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Enhancing Gas Production in Landfill Bioreactors by Leachate Augmentation

by

Poornima Arundathie Jayasinghe

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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Abstract

Operation of waste cells as bioreactors is an attractive option for managing municipal solid waste. Among various techniques, leachate recirculation is one of the most promising techniques for accelerating waste degradation in landfill bioreactors. Although leachate recirculation has been practised in some landfills worldwide, leachate augmentation with enzymes, prior to its recirculation, to enhance the waste degradation and gas production in anaerobic landfill bioreactors is a relatively new concept, and little is known about its applicability. This research was undertaken to determine the viability of enzymatic augmentation of leachate with peroxidase enzymes to enhance the waste degradation rates at later stages of anaerobic bioreactor operation. A comprehensive set of laboratory experiments were conducted to assess the viability of this process and to identify the process parameters. The results showed that there was a significant increase in the cumulative methane production in enzyme-added batch reactors and flow-through columns compared to the corresponding control operation. This observation is attributed to an increase in the fraction of waste being degraded as indicated by the increasing levels of dissolved organic carbon and decreased lignin levels in the waste.

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List of Abbreviations and Nomenclature

ANOVA	Analysis of Variance
BOD	Biochemical Oxygen Demand
CI	Confidence Interval
CL	Confidence Level
COD	Chemical Oxygen Demand
CRD	Construction, Renovation and Demolition
DO	Dissolved Oxygen
DOC	Dissolve Organic Carbon
DS	Dry Solid
E	Enzyme
FC	Field Capacity
HRP	Horseradish Peroxidase
ICI	Industrial, Commercial and Institutional
LFG	Landfill Gas
LiP	Lignin Peroxidase
MC	Moisture Content
MnP	Manganese Peroxidase
MPN	Most Probable Number
MSW	Municipal Solid Waste
S	Substrate
SbP	Soybean Peroxidase
SD	Standard Deviation
SEM	Standard Error of Mean
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorous
TS	Total Solid
VS	Volatile Solid
VFA	Volatile Fatty Acids
WRF	White Rot Fungi

BOD_5	five day BOD (mg/L)
b	biocell
c	column
DO_1	DO at day 1 (mg/L)
DO_5	DO at day 5 (mg/L)
v	volume of gas phase (mL)
v_{CH_4}	volume of CH_4 (mL)
$x^o_{N_2}$	initial of N_2 gas
x_{CH_4}	mole fraction of CH_4 at time t
x_{CO_2}	mole fraction of CO_2 at time t

$\overline{X}$	sample mean
n	number of repetitions
$\Delta X_{\max}$	maximum error
$X_{\max}$	maximum value of a data set
$X_{\min}$	minimum value of a data set
Re_{column}	Reynolds number - Column
$\mathrm{Re}_{biocell}$	Reynolds number - Biocell
ρ	density of leachate (kg/m^3)
d	average diameter of waste particles (m)
V	superficial velocity of leachate flow (m/s)
μ	viscosity of leachate (kg/m.s)
ε	void fraction in packed waste
Q	leachate flow rate (m^3/s)
A	cross sectional area (m^2)
v'	velocity of the enzyme catalyzed reaction (g_{TOC}/g_{DS}.day)
v''	velocity of the un catalyzed reaction (g_{TOC}/g_{DS}.day)
$V_{\max}$	maximum rate of reaction (velocity) (g_{TOC}/g_{DS}.day)
K_M	Michaelis constant (g_{TOC}/g_{DS})
k_{cat}	catalytic rate constant (1/day)
$[S_o]$	initial concentration of substrate at t_o (g_{TOC}/g_{DS})
$[S_t]$	concentration of substrate at time, t_t (g_{TOC}/g_{DS})
k	un catalyzed rate constant (1/day)
k_E	enzyme decay constant (1/day)
$[E_o]$	initial concentration of enzyme at time 0, t_o (U/L)
$[E_t]$	concentration of enzyme at time, t_t (U/L)
E_a	activation energy (J/mol)
A'	pre exponential factor
R	universal gas constant (J/mol. K)
T	absolute temperature (K)
X_s	substrate conversion ratio
$a(t)$	activity of enzyme at any time *t*
W	weight of enzyme (mg)
U	flow rate of enzyme in leachate (mg/day)
$[L]$	aqueous organic carbon concentration (g_{DOC}/g_{DS})
Y_{S-L}	yield coefficient (ratio) of converting solid carbon to aqueous carbon
K_L	aqueous carbon degradation rate constant (1/day)
$[G]$	gaseous (methane) carbon concentration ($g_{CH4\text{-}C}/g_{DS}$)
Y_{L-G}	yield coefficient (ratio) of converting aqueous carbon to methane carbon

Chapter One: Introduction

1.1 Problem Statement

Sanitary landfilling is an engineering method for disposal of solid waste in a safe manner. The oldest practice of waste disposal, open dumps, has created negative impacts on the environment and has also been unsustainable in terms of resource utilization (Hettiaratchi *et al.,* 2007). Dry tomb landfills were the next step towards the land disposal of solid waste, in which the waste was kept dry to minimize leachate production. In contrast to both open dumps and dry tomb landfills, landfill bioreactors use specific design and operational practices to enhance waste biodegradation together with gas production rate, while minimizing environmental impacts (Yuen, 2001; Reinhart *et al.*, 2002). Enhancing the rate of waste biodegradation in landfills provides a number of benefits, including increased gas production and utilization opportunities as an energy source, cost effective leachate management, and the potential to reuse the landfill space (Pacey *et al.*, 1999; Pohland *et al.*, 2003). Landfill bioreactors could be operated either in anaerobic or aerobic mode or in hybrid mode (sequential or simultaneous anaerobic/aerobic modes). Sequential operation has the advantage of energy recovery as well as resource and space recovery, if the stabilized waste is mined at a later stage (Hettiaratchi *et al.,* 2007).

The rate of gas production from anaerobic waste degradation is not constant. As shown in Figure 1-1, the gas production rate decreases over time (Pohland *et al.*, 2003). One of the main reasons for this observation could be the lack of available carbon for microorganisms. At later stages of anaerobic bioreactor operation, the majority of the organic fraction is composed of compounds such as lignocellulosic materials, rich in lignin, which are somewhat resistant to anaerobic microbial degradation (Higuchi, 2004). In order to maximize the amount of gas produced from a given waste mass during landfill bioreactor operation, it is necessary to investigate methods to prolong waste degradation and gas production. Employing novel methods to assist the resident microbial populations to metabolize lignin would allow for an increase in the available carbon, which would translate into increased CH_4 gas production by methanogenic activity. The increased

lignin degradation would also yield higher waste stabilization rates, which reduces both the time and space requirements for a landfill bioreactor based waste management system.

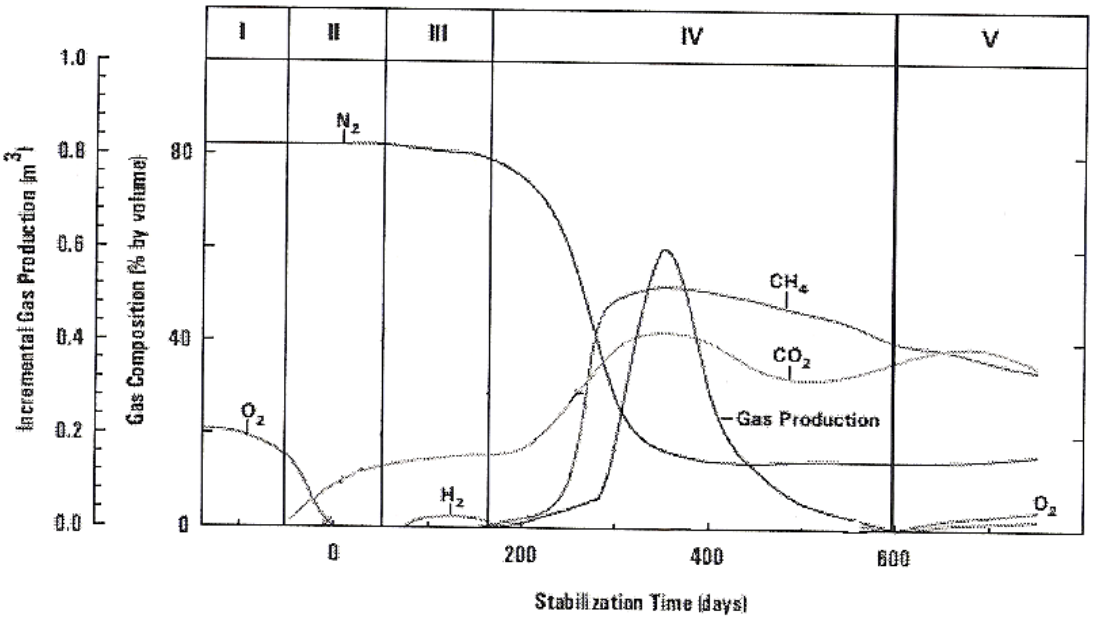


Figure 1-1: Gas production in landfills (Pohland *et al.*, 2003, reproduced with permission from IWA publishing)

There are several issues related to the impact of landfills on the environment. The two most important ones are the release of landfill gas (LFG) to the environment and the production and escape of leachate into the sub-surface. The capture of LFG and use it as an energy source, minimizes LFG related issues, but this is viable only when a sufficient quantity of gas is available throughout the landfill operation period.

Modification of leachate by adding supplemental materials before recirculation is a promising technique for increasing the methane production rate during the gas declining phase of anaerobic bioreactor operation. The leachate acts as a carrier medium of supplemental materials into the landfill bioreactor. Many of the past research efforts were

focused on nutrient balance and pH modification (Barlaz *et al.*, 1990; Warith *et al.*, 1999). The methane production from fresh waste was most consistently enhanced by nutrient addition and pH neutralization (Barlaz *et al.*, 1990). Few past studies have investigated enzyme enhancement of leachate before recirculation (Lagerkvist & Chen, 1993; Cirne *et al*., 2008). These studies focused on the use of cellulase enzymes to enhance the degradation of cellulose-rich waste. Enzymatic augmentation of leachate before recirculation that could potentially enhance the gas production in later stages of landfills (old waste or lignin-rich waste) is a new concept and not much is known about its applicability.

