong

correlation between enzymatic activities and COD/sCOD removal efficiencies was revealed from all species, which illustrates lignocellulosic enzymatic activities were the main driving force for higher COD/sCOD reduction efficiency. Therefore, lignocellulosic enzymatic activities acted as the predictors to estimate the percentage of COD/sCOD removal efficiency.

Among the four locally isolated native fungal strains, *Penicillium* sp. and *T. chioneus*, proved to be more efficient, even more effective than previously investigated WRF: *B. adusta* and *P. chrysosporium*, in terms of COD/sCOD reduction and ligninolytic enzymes secretion. *Tyromyces chioneus* is the first ever reported species in this research showing significant lignocellulosic enzymatic activity. This finding is promising, and further investigation could explore the potential of this species to remediate toxic contaminants and problematic wastewater.

Considering the COD/sCOD reduction efficiency, the fungal strains can be arranged in the order: *Penicillium* sp. > *T. chioneus* > *B. adusta* > *P. chrysosporium* > *T. asperellum* $\geq$ *Cladosporium* sp., where *Penicillium* sp. seems to be the best species among the six selected fungi, which suggests that some native grown filamentous fungal strains could be more effictive than the WRF in terms of same contaminants' treatability and further investigation could explore even more efficient fungal strain.

Aerobic activated sludge sequencing batch reactor (SBR) feeding with 20% v/v diluted MLL (80% MLL and 20% wastewater) was used evaluate the MLL treatability and observed maximum 10% - 12% of COD reduction and 99%-100% of NH_4^+–N removal efficiency. The SBR was effective to remove ammonia nitrogen but not effective to remove organic contaminants, and therefore considered as the pre-treatment option.

The pretreated and raw leachate with initial NH_4^+–N concentration ranged of 0, 105, 352, and 914 mg/L was used to evaluate the effect of initial NH_4^+–N concentration on COD/sCOD reduction

and enzymes secretion by *Penicillium* sp. Maximum 41% COD reduction (enzymatic activity: 193, 37, and 25 U/L of Lac, LiP and MnP) was observed with 352 mg/L of NH_4^+–N which was then considered as the best feed for the fungal bioreactor. Very low or very high ammonia nitrogen concentration inhibited the fungal performance possibly due to nutrient deficiency or high ammonia toxicity.

Penicillium sp. was utilized in the fungal bioreactor and COD, sCOD, total carbon (TC), total organic carbon (TOC), and color removal efficiencies were evaluated in accordance with the lignocellulosic enzymatic activities. The contaminants removal efficiency (COD, TC, TOC, and color) successively increased with increases of hydraulic retention time (HRT). The fungal bioreactor exhibited maximum 52%, 60%, 58%, and 75% of COD, TC, TOC, and color removals efficiency in accordance with the 149, 27, and 16 U/L of Lac, LiP, and MnP enzymatic activity at HRT 120 h. The sequential treatment options using SBR pre-treatment and fungal bioreactor achieved net 64% COD and 100% ammonia removal efficiency.

The findings of this research seem to be promising and lignocellulosic fungal biomass could be a sustainable treatment option to remove refractory components from the problematic wastewater. The integration of possible advanced biological process could be evaluated to explore further enhancement of this process.

Chapter 6: Engineering Significance

6.1. Importance of the research

Mature landfill leachate (MLL) is a highly polluted toxic liquid which contains mainly four basal groups of contaminants: organic pollutants, inorganic salts, heavy metals and xenobiotic compounds in addition to the wide range of pathogens (Cheng et al., 2017). Due to the higher content of toxic pollutants, little amount of MLL can pollute large volumes of water and poses significant potential risk for the environment, biota and human health as well (Gajski et al., 2012). Therefore, effective treatment of MLL is must before discharging into the environment. Although, several physical-chemical treatment methods have been reported to be effective, these high-tech treatment options are not sustainable approach due to the potential risk of secondary pollution and high operation costs (Gao et al., 2015). In contrast, utilization of biological process is considered as more sustainable approach. Consequently, conventional biological process (activated sludge) is widely used around the world to treat leachate together with wastewater in the municipal wastewater treatment plant, but this process is not effective to remove diverse recalcitrant compounds from the MLL and to meet the straighten regulation. So, developing advanced biological process has become a greater interest for the environmental scientist. Several advanced biological process has effectively remediated young or intermediate leachate, for example, Sun et al., (2015) reported 93.5%, 99.5%, and 99.1% of COD, TN, and NH_4^+−N removal efficiencies from a combination of aerobic sequencing batch reactor (SBR) and up-flow anaerobic sludge bed (UASB), which was operated in a simultaneous anaerobic and anoxic mode. Satisfactory biological remediation of MLL still remains challenges for the scientists.