1.2 Research Objectives and Research Approach

The main objective of this research was to investigate a leachate manipulation technique for accelerating anaerobic waste degradation and gas production at later stages of landfill bioreactor operation.

The research approach used in this study is shown in Figure 1-2.

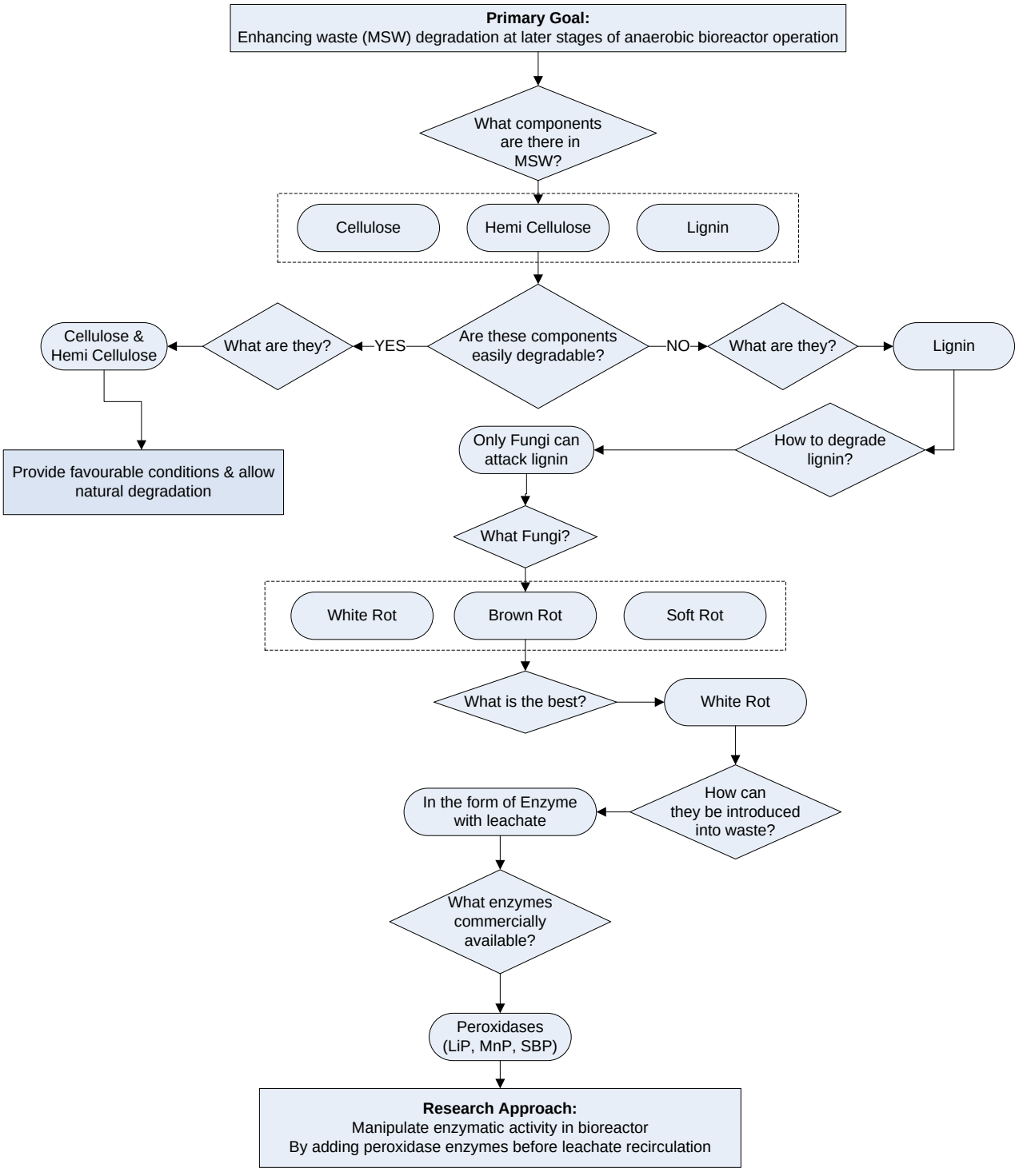


Figure 1-2: Research approach flow chart

The target waste in this study included partially degraded municipal solid waste (MSW). Lignocellulose, which consists mainly of three types of polymers, cellulose, hemicellulose, and lignin, is the major component of MSW. Many microorganisms are capable of degrading and utilizing cellulose and hemicellulose as carbon and energy sources while lignin is highly resistant to degradation (Higuchi, 2004). Therefore, cellulose and hemicellulose degradation in waste occurs at early stages of bioreactor operation under favourable and controlled conditions such as optimum moisture content, nutrients, and temperature. Therefore, at later stages of bioreactor operation, most of the undigested MSW could be lignin-rich waste materials.

Lignin, the major component of the target waste in this research, is a three dimensional polymer connected by several acid resistant C-C linkages. Lignin could be only partly degraded to monomeric compounds by hydrolysis and is mostly degraded by oxidative attack on the C-C bonds (Higuchi, 2004; Martinez *et al*., 2005). White rot fungi are a well-studied group with the ability of degrading lignin by oxidation (Higuchi, 2004; Sanchez, 2009). A special category of commercially available enzymes made from white rot fungi are peroxidases. Peroxidases could potentially catalyze the lignin degradation process.

There is considerable interest in using peroxidases in contaminated site remediation (Husain *et al.*, 2009) and sludge dewatering (Neyens & Baeyens, 2003). Recent studies have shown that the use of peroxidases in waste management processes could be effective in breaking down many organic pollutants. However, enhancing anaerobic degradation of lignin-rich waste materials by adding peroxidase enzymes has not been studied in the past.

Therefore, this study focused specifically on establishing the feasibility of augmenting leachate with different peroxidase enzymes before recirculation to increase the degradation rates of partly degraded waste (i.e. lignin-rich waste materials) at later stages of anaerobic landfill bioreactor operation.

In order to accomplish the main objective, this study was divided into four main stages.

1. In the first stage, laboratory batch experiments were conducted to determine the viability of using enzymes in enhancing the waste degradation and gas production, to identify the most suitable enzyme type, to determine various factors affecting this process, and to obtain the reaction kinetic data.

2. The second stage included laboratory experimental study using flow-through columns to aid the scaling up of the process and to study the impact of enzyme augmented leachate recirculation.

3. In addition to the two experimental stages, this study also included mathematical modeling. The experimental data were fitted to an existing kinetic model and two models, scale up model and gas production predictive model, were developed and validated using the results obtained through experimental stages.

4. The final stage of this study included developing a comprehensive methodology for scaling up the identified novel technology to the field-scale application.

1.3 Outline of the Thesis

The thesis is organized as follows:

Chapter One: Introduction provides the background information related to the research study and the scope and research objectives.

Chapter Two: Literature Review is a comprehensive review of literature on waste degradation.

Chapter Three: Materials and Methods describes the materials used and the experimental test methods used to evaluate the effect of enzyme addition on enhancing anaerobic waste degradation at later stages of bioreactor operation.

Chapter Four: Laboratory Batch Experiments provides an in-depth analysis of laboratory batch experimental study undertaken to identify the various factors affecting the process. It includes of the methodology, experimental design, and results obtained in detail.

Chapter Five: Laboratory Flow-Through Column Experiments provides the methodology, experimental design, and results obtained from laboratory flow-through column study developed to aid in the scale up process.

Chapter Six: Mathematical Models presents the evaluation of the mathematical models used to represent the system behaviors.

Chapter Seven: Development of Methodology for Field Application represents the methodology developed for scaling up this process to the field application, with the potential possibility to apply it to the Calgary biocell.

Chapter Eight: Conclusions and Recommendations summarizes the major conclusions of this study along with recommendations for future work.

Chapter Two: Literature Review

This chapter includes a comprehensive review of literature related to anaerobic waste degradation enhancement at landfills. The literature reviewed covered the theoretical background related to the subject area and a review of the state of current knowledge and developments in waste degradation enhancements.

2.1 Solid Waste

2.1.1 Waste generation and classification

Waste can be simply defined as 'a matter in the wrong place'. Waste is mainly a by product of human activity and the increase in waste generation relates to the rates of population expansion, urbanization, types and patterns of consumption, household revenue, and lifestyles. In 2008, Canadians produced 1,031 kg of waste per person (Statistics Canada, 2010). Canada's per capita income has been steadily increasing since 1980, leading to an increasing household consumption rates (Conference Board of Canada, 2011). In 1980, Canadians generated about 510 kg of waste per capita, since then it has been increased by more than 100 percent (Conference Board of Canada, 2011).

There are 3 main types of waste, solid, liquid, and gaseous. They can be hazardous or non-hazardous and classified by source such as residential, commercial, and industrial, and by composition such as organic and inorganic. The current research focuses on non-hazardous solid waste generated by municipalities, the municipal solid waste (MSW). It is primarily a mixture of residential; industrial (selected), commercial and institutional (ICI); and construction, renovation and demolition (CRD) waste. The waste is highly heterogeneous; the proportion of the various materials present in MSW could vary from one site to another and also within a site. In 2008 in Canada, one-third of waste (nearly 13 million tonnes of waste) for disposal came from residential sources while the other two-thirds came from non-residential sources (Statistics Canada, 2010).

2.1.2 Solid waste management

Managing MSW has been a challenge. The increase in solid waste generation rates and its heterogeneous nature create numerous questions on the waste management strategies and their effects on environment and human health.

The waste management hierarchy refers to 5 R's of reduction of waste at source, reuse of products, recycling of materials, recovery of energy, and residual management (Vesilind *et al.*, 2002). One activity alone may not achieve the objective of minimizing the risks associated with waste; using a combination of these activities could be the most effective way to manage waste to protect human health and the environment.

The disposal in landfill is still the most commonly used MSW management method in Canada (Statistics Canada, 2010). Of Canada's per capita waste generation in 2008, 777 kg went to landfills or was incinerated while 254 kg was diverted from landfill. Overall, this translated into 34 million tonnes of waste handled by the waste management industry; 26 million tonnes of that waste was disposed of in landfills or was incinerated and over 8 million tonnes of waste was diverted or processed through material recovery facilities or centralized composting operations (Statistics Canada, 2010).

2.2 Landfills

Landfilling of solid waste is an important and essential practice of integrated solid waste management because there will always be a need to dispose of waste that cannot be economically reused, recycled, or incinerated. One of the most important impacts of landfills on the environment is the release of landfill gas (LFG) to the environment. The largest anthropogenic source of atmospheric methane in many developed countries is from landfill emissions. For example, LFG represents 30%, 24%, and 25% of the anthropogenic emissions of methane into the atmosphere in Europe, United States, and Canada, respectively (Nikiema *et al.*, 2007). One obvious option for managing landfill gas is to capture the produced biogas and use it as an energy source. However, this is viable only when a sufficient quantity of gas is available during the landfill process.

Landfills could be mainly categorized as open dumps, sanitary landfills, and landfill bioreactors.

2.2.1 Open dumps

The oldest practice of waste disposal was open dumps. In open dumps, all types of waste are stacked on top of each other and they are in contact with the environment. Therefore, open dumps did not provide adequate environmental protection and have not been accepted as a good waste management method. The problems related to open dumps include the ground water and surface water contamination with leachate, uncontrolled production and release of greenhouse and toxic gases, and slow rate of waste degradation and stabilization (Pacey *et al.*, 1999; Yuen, 2001).

2.2.2 Sanitary landfills

Sanitary landfills are the most predominant MSW disposal option in landfilling. They are designed and constructed to eliminate problems associated with open dumps. For example; the sanitary landfills include different features to minimize contamination of groundwater from landfill leachate, including bottom liners, leachate collection and removal systems, and final covers to minimize infiltration of precipitation that contributes to the leachate production. However, sanitary landfills could not control the issues associated with landfill gas emissions and land space (Yuen, 2001). Sanitary landfills are operated as a permanent facility and therefore municipalities have to look for new land space to accommodate the increasing waste capacity.

2.2.3 Landfill Bioreactors

According to Pacey *et al*. (1999), the landfill bioreactor can be defined as "a sanitary landfill that uses enhanced microbiological processes to transform and stabilize the readily and moderately decomposable organic waste constituents within 5 to 10 years of bioreactor process implementation. The landfill bioreactor significantly increases the extent of organic waste decomposition, conversion rates and process effectiveness over what would otherwise occur within the landfill." According to Reinhart *et al*. (2002), the

benefits of landfill bioreactor operation have been first proved through laboratory studies during 1970's and pilot and full scale demonstrations in 1980's.

Advantages of landfill bioreactors can be classified in the form of environmental, regulatory, monetary, and social benefits (Pacey *et al.*, 1999). Some of the key benefits include rapid organic waste degradation and stabilization, maximized landfill gas production, increased landfill space reuse due to rapid settlement during operational period, improved leachate treatment, reduction in greenhouse gas emissions and other environmental impacts, reduction in post-closure care, maintenance and risk, and overall reduction of landfilling cost.

Landfill bioreactors use specific design, operation, and construction practices to enhance the biodegradation of waste and hence the gas production rate. The enhancement techniques used to accelerate the waste degradation and gas productions include leachate recirculation (Reinhart & AlYousfi, 1996; Warith *et al.*, 1999; Morris *et al.*, 2003; Bilgili *et al.*, 2004; Francois *et al.*, 2007), pre-treatment of waste such as shredding, compaction, source separation, waste pre-disposal and post-disposal conditioning and some physico-chemical pre-treatments, such as wet oxidation and ozonation (Delgenes *et al.*, 2002), leachate modifications through addition of nutrients and biosolids (Barlaz *et al.*, 1990; Warith *et al.*, 1999; Suna-Erses & Onay. 2003), temperature modifications in landfills (Ferrer *et al.*, 2008), leachate recirculation at different rates and different frequencies (Suna-Erses & Onay. 2003), and implementation of cover and liner systems (Komilis *et al.*, 1999). However, among the above-mentioned techniques, leachate recirculation is by far the most promising and most effective process based management option for accelerating waste degradation in landfill bioreactors (Francois *et al.*, 2007).

The landfill bioreactors can be operated as aerobic, anaerobic or hybrid mode (sequential or simultaneous anaerobic/aerobic modes). In aerobic bioreactors, air is injected into the waste matrix to accelerate the activity of aerobic bacteria and hence the waste degradation. Carbon dioxide is the main product of aerobic waste degradation process.

The anaerobic bioreactors operate in the absence of oxygen. In anaerobic bioreactor operations, groups of anaerobic bacteria work together to produce methane and carbon dioxide. The hybrid bioreactor landfill process combines elements of both aerobic and anaerobic systems to accelerate the waste degradation. Some of the advantages and disadvantages of the aerobic and the anaerobic bioreactor operation (McCarty, 1964; Rendra *et al.*, 2008) are tabulated in Table 2-1.

Table 2-1: Aerobic VS anaerobic landfill bioreactors

Aerobic Bioreactor Operation	Anaerobic Bioreactor Operation
Advantages	Advantages
✓ Higher rate of waste decomposition and settlement than anaerobic process. ✓ Low landfill leachate production due to evaporation. ✓ Reduction of odour problems. ✓ Potential for landfill mining and sustainability.	✓ Methane is a useful end product; thus suitable for waste to energy programs. ✓ High degree of waste stabilization is possible. ✓ No oxygen requirements, therefore, less energy intensive. ✓ Low production of sludge. ✓ Lower post closure costs. ✓ Lower nutrient demand.
Disadvantages	Disadvantages
✓ Additional cost for injecting air to the landfill. ✓ Risk of fire and explosive gas mixtures through addition of air to landfill. ✓ Hazardous and noxious chemicals may be emitted.	✓ The generation of methane can lead to more environmental problems if not properly handled. ✓ Much more sensitive to many environmental parameters. ✓ Increased potential for production of odours and corrosive gases. ✓ Longer start up time.

2.2.4 Biocell

A novel concept of landfill waste disposal is known as "sustainable landfill biocell concept" or the biocell (Hettiaratchi *et al.*, 2007). The biocell is a variation of the landfill bioreactor approach and involves the operation of a landfill cell under sequential anaerobic - aerobic conditions with leachate recirculation to take advantage of both forms of biodegradation.

The biocell operation consists of three stages. In the first stage, the biocell works as an anaerobic bioreactor and it enhances methane (CH_4) production. In the second stage, the biocell is converted to an aerobic bioreactor. The aerobic biodegradation is carried out up to the point where all the waste is decomposed. The third stage is cell mining to recover resource and space thus making the landfill operation sustainable. Considering the difficulties in locating new landfills in or near urban centers, reusing of space is one of the key benefits of the biocell (Hunte, 2010). The biocell is a holistic approach to waste disposal; some of the direct benefits include energy recovery, CH_4 emission control, minimization of groundwater contamination, and space recovery.

The 'Calgary Biocell' is a full scale landfill bioreactor which is located in Calgary Shepard landfill (Hettiaratchi *et al.*, 2007; Hunte, 2010). It is currently being operated in later stage of its first stage, the anaerobic operation. Further details of the Calgary Biocell will be discussed in Chapter 7.

2.3 Anaerobic Waste Degradation

2.3.1 Waste degradation process

When the waste is placed in a landfill, many different physical, chemical, and biological processes are taking place and result in the release of gaseous and dissolved compounds. When a landfill contains some organic waste, the microbial processes will dominate the stabilization of waste and it leads to the generation of landfill gas and the leachate.

Organic biodegradable components in solid waste begin to undergo bacterial decomposition soon after they are placed in the landfill cell. In this stage, aerobic decomposition occurs because a certain amount of air is trapped within the landfill. The aerobic digestion process can be represented as,

$$C_aH_bO_cN_d + (a + \frac{b}{4} - \frac{c}{2} - \frac{3d}{4})O_2 \rightarrow aCO_2 + (\frac{b}{2} - \frac{3d}{2})H_2O + dNH_3 \qquad \textbf{Equation 2-1}$$

Oxygen may diffuse into the waste from the atmosphere, and aerobic bacteria in the top layers of the landfill will readily consume the oxygen and limit the aerobic zone.

Once the oxygen is consumed, the anaerobic waste degradation process begins. The anaerobic waste degradation is a process by which biodegradable waste is decomposed by anaerobic microorganisms in the absence of oxygen, producing methane and carbon dioxide. The simplified theoretical waste degradation pathways of anaerobic process are presented in Figure 2-1.

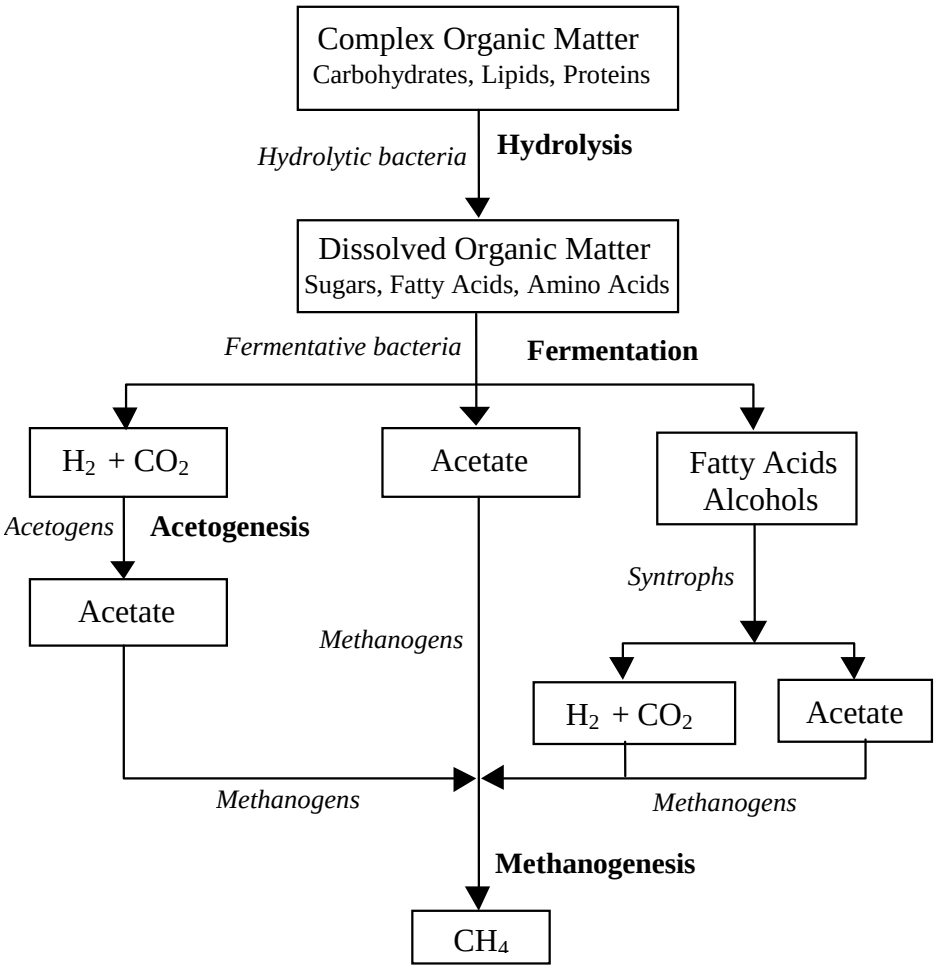


Figure 2-1: Simplified substrate degradation pathways in anaerobic waste degradation process

As shown in Figure 2-1, the anaerobic waste degradation process can be divided into three main stages.