The lignocellulosic enzymatic activities of some fungal strains attracted the attention of scientist in recent decades due to their capability of degrading diverse toxic compounds. To address the

challenges of sustainable environmental remediation of MLL, this research employed advanced biological process utilizing potential fungal strains through batch experimental tests and sequential treatment options with a combination of aerobic activated sludge SBR and a packed bed continuous flow fungal bioreactor under non-sterile condition.

6.2. Engineering significance and implications

This study explored that neither very limited nor very excess amount of ammonia nitrogen concentration is favorable for better fungal performance and enzymatic activities (due to nutrient deficiency/ammonia toxicity). This information could be beneficial for the researchers to find the optimum fungal growth condition for other type of fungi and to investigate and choose best feed condition for the fungal bioreactor.

WRF are well known for their extracellular enzymatic activities and got greater attention for fungal bioremediation, while little interest has been observed on other types of fungi such as filamentous fungi from the researchers. This study showed that one filamentous fungus and one WRF strain, which were locally isolated from MLL, expressed to be more effective among the six selected fungi. Thus, the current study provides some useful information that there could be some fungal strains other than WRF which would be also very effective for bioremediation.

Among the three co-substrates studied, wood-chips seemed to be more effective acting as the carbon source by slowly solubilization and providing attach growth media on it's stable and integrated surface. The performance of the present study could be enhanced and optimized by utilizing different size and types of wood matters or matrices, for instance sawmill dust and bio-bed, which could simultaneously act as the fungal support media and potential carbon source to obtain best performance.

The higher removal efficiency of recalcitrant compounds from this sustainable approach of advanced biological process using sequential treatment options through activated sludge SBR and fungal bioreactor systems would be inexpensive with low operating and maintenance cost in compared with physicochemical systems. No chemical addition is required as the whole system is based on two biological process, therefore, very negligible chances of secondary pollution.

Advanced biological process is a small-footprint technology for the effective treatment of organics and nutrients from the MLL.

Chapter 7: Recommendations for Future Studies

Although, many of the fungal strains, especially from the group of WRF, have widely been employed to degrade diverse problematic wastewaters, but very limited number of fungal strains have been utilized to treat MLL. One fungal strain may be effective to treat certain type of wastewater or pollutants but might not be effective for MLL and vice versa. Therefore, it is recommended to continue further screening of new fungal strains from the MLL and to continue evaluation of the MLL treatment efficiency.

The newly isolated fungal strain, *T. chioneus,* exhibited better performance but many of its aspects are not known such as toxicity, response with other types of contaminants, and other type of enzymatic activities etc. Therefore, further investigation of these species is highly recommended to explore environmental remediation and the potential of other type of important enzymatic activities.

This research utilized six fungal strains and only the *B. adusta* strain of them has previously been investigated to treat the same MLL, where no or very little amount of enzymatic activity was detected. The enzymatic activities of this species in this research is much higher than the previously reported amount suggesting that same fungal strain could behave differently in different condition.

Therefore, it is recommended to extend this research employing these fungal strains to treat diverse types of contaminants and MLL from different sources and to evaluate the treatment efficiency and enzymatic activities.

This research investigated the contaminants removal efficiency in terms of COD, sCOD, TC, TOC, NH_4^+–N, and color removal. It is recommended to extend this study to investigate the biodegradation of individual component for deeper insight knowledge and to identify which groups of contaminants are more susceptible to this fungal treatment.

Higher removal efficiency of recalcitrant compounds was obtained from this sustainable sequential treatment using activated sludge SBR and fungal bioreactor system. It is recommended to evaluate this system 1) through utilizing different types of fungal strains, 2) for the treatment efficiency with other types of pollutants, 3) for further optimization, and 4) in combination with one or more additional physicochemical or biological treatment steps to obtain higher removal efficiency.

Appendix

List of Abbreviations

Acronym	Expansion	Acronym	Expansion
°C	Degree Celsius	min	Minute
µl	Microliter	mL	Milliliter
µm	Micrometer	MLL	Mature landfill leachate
µM	Micromolar	MLTSS	Mix liquor total suspended solid
AAS	Aerobic activated sludge	MLVSS	Mix liquor volatile suspended solid
ABTS	2,2'-azino-bis-(3-ethylbenozthiazoline-6-sulfonic acid	mM	Millimolar
AC	Activated carbon	MnP	Manganese-peroxidase
AIC	Akaike information criterion	MSW	Municipal solid waste
ANOVA	Analysis of variance	MUT	Mycotheca Universitatis Taurinensis
AOB	Ammonia-oxidizing bacteria	N	North (direction)
AOP	Advanced oxidation process	NCBI	National Center for Biotechnology Information
APHA	American Public Health Association	NO	No co-substrate
AS	Activated sludge	NOB	Nitrite-oxidizing bacteria
BA	Bjerkandera adusta	NSERC	Natural sciences and engineering research council
BTEX	Benzene, toluene, ethylbenzene and xylene		
BIC	Bayesian information criterion	p	Probability
BLAST	Basic local alignment search tool	PAH	Polycyclic aromatic hydrocarbons
BOD	Biochemical oxygen demand	PC	*Phanerochaete chrysosporium*
BT	β-tubulin gene	PCR	Polymerase chain reaction
cm	Centimeter	PEF	Photo-electro-Fenton
CMD	Calmodulin gene	PLS	Partial least square