1. The first stage of anaerobic waste degradation is called hydrolysis; the complex organic compounds such as proteins, carbohydrates, and lipids are broken down into simple organic matter, such as amino acids, sugars, and fatty acids, respectively, by the action of extracellular enzymes excreted by hydrolytic

bacteria. Hydrolysis is considered to be the rate limiting step in the solid waste biodegradation process (Haarstrick *et al.*, 2001; Garcia-Heras, 2003).

2. The second stage can be explained in terms of two sub stages. In fermentation, the products from the first stage are further broken down into simple molecules such as short-chain carboxylic acids, acetate, CO_2, and H_2, by fermentative bacteria. In acetogenesis stage, the simple molecules from fermentation are further digested by acetogens to produce H_2, CO_2, and acetate. The fermentation of some intermediate products such as fatty acids (eg: propionate and butyrate), to acetate, CO_2, and H_2 is thermodynamically favorable only at very low H_2 concentrations. Therefore, for acetogenesis to effectively proceed, the methanogens must sufficiently scavenge the available H_2. This phenomenon is referred to as 'syntrophy' (Zehnder, 1978; Mormile *et al*., 1996; Barber, 2007).

3. In the methanogenesis stage, methane is produced by the methanogens. Two most common substrates for methanogens are H_2/CO_2 and acetate. In this stage, acetotrophic microbesconvert acetate to CH_4 and hydrogenetrophic microbes convert H_2 and CO_2 to CH_4.

If the chemical composition of the waste is known, the theoretical methane yield can be predicted using Buswell Equation (Mata-Alvarez, 2003). The overall process of converting organic compound to methane and carbon dioxide may stoichiometrically be expressed by,

$$C_nH_aO_bN_c + (n - \frac{a}{4} - \frac{b}{2} + \frac{3c}{4})H_2O \rightarrow (\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8})CH_4 + (\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8})CO_2 + cNH_3$$

Equation 2-2

However, the anaerobic waste degradation process may fail if one group of microorganisms or one process is inhibited. Therefore, well controlled environmental

parameters, such as moisture content, pH, temperature, nutrients, need to be maintained throughout the process.

2.3.2 Factors affecting anaerobic waste degradation

The major factors affecting the anaerobic waste degradation process include pH, moisture content, temperature, nutrients, and inhibitors.

pH: Methanogens are more susceptible to pH variation than other microorganisms in the microbial community (Khanal, 2008). Therefore anaerobic waste degradation process is affected by slight variation of pH from optimum. The optimum pH for acetogens is 5.5-6.5 and for methanogens is 6- 8 (Jones *et al.*, 1983; Barlaz *et al.*, 1990). However, the optimum pH for the combined culture ranges from 6.8 to 7.4 with neutral pH being the ideal (Khanal, 2008).

Moisture Content: Having adequate moisture is an essential requirement in a functional bioreactor. The availability of moisture promotes microbial degradation in two ways: transfer the nutrients and microbes through solid waste, and flush out soluble pollutants and degradation products. The methane production rate increases as the moisture content of the waste increases. Research findings suggest that an exponential increase in gas production rate occurs between 35% and 60% moisture content of waste (Barlaz *et al.*, 1990, Hamoda *et al.*, 1998).

Temperature: The anaerobic waste degradation process is affected by temperature from both kinetic and thermodynamic point of view; the rate and yield are increasing with the temperature (Mata-Alvarez, 2003). The methanogens mainly contains a mesophilic group with a maximum rate of gas production at around 35-40 ^{0}C and thermophilic group with a maximum around 55 ^{0}C (Mata-Alvarez, 2003; Khanal, 2008). However, anaerobic digestion can function over a large range of temperatures from around 10 ^{0}C to 65 ^{0}C (Scherer *et al.*, 2001; Sternenfels, 2012).

Nutrients: Organic and inorganic substances are required for anaerobic waste degradation. In addition to the organic carbon substrate, there is a need for macro nutrients such as nitrogen and phosphorous and micro nutrients such as calcium, magnesium, potassium, nickel, iron, zinc, copper, cobalt, and some vitamins. Various researchers have suggested different ratios of C (expressed as COD), N, and P based on biodegradability of waste, for example, COD:N:P ratio of 100:0.44:0.08 (McCarty, 1964; Christensen and Kjeldsen, 1989) and 350:7:1 (Khanal, 2008). However, an average ratio of COD:N:P of around 100:1.2:0.2 can be recommended for a substrate to be anaerobically degraded (Mata-Alvarez, 2003).

Inhibitors: The absence of oxygen is an essential condition for the anaerobic bacteria to grow. There are some substances that at a given concentration inhibit the bacterial activity; ammonia-nitrogen above 1500 mg/L (Khanal, 2008) and hydrogen sulphide above 200 mg/ L (Mata-Alvarez, 2003) are inhibitors. However, the presence of some substances can act as stimulants at their low concentrations or inhabitants at high concentrations (McCarty, 1964). For example, sodium is a stimulant when the concentration ranges between 100-200 mg/L while it is an inhibitor when the concentration is higher than 3000 mg/L.

2.4 Anaerobic Waste degradation Enhancements

Since the anaerobic waste degradation process is affected by various parameters, a well controlled environment needs to be maintained throughout the process. While controlling the existing conditions, it is also important to identify various methods which could stimulate the process in order to increase the waste degradation and gas production.

Two of the most important process-based techniques used to accelerate the gas production from bioreactor landfills are pre-treatments of waste (Mata-Alvarez, 2002) and leachate recirculation and enhancements (Reinhart & AlYousfi, 1996; Warith *et al.*, 2001; San & Oney, 2001; Morris *et al.*, 2003; Bilgili *et al.*, 2004; Ozkaya *et al.*, 2004; Francois *et al.*, 2007). The waste pre-treatment strategies include waste shredding and

compaction, waste pre-disposal conditioning (eg: thermal conditioning of waste), and physico-chemical pretreatments, such as wet oxidation and ozonation (Delgenes *et al.*, 2002). The process of waste pre-treatment has to be undertaken before the waste placement in the landfill. The process of leachate modifications and recirculation can be continued as the waste degradation process continues. However, among these two techniques, leachate recirculation offers the best potential in terms of accelerating waste degradation and gas production (Francois *et al.*, 2007).

2.4.1 Leachate recirculation

Landfill leachate is formed when water percolates through the waste matrix. The leachate picks up the organic and inorganic components from waste by physical, hydrolytic and fermentative processes. During leachate recirculation, leachate collected at the base of the landfill is recirculated through waste matrix several times instead of a single pass. Leachate recirculation enhances the microbial activity by supplying the optimum moisture requirements, reintroducing the nutrients, homogenizing the environment allowing better contact between the microbes and substrate, and diluting the inhibitory compounds (Suna-Erses & Onay, 2003). As a result, the process of conversion of organic matter to methane is enhanced.

Many researchers consider leachate recirculation alone as a method to increase the moisture content of the waste. This only accelerates early hydrolysis and acidogenesis stage, which results in a high acid concentration in leachate (Yuen, 2001). The modification of leachate before recirculation, that may aid the biodegradation process, has received relatively less attention.

2.4.2 Leachate enhancement and recirculation

There are some laboratory and field scale investigations on manipulation of leachate before recirculation to enhance biodegradation. The following section reviews these leachate enhancement techniques identified through different studies.

The available studies related to leachate manipulation process include addition of sludge (Barlaz *et al.*, 1990; Warith *et al.*, 1999; Alkaabi *et al.*, 2009), addition of supplemental nutrients and buffer (Warith *et al.*, 1999), replacement of present landfill leachate with old landfills leachate (Suna-Erses & Onay. 2003), accumulation of potentially toxic components from leachate nitrification (Vigneron *et al.*, 2005; Tallec *et al.*, 2009) and augmentation of leachate with potential enzymes (Lagerkvist & Chen, 1993; Sah *et al.*, 2006; Cirne *et al.*, 2008). Among these techniques, the addition of sludge is shown to be the most common and oldest practice.

Warith *et al.* (1999) conducted a pilot scale experimental study with simulated landfill cells filled with MSW over a period of 65 weeks to study the effect of addition of supplemental materials to leachate during recirculation. In this study, the recirculated leachate was supplemented in two ways; addition of nitrogen and phosphorus with buffer as supplemental nutrients to balance the nutrient deficiency within the solid waste matrix and addition of primary sludge to increase the microbial population within the waste. The effectiveness of adding these materials was determined by analyzing the characteristics of effluents, such as BOD, COD, TOC, and heavy metal concentrations. Experimental results indicated that the addition of supplemental materials to leachate during recirculation significantly enhances the rate of biodegradation of solid waste. BOD:COD ratio in sludge-added columns was about 0.9, while it was about 0.7 in nutrient-added columns. In the control column, the BOD:COD ratio was fluctuated around two of these values over the monitoring period. Towards the end of the experiments, this ratio decreased to about 0.4 in both cases, indicating a decrease of biodegradable components of waste over time. Comparing the effectiveness of the two supplemental methods, the waste stabilization in sludge-added columns was faster than the buffer and nutrient-added columns.

A laboratory study carried out by Alkaabi *et al.* (2009) showed that the addition of sludge under saline conditions enhances the biodegradation of MSW. Two groups of laboratory scale bioreactor cells were used, one group was used to study the effect of salinity of

water on waste degradation under different operating conditions and other group was used to study the impact of sludge addition under saline conditions. The methane yield was about 14% more in the bioreactors with sludge addition at different salt concentrations compared to the bioreactors operated without sludge addition. High salt concentrations (3% W/V) inhibited the waste biodegradation as evidenced by the methane production, the percentage reduction in leachate concentration, and the settlement that occurred during the study. In terms of leachate quality, the percentage reductions of COD, BOD, and VFA were higher in the bioreactors operated with sludge addition and showed a significant decrease with increasing the salt concentrations.

Studies have been conducted on recirculating methanogenic leachate in the early stages of landfills, i.e. young landfills. The utilization of leachate from mature landfills provides additional substrate and nutrients to young landfills. Both laboratory and field studies have shown that there are many benefits of this practice, including early methane production, accelerated waste stabilization and rapid reduction of leachate strength (Yuen, 2001; Suna-Erses & Oney, 2003). Suna-Erses & Oney (2003) studied this leachate management strategy in laboratory by recirculating an anaerobically digested mature landfill leachate into a young landfill together with the addition of buffer solutions. During old landfill leachate recirculation in young landfills, COD value in the young landfill decreased by about 43%. Moreover, introducing methanogens through old leachate showed a significant improvement in methane production in young landfills.

A study was conducted by Vigneron *et al.* (2005) to develop long term nitrogen management strategy in bioreactor landfills. In general, existing landfill leachate recirculation process does not involve ammonia elimination. Therefore, in landfill bioreactors, NH_4^+ may accumulate to higher levels than during conventional landfilling, which could cause an inhibition of waste degradation. To avoid this inhibition, Vigneron *et al.* (2005) proposed to carry out the aerobic treatment of leachate outside the landfill. This is one of the new strategies for NH_4^+ removal from bioreactor landfills leachate. The aerobic treatment converts NH_4^+ to NO_3^-, so that when nitrified leachate is recirculated,

the landfill is used as an anoxic bioreactor for the reduction of NO_3^- to N_2 gas by denitrification (Vigneron *et al.*, 2005).

The leachate manipulation with enzymes before recirculation was first studied by Lagerkvist & Chen (1993). Laboratory column experiments were carried out to investigate the effect of addition of cellulolytic enzymes to fresh MSW. The cellulase enzyme used was Econase. The effect of enzymes on waste degradation was studied during acidogenic and methanogenic degradation stages separately. It was observed that there was an enhanced degradation of waste by adding enzymes compared to the control cell. Success of this manipulation was measured by cellulose content and conversion of volatile solids (VS). The observed conversion of cellulose was 42-70% in cells with enzymes addition and 29% in cells without enzyme addition. The conversion of VS was approximately 40% to 50% in enzyme added cells. Furthermore, the enzymes were added a second time during the decline of gas production, but it did not result in any increase in gas production rates. This leads to the conclusion that further degradation could be limited by other factors such as end product concentrations (Lagerkvist & Chen, 1993).

Sah *et al.* (2006) investigated the impact of enzyme addition on waste paper degradation. The enzyme used was Soyabean Peroxidase. Three closed loop leachate recirculating columns were operated for 180 days. In order to compare the effect of enzymes, one column was operated as a control column, where there was no leachate manipulation with enzymes before recirculation. In the other two columns, the leachate was enhanced with enzymes prior to recirculation in anaerobic stage of operation. They monitored the effect of enzymes on waste degradation in terms of leachate characteristics, such as COD, BOD, TS, and settlement behaviours of laboratory simulated landfills. Findings of this study showed that the columns with enzymes exhibited faster decomposition than the control column. They also compared the results obtained from enzymes-amended experiments with the results obtained from biosolids-amended experiments. The addition of biosolids showed faster biodegradation than natural biodegradation. However, the bioreactors with enzyme exhibited faster biodegradation than the bioreactors with

biosolids. The COD reduction efficiency in bioreactors with enzyme was about 2 times higher than the bioreactors with biosolids. The BOD reduction efficiency in bioreactors with enzyme was about 1.6 times higher than the bioreactors with biosolids.

The most recent study among the existing studies related to enzyme addition was conducted by Cirne *et al.* (2008). They suggest that the degree of solubilization of organic compounds in waste digestion systems could be increased by addition of cellulolytic enzymes. The first stage of this study was the aeration of leachate before recirculation while the second stage was the enzyme addition under anaerobic conditions. Of these two stages, the second stage showed the better performance in terms of degree of solubilization, with the increase of degree of solubilization by about 34%.

According to the above discussion on existing studies, it is clear that a significant increase of waste degradation could be achieved through these practices. However, further research is required in order to better understand the importance of leachate manipulation methods.

2.5 Enzymatic Enhancement of Lignin-rich Waste Degradation

As described before, one of the possibilities for stimulating the anaerobic waste degradation process of lignocellulosic materials is to manipulate the natural enzymatic activity by supplying additional enzymes into the system. However, the available studies related to enzyme supplementation through leachate recirculation consider enhancing the degradation of fresh waste at early stages of bioreactor operation. Enhancing waste degradation at later stages of anaerobic bioreactor landfills consisting of lignin-rich waste materials landfills has not been studied in the past. Therefore, this study intends to determine the viability of augmenting leachate with different peroxidase enzymes to increase the lignin-rich waste degradation rates at later stages of anaerobic bioreactor operation. This section of the thesis, therefore, discusses the theoretical background related to the functional behaviour of lignin and enzymes and the mechanism of lignin degradation by enzymes.

2.5.1 The Structure of lignin

A representative structure of lignin, the major component of the target waste in this research, is shown in Figure 2-2. Lignin is a complex three dimensional polymer consisting of non-repeating phenyl propanoid units linked by various C-C and ether bonds. The stereo irregularity of lignin makes it very resistant to be absorbed or degraded by intracellular enzymes (Barr & Aust, 1994).

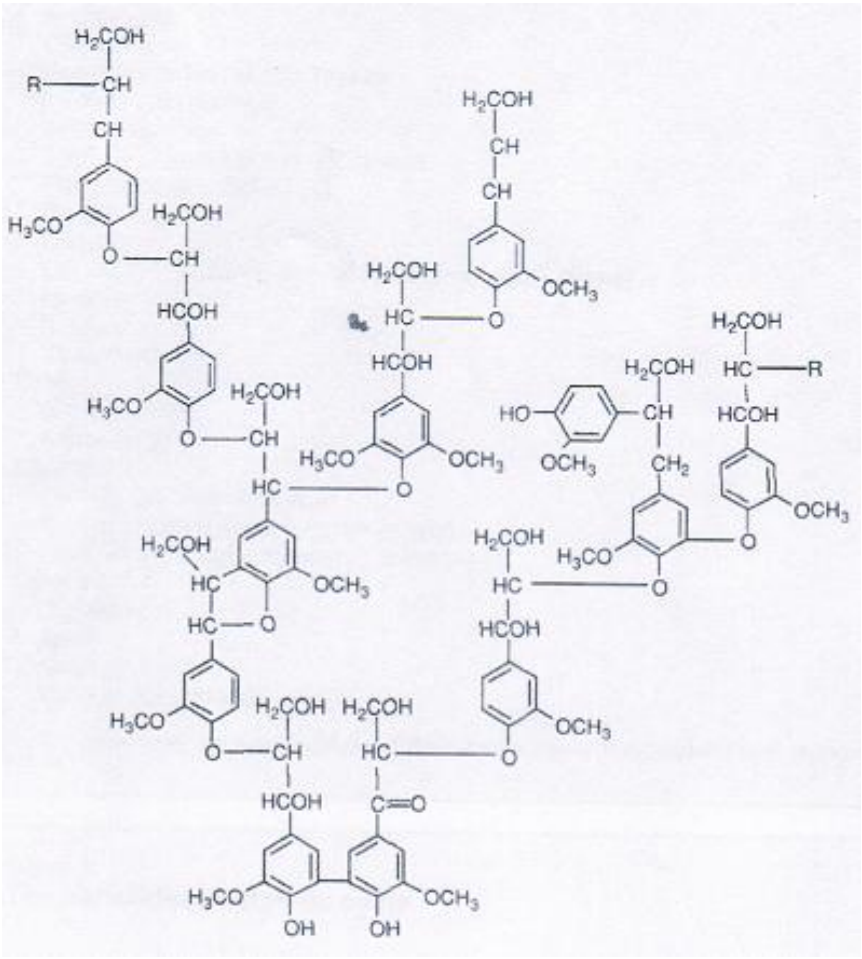


Figure 2-2: Representative structure of lignin (Adapted with permission from Barr & Aust, 1994, American Chemical Society)

Lignin could be only partly degraded to monomeric compounds by hydrolysis and is mostly degraded by oxidative attack on the C-C bonds (Higuchi, 2004; Martinez *et al*.,

2005). White rot fungi (WRF) are a well studied group with the ability of degrading lignin by oxidation (Higuchi, 2004; Sanchez, 2009). WRF have a unique mechanism in degrading lignin; these fungi do not use lignin as a carbon source for growth, instead, they degrade the lignin to obtain cellulose, which is their actual carbon and energy source (Have & Teunissen, 2001).

2.5.2 Peroxidase enzymes

Enzymes are macromolecules, mostly of protein nature, that occur in all living organisms. They are biological catalysts or assistants that can either launch a reaction or increase the reaction rate by decreasing the activation energy of the particular reaction. Peroxidases are a special category of enzyme made from WRF which have the unique ability of assisting lignin degradation. Peroxidases catalyze a variety of reactions but they all require the presence of peroxides such as hydrogen peroxide (H_2O_2) to activate them. Hydrogen peroxide first oxidizes the enzyme, which in turn oxidizes the substrate (Barr & Aust, 1994).

Once activated by H_2O_2, peroxidase enzymes can catalyze the oxidation of a wide variety of toxic aromatic compounds including phenols, biphenols, anilines, benzidines, and heteroaromatic compounds (Neyens & Baeyens, 2003; Husain *et al.*, 2009). Therefore, peroxidase can be used in many industrial and commercial processes, such as wastewater treatment, soil remediation, sludge dewatering, and pulp and paper bleaching, etc. Compared to the conventional waste treatment methods, the high cost of the enzyme required to treat the contaminant in large scale could be the main prohibitive factor of applying this technology in waste treatment.