COD	Chemical oxygen demand	PPCP	Pharmaceutical and personal care products
CS	*Cladosporium* sp.	PS	*Penicillium* sp.
CT	Control	PUF	Polyurethane foam
CTAB	Cetyl-trimethyl-ammonium bromide	RBC	Rotating biological contactor
CV	Cross validation	RO	Reverse osmosis
DDF	Denominator degrees of freedom	rpm	Rotation per minute
DDT	Dichloro-diphenyl-trichloro-ethane	rRNA	Ribosomal ribonucleic acid
DMAB	3-dimethylaminobenzoic acid	s	Second
DNA	Deoxyribonucleic acid	SBR	Sequencing batch reactor
dNTP	Deoxyribonucleotide triphosphate	sCOD	Soluble chemical oxygen demand
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen	SMEW	Standard methods for examination of water and wastewater
E. coli	*Escherichia coli*	SPEF	Solar PEF
EAOP	Electrochemical advanced oxidation process	SSrRNA	Small subunit rRNA
EDTA	Ethylene-diamine-tetraacetic acid	Susp	Suspended biomass
EF	electro-Fenton	SVI	Sludge volumetric index
F	Fungal (*Penicillium* sp.)	T	Transition phases
FIA	Flow inject analyzer	TA	*Trichoderma asperellum*
g	Gram	TC	Total carbon
GETS	Graduate enhancement of tri-council stipends	TC	*Tyromyces chioneus*
GL	Glucose	TOC	Total organic carbon
GLY	Glucose and yeast extract (broth)	U/L	Unit / Liter
h	hour	UASB	Up-flow anaerobic sludge blanket
HRT	Hydraulic retention time	US	Ultrasound (sonication)
ITS	Internal transcribed spacer	UV	Ultraviolet ray/light
Lac	Laccase	v/v	Volume by volume percentage
LiP	Lignin-peroxidase	VFA	Volatile fatty acid

LL	Landfill leachate	VIP	Variable importance in projection
M	Molar (molarity)	VP	Versatile peroxidases
MAP	Magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$)	VSS	Volatile suspended solids
MBBR	Moving-bed biofilm reactor	W	West (direction)
MBR	Membrane bioreactor	WC	Woodchips
MBTH	3-methyl-2-benzothiazolinone hydrazone hydrochloride	WRF	White rot fungi
MEA	Malt extract agar (media)	WS	Wheat-straws
mg/L	Milligram / Liter	WWTP	Wastewater treatment plant

List of Chemical Formula or Symbols

Symbol	Name	Symbol	Name
2, 4-D	2, 4-Dichlorophenoxyacetic acid	MnO_4^+	Permanganate
·OH	hydroxyl radical	$MnSO_4$	Manganese sulphate
C	Carbon	N	Nitrogen
Ca	Calcium	Na	Sodium
$CaCO_3$	Calcium carbonate	Na_2HPO_4	Disodium hydrogen phosphate
Cd	Cadmium	NaCl	Sodium chloride
CH_4	Methane	NH_4^+–N	Ammonia nitrogen
Cl^-	Chloride	NH_4Cl	Ammonium chloride
ClO^-	Hypochlorite	NH_4NO_3	Ammonium nitrate
CO_2	Carbon dioxide	Ni	Nickel
Cr	Chromium	NO_2^-–N	Nitrite nitrogen
Cu	Copper	NO_3^-–N	Nitrate nitrogen
Fe	Iron	NOx	Total NO_2^-–N and NO_3^-–N
Fe^{2+}	Ferrous ion	O_3	Ozone
H_2O_2	Hydrogen peroxide	P	Phosphorus
H_2SO_4	Sulfuric acid	Pb	Lead
Hg	Mercury	PO_4^+–P	Orthophosphate phosphorus
K	Potassium	$S_2O_8^{2+}$	Persulfate
KH_2PO_4	Potassium dihydrogen phosphate	TiO_2	Titanium dioxide
MCPA	2-methyl-4-chlorophenoxyacetic acid	TN	Total nitrogen
Mg	Magnesium	Zn	Zinc
Mn	Manganese		

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