Examples of commercially available peroxidases are lignin peroxidase (LiP), manganese peroxidase (MnP), soybean peroxidase (SbP), horseradish peroxidase (HrP), and laccases. Of these peroxidases, LiP and MnP are described as true lignin degraders because of their high potential redox value (Martinez *et al.*, 2005).

LiP, also known as ligninase, is a part of the extracellular enzyme system of white rot fungi *Phanerochaete chrysosporium*. LiP is a glycol-protein with molecular weight of ~ 38-46 kDa (Cullen & Kersten, 2004). LiP is mainly responsible for C-C bond cleavage and ring opening in lignin (Gronqvist *et al.*, 2003). LiP was also shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds (Cai & Tien, 1991).

MnP produced by *Phanerochaete chrysosporium* are somewhat larger heme-protein than LiP with molecular mass of 47-60 kDa (Hatakka *et al.*, 2003). The principle function of MnP is to oxidize Mn^{+2} to Mn^{+3}, using H_2O_2 as an oxidant (Gold *et al.*, 1991). The free radical forms during MnP reaction oxidizes both phenolic and non-phenolic subunits in lignin and form phenoxy radicals, which lead to further decomposition of lignin molecule (Hatakka *et al.*, 2003).

SbP is an enzyme found in the husk or seed of the soybean plant. SBP is an anionic peroxidase that has a pH of 4.1 and molecular weight of ~37 kDa (Sah *et al.*, 2006). HrP is one of the most studied enzymes among the available commercial enzymes in waste treatments. HrP is particularly suitable for wastewater treatment because it retains its activity over a broad range of pH and temperature compared to other peroxidases (Karam & Nicel, 1997). Laccases are copper oxidases that oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl-C bond cleavage (Cullen & Kersten, 2004).

2.5.3 Enzyme-Substrate reaction mechanism

The simplest mechanism of an enzyme with a single substrate can be explained using a lock and key analogy which was first proposed by Emil Fischer in 1894 (Ophardt, 2003). As shown in Figure 2-3, a substrate molecule binds to the active site on the enzyme to form an enzyme-substrate complex. The complex goes through a transition state and leads to a molecule with a covalent bond between the enzyme and the substrate. After decomposition, the substrate is converted to stable product and the original enzyme becomes available for a new substrate. This process is continued until complete

decomposition of the substrate or the inactivation of the active site of the enzyme. However, not all experimental evidence could be adequately explained by using a rigid enzyme model like lock and key model, the models may need to be modified (Ophardt, 2003)

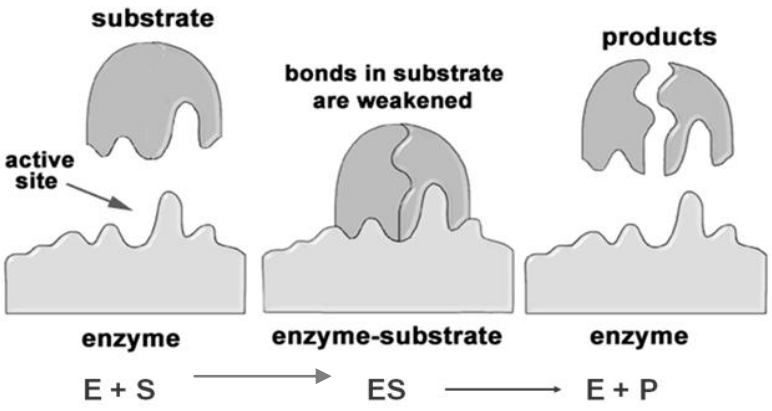


Figure 2-3: Enzyme-substrate reaction (Adapted with permission from Armstrong, 2012, Wayne P. Armstrong)

There are various conditions in which the peroxidase enzymes become inactivated. The main factors affecting the enzyme activity are the concentration of substrates, temperature, pH, concentration of H_2O_2, and the presence of inhibitors. Depending on the type of the enzyme, these parameters need to be maintained at their optimum conditions.

2.5.4 Enzyme Catalytic Cycle

The peroxidase catalytic cycle is shown in Figure 2-4.

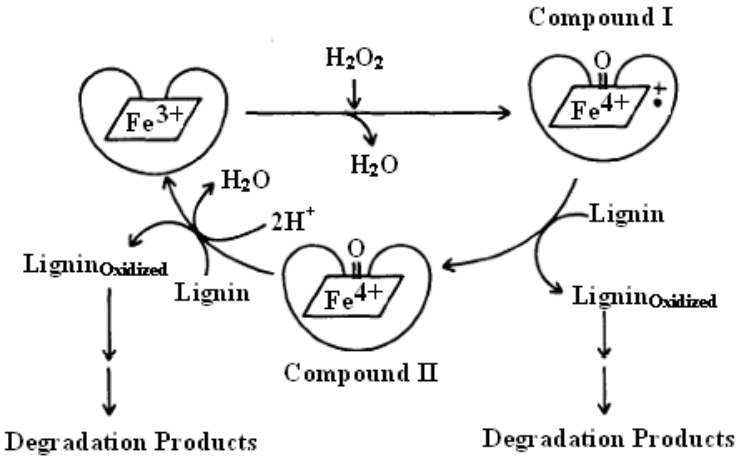


Figure 2-4: Peroxidase catalytic cycle (Adapted with permission from Barr & Aust, 1994, American Chemical Society)

Peroxidases use H_2O_2 to promote the one electron oxidation of lignin to free radical. In the resting state, the heme ion, the functional group of the peroxidase, is in the ferric state (Fe^{+3}). The catalytic oxidation of lignin by peroxidases is a three-step process (Barr & Aust, 1994).

1. In the first step, H_2O_2 oxidizes the ferric enzyme (by two electrons) to water and Compound I. Compound I is also an enzyme, which contains an oxyferryl (Fe^{+4}- O) cation radical.

Native (ferric) peroxidase enzyme + H_2O_2 → Compound I + H_2O

2. In the next step, Compound I oxidizes lignin substrate (S) (by one electron) to a substrate radical (S*) and compound I can be reduced to Compound II (by one electron).

Compound I + S → Compound II + S*

3. In the final stage, Compound II again oxidizes another lignin substrate to become reduced to the original enzyme. Substrate is oxidized to a substrate radical.

Compound II + S → Native (ferric) peroxidase enzyme + S*

2.5.5 Lignin Degradation Mechanisms

Lignin degradation by peroxidases is an oxidation process. It requires oxidative attack on the carbon-carbon (C-C) and ether (R-O-R) inter-unit bonds. The simplified lignin degradation pathways by peroxidase enzymes are summarized in Figure 2-5.

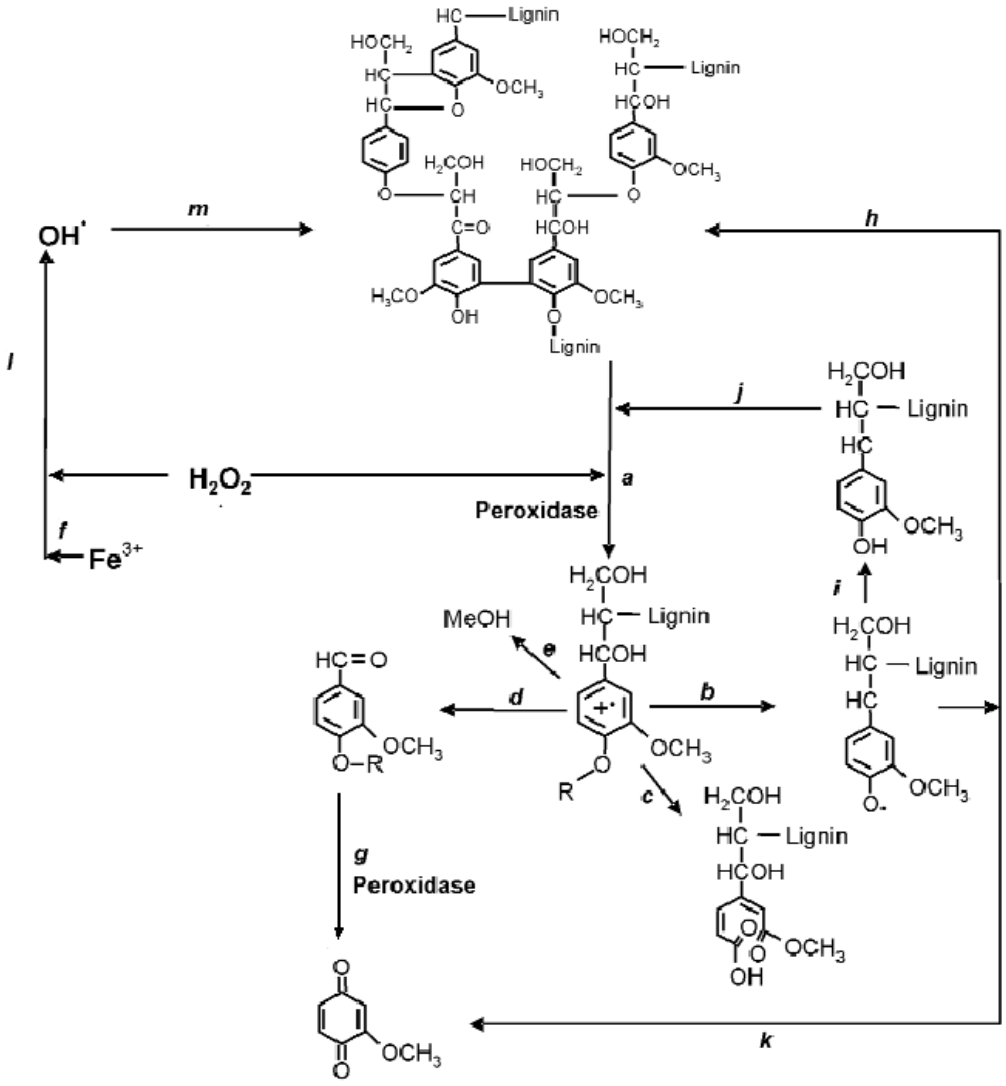


Figure 2-5: Lignin degradation pathways by peroxidases (Sources: Simplified from Martinez *et al.*, 2005)

Related to Figure 2-5,

(**a**) Enzyme oxidizes the lignin polymer generating aromatic radicals. These evolve in different non-enzymatic reactions,

(**b**) C_4-ether breakdown

(**c**) aromatic ring cleavage

(**d**) Cα-Cβ breakdown

(**e**) demethoxylation

Phenoxy radicals from C_4-ether breakdown (**b**) can repolymerize on the lignin polymer (**h**) if they are not reduced by peroxidases to phenolic compounds (**i**). The phenolic compounds formed can be again reoxidized by peroxidases (**j**).

Phenoxy radicals (**b**) can also be subjected to Cα-Cβ breakdown (**k**), yielding *p*-quinones. Quinones can also be formed by aromatic aldehydes (**g)**. Ferric iron present in lignin or peroxidase are reoxidized (**f**) with H_2O_2 to form hydroxyl free radical (OH^*) (**l**). The hydroxyl radical is a very strong oxidizer that can initiate the attack on lignin (**m**) in the initial stages of lignin degradation. Then, lignin degradation proceeds by oxidative attack of the enzymes as described above.

In summary, the oxidative reactions of lignin with peroxidase enzyme often result in demethylation, hydroxylation and dimerization of lignin. The products are simple molecules compared to the lignin polymer, which could lead to further degradation. For example, the reaction of lignin and peroxidases may produce several fatty acids which could enter the metabolic pathways ultimately leading to methane generation (Have & Teunissen, 2001).

2.6 Reaction Kinetics

2.6.1 Waste degradation kinetics

Studying the kinetics of solid waste biodegradation is important, because of the practical importance of being able to predict how long it takes to achieve stabilization of solid

waste in landfills and the study of reaction rates leads to an understanding of mechanisms of the reaction (Wang, 2004; Abu Qdais & Alsheraideh, 2008).

Most of the reactions taking place in the waste degradation landfills are catalyzed by naturally occurring microorganisms. Therefore, the common practice to measure the performance of the solid waste degradation process is to measure the rate at which the microorganisms metabolize the waste, which, in turn, is directly related to their rate of growth (Smith, 1981; Haarstrick *et al.*, 2001; Bizukojo *et al.*, 2002; Wang, 2004).

The most commonly used model relating the microbial growth to the substrate utilization is the Monod equation (Wang, 2004). It is expressed as;

$$\mu = \mu_{\max} \frac{C}{K_s + C} \qquad \textbf{Equation 2-3}$$

Monod equation describes the relationship between the concentration of the substrate (C) and the specific growth rate of biomass (μ). $\mu_{\max}$ is the maximum specific growth rate and K_s half velocity constant, the substrate concentration at which the specific growth rate is one half $\mu_{\max}$. This equation describes that the overall rate of metabolism is controlled by the substrate concentration and also this model is most rigorously applicable to soluble substrates under conditions where the concentration of microorganisms involved can be evaluated (Smith, 1981).

In relating the microbial change to the substrate utilization or the gas production, the net rate of microbial change needs to be accounted for considering both the microbial growth and the decay rate as below.

$$\frac{dC}{dt} = -\frac{1}{Y}(\mu_{\max} \frac{C}{K_s + C} - k_d)X \qquad \textbf{Equation 2-4}$$

where, k_d is the specific decay rate which includes respiration and death, X is the microbial concentration, and Y is the yield coefficient.

In general, waste degradation follows first order kinetics (Bizukojo *et al.*, 2002; Vavilin, 2008). The half-life (the time required for the concentration/quantity of waste to drop to one-half of its original value) for the first order reactions can be determined using,

$$k = \frac{0.693}{t_{1/2}} \qquad \textbf{Equation 2-5}$$

where, $t_{1/2}$ denotes the half-life and k denotes the first order rate constant.

2.6.2 Enzyme reaction kinetics

The study of the chemical reactions rates and the mechanisms that are catalyzed by enzymes is called enzyme kinetics. The kinetics of enzyme reactions are similar to those non-enzymatic reactions except the enzymes have a unique way of accelerating the reaction (Grunwald, 2009). Kinetics of enzyme catalyzed reactions is referred to as Michaelis-Menten kinetics (Shuler & Kargi, 2002, Yang *et al.*, 2005).

For example, the Michaelis-Menten kinetic model for a mono substrate reaction is shown below. A single molecule of an enzyme (E) combines reversibly with a single molecule of substrate (S) to form an enzyme-substrate complex (ES), which is then transformed in to a molecule of a product (P) and the free enzyme (E).

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_3} E + P \qquad \textbf{Equation 2-6}$$

The Michaelis-Menten equation of this reaction mechanism is,

$$v' = -\frac{d[S]}{dt} = \frac{V_{\max}[S]}{K_M + [S]}$$ **Equation 2-7**

where,

k_1, k_2, k_3 are the rate constants

v' is the velocity of the reaction

$V_{\max}$ is the maximum rate of reaction (velocity), $V_{\max} = k_3[E_o]$

K_M is Michaelis constant, $K_M = \left(\frac{(k_2 + k_3)}{k_1}\right)$

$[S]$ is the concentration of substrate

$[E_o]$is the initial concentration of enzyme

At low substrate concentrations, the rate of reaction (v') is proportional to the substrate concentration ($[S]$); hence the reaction is first order. At high substrate concentrations, the rate of the reaction becomes constant and independent of substrate concentration; the reaction is zero order. In practice, initially the rate of the reaction is only restricted by the ability of the enzymes to utilize the substrate which is in excess, thus the reaction kinetics are zero order. However, as the substrate is utilized the reaction begins to become substrate limited, and thus the reaction becomes first order (Wang, 2004).

The Michaelis-Menten equation shown in Equation 2-7 can be solved in several linear transformations; the most popular forms are as follows (Leskovac, 2003).

Lineweaver-Burk plot $$\frac{1}{v'} = \frac{1}{V_{\max}} + \left(\frac{K_M}{V_{\max}}\right)\frac{1}{[S]}$$

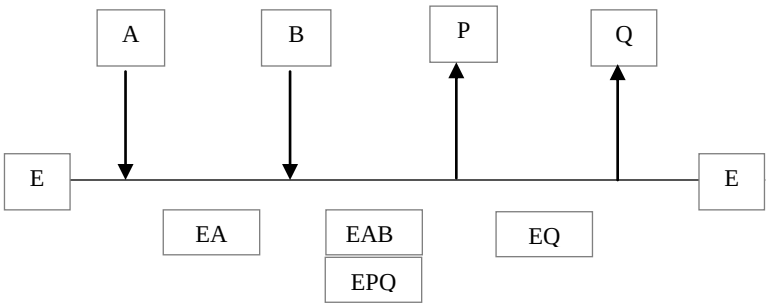
Hanes plot $$\frac{[S]}{v'} = \frac{K_M}{V_{\max}} + \left(\frac{1}{V_{\max}}\right)[S]$$

Eadie and Hofstee plot $$v' = V_{\max} - K_M\left(\frac{v'}{[S]}\right)$$

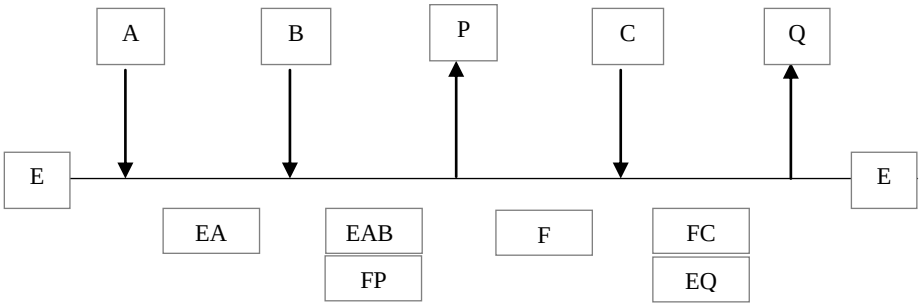
$V_{\max}$ and K_M can be determined by plotting the experimental results of reaction rate and substrate concentration in one of these three forms.

The mechanisms in which the substrate binds to an enzyme's active site and product formation can be divided into single (mono)-substrate and multiple-substrate mechanisms. Most enzymes of known function catalyze reactions involve more than one substrate. The mechanisms of those multi substrate may be quite complex, yet it usually follows the same form of Michalis-Menten model (Plowman, 1972). The rate of reaction of those multi substrate reactions is measured by varying one substrate at a time while keeping the other substrates constant.

Two well studied reaction mechanisms for enzyme catalyzed reactions are the sequential mechanism and the ping-pong mechanism (Plowman, 1972; Leskovac, 2003; Grunwald, 2009). The mechanism can be further described as uni, bi, tri, etc., to indicate the number of substrate addition and product release in each group. In the sequential mechanism, all substrates must be present on the enzyme before any product can leave. The sequential mechanism could be designed as ordered or random, depending on whether the substrates add and the products release in an obligatory sequence or in a non-obligatory sequence (Plowman, 1972). In the ping-pong mechanism, one or more products are released during the substrate addition sequence, thereby breaking the substrate addition sequence into two or more smaller segments. For example, some of those mechanisms can be systematically represented as shown in Figure 2-6.



(a)



(b)

Figure 2-6: The reaction sequence of enzyme-catalyzed reaction (a) Ordered Bi Bi (b) Bi Uni Uni Uni Ping-Pong

where,

E = Enzyme

A, B, C = Substrate

P, Q = Products

EA, EAB, EPQ, EQ, FP, FC = Enzyme-Substrate Intermediates.

Writing the mechanisms of complex reactions in ordinary form as chemical equations is complex and time consuming (Plowman, 1972). The steady state rate equations for mechanisms with multi substrate or products can be easily obtained by King Altman method (King & Altman, 1956). This method has been used extensively in deriving

enzyme kinetic equations for decades, and is probably the standard by which other methods are validated (Taylor, 2002).

For the application of the King Altman method, it is necessary to write a master pattern, which represents the mechanism of the enzyme catalyzed reaction including all the enzyme species in a closed loop format without any loose ends. All the reactions are treated as first order reactions, for example, the second order reactions are replaced with pseudo-first order reaction constants. The partial patterns for each species need to be identified from the master pattern. Partial patterns consist only of lines from the master patterns, connecting every enzyme species in an open loop. For each partial pattern and for each enzyme species, the product of the rate constant is written down. The ratio of concentration of each enzyme species to the concentration of the total enzyme is the sum of all its products obtained from all partial patterns of that particular enzyme species, divided by the sum of all products for all of the enzyme species (Leskovac, 2003).

2.7 Bioreactor Scale Up

The basic concept of scaling up is to provide a larger system that duplicates exactly the same heterogeneity in environment that exists at a smaller scale. At a smaller scale, many parameters can be tested more quickly and inexpensively than at the larger scale. Also, such a small system can be used to evaluate proposed process changes for an existing process (Shuler & Kargi, 2002). When knowledge of the small scale process is incomplete, it is necessary to employ a pilot plant of larger size before scale up into the industrial scale.

According to Sweere *et al.* (1987), scale up methods can be classified as,

- ✓ Fundamental methods
- ✓ Dimensional methods
- ✓ Rules of thumb
- ✓ Scale down approach
- ✓ Trial and error

This classification allows a certain organization of complex reality. The development of mathematical models able to describe the key characteristics of the system is perhaps the most effective tool for successful scale up (Sola & Godia, 1995). However, a combination of these methods must be used in order to obtain a successful translation of a process form laboratory to a full scale (Trambouze *et al.*, 1988).

The use of similarity theory in scale up processes is the most common approach (Sola & Godia, 1995). The basis of similarity is expressed in general as a linear relationship between the values of any magnitude, being an independent variable in both systems, in small scale (m) and in large scale (m'); k is the scale factor.

$$m' = k\,m \qquad \textbf{Equation 2-8}$$

Depending on whether the Equation 2-8 is valid for all the variables implied or only for some of them, similarity between systems will be total or partial. Similarity can be divided into classes according to the nature of variables obeying Equation 2-8:

- ✓ Geometric similarity
- ✓ Dynamic similarity
- ✓ Thermal similarity
- ✓ Mass (concentration) similarity
- ✓ (Bio) Chemical similarity

Examples of commonly used scale up parameters in bioreactor include height to diameter ratio (H:D), power input, oxygen transfer rate, liquid circulation rate, Reynolds number, mixing time, and agitator tip speed.

In fact, the underlying idea in the concept of similarity is: if two systems are described by the same differential equation, having the same contour constraints, then both systems behave identically (Sola & Godia, 1995). In the case that the set of equations is not

amenable to solve mathematically, a satisfactory scale up may require an empirical procedure to see how it behaves in a pilot scale experiment.

However, transferring to an industrial scale processes successfully developed at the laboratory scale is not a simple procedure (Sola & Godia, 1995). When a new process or changes in some parts of process moves from a laboratory to a large scale operation, especially when dealing with biochemical reactions in which the physical, chemical, and biological processes are interconnected, unexpected problems are often encountered: they may be of a physical nature, chemical nature, or involve some aspects of both (Bisio & Kabel, 1985).

Chapter Three: Materials and Methods

This chapter describes the materials, apparatus, and experimental methods used to undertake the laboratory experiments to determine the viability of augmenting leachate with enzymes before recirculation to enhance the gas production in later stages of anaerobic waste degradation.

3.1 Waste Materials

Samples of partly degraded MSW used in laboratory experiments were collected from a 30 year old landfill cell located at the city of Calgary Shepard landfill site. The average depth of the sampled cell was about 12 m, the area of the cell was 100 m x 100 m and the cover thickness was 1 m.

3.1.1 Representativeness of the waste sample

Samples were taken from 3 boreholes, at different depths (every 1 m) up to a total depth of 8 m.

As described previously, the moisture content is an important factor affecting the rate of waste degradation. Therefore, the moisture content of the collected discrete samples was measured. The results are shown in Table 3-1.

Table 3-1: Moisture content (%) for waste samples collected at different boreholes at different depths

Sample	1m	2m	3m	4m	5m	6m	7m	8m
Borehole 1	23.1	20.1	18.1	15.0	15.1	15.2	16.8	22.2
Borehole 2	22.1	19.1	17.6	14.8	15.4	17.0	16.9	21.6
Borehole 3	22.4	19.6	17.2	14.8	15.8	16.0	16.3	21.0

As expected, the moisture content of the samples varied significantly in the vertical direction along the depth of the landfill cell. The aerial variation (in a horizontal direction) of the moisture content was less significant compared to the vertical variation. Even though taking a representative sample from a heterogeneous waste landfill is a difficult task, it is reasonable to make a sampling plan to collect the waste samples from 3 boreholes aerially, every 1 m deep, up to 8 m.

In order to obtain samples representative of the entire landfill cell, ASTM standard D4687 procedure was followed during the sample collection.

Figure 3-1 shows a picture taken during the waste sampling at Shepard landfill site.

Figure 3-1: Waste drilling and sampling at Shepard landfill site

3.1.2 Waste characteristics

Figure 3-2 shows a picture of waste samples collected from the site. Once collected, the moisture content of each individual sample was measured as mentioned previously (Table 3-1) and then the waste samples were mixed according to ASTM D4687. The composite samples were shredded using a cement grinding machine (EIRICH R07) and

sieved (using Canadian standard sieve series - No 8 sieve) to an average particle size of about 2 mm prior to use in batch experiments.

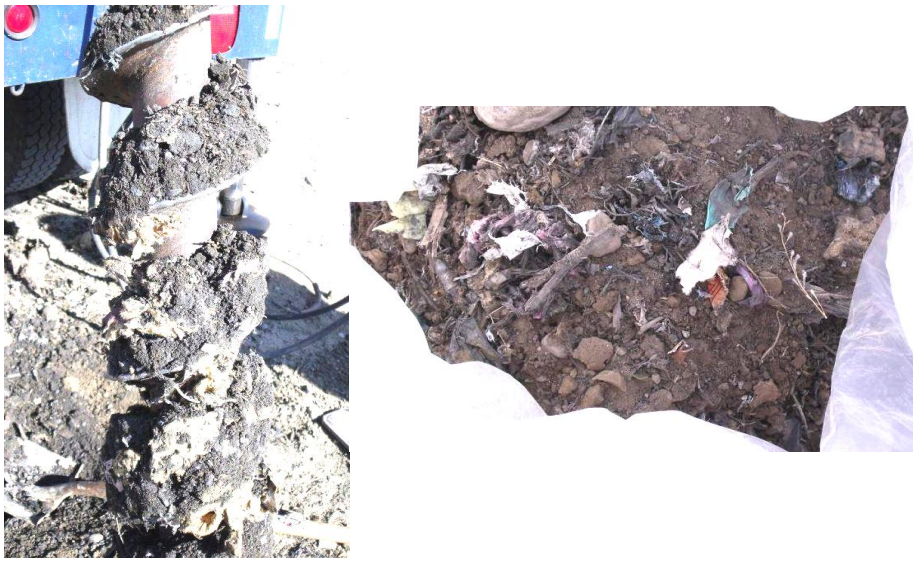


Figure 3-2: Waste samples

The characteristics of composite waste samples were determined experimentally according to the standard test methods (adapted from APHA, 2005 and described in Section 3.3), and are shown in Table 3-2.

Table 3-2: Waste characteristics

Parameter	Value
Moisture Content (% of TS)	18.0
Dry Solids (% of TS)	82.0
Volatile Solids (% of DS)	18.6
Lignin Content (% of VS)	81.9
Field Capacity (%)	48.0
Cellulose and Hemicellulose to Lignin ratio, (C+H)/L ratio	0.2

3.1.3 Estimating a molecular formula for waste

Elemental analysis of waste samples was conducted using CHN analyzer - Perkin Elmer Model 2400 series II. The results are shown in Table 3-3.

Table 3-3: Elemental analysis of waste

(* including water)

Parameter	mass %*
Carbon (%)	44.2
Hydrogen (%)	5.8
Nitrogen (%)	0.6
Oxygen (%)	39.4
Ash (% assumed)	10.0

Based on the elemental analysis, an approximate molecular formula for the waste was estimated as $C_{86}H_{135}O_{57}N$. Molecular weight of the waste was then calculated as 2093 g/mol.

The overall process of converting waste to CH_4 and CO_2 may stoichiometrically be expressed in the form of Buswell Equation (Mata-Alvarez, 2003) as follows.

$$C_{86}H_{135}O_{57}N + 24.5H_2O \rightarrow 45.25CH_4 + 40.75CO_2 + NH_3 \qquad \textbf{Equation 3-1}$$

Assuming that all VS are biodegradable, theoretical methane yield could be calculated from Equation 3-1 as 0.35 g_{CH4} per g of wet solid.

3.2 Enzymes and Other Chemicals

Examples of commercially available peroxidases are lignin peroxidase (LiP), manganese peroxidase (MnP), soybean peroxidase (SbP), horseradish peroxidase (HRP), and laccases. Of these peroxidases, LiP and MnP are described as true lignin degraders because of their high potential redox value (Martinez *et al.*, 2005).

In this study, three types of peroxidase enzymes, LiP, MnP, and SbP were selected to test their ability to further degrade partly degraded MSW. The enzymes were purchased from Sigma Aldrich Canada Ltd. The product details are shown in Table 3-4.

Table 3-4: Enzyme product details

Enzyme	Product Number	Description	Enzyme Commission (EC) Number
LiP	42603	Lignin Peroxidase	1.11.1.14
MnP	93014	Manganese peroxidase from white rot fungus (*Phanerochaete chrysosporium*)	1.11.1.13
SbP	P1432	Peroxidase from *Glycine max* (soybean)	1.11.1.7

Peroxidase enzymes needed to be activated using hydrogen peroxide (H_2O_2). 30% H_2O_2 solution (Product number: 14411) was purchased from Hach Company (Canada).

3.3 Equipment and Analytical Test Methods

The quality of the produced gas, leachate, and waste samples was measured.

In batch experiments, the gas compositions were measured for each sample every day during the monitoring period to calculate the volume of methane produced. Total organic carbon (TOC) in degraded waste samples for some selected batches was measured on regular basis to determine substrate utilization. The lignin content of the waste samples before and after the batch experiments was measured for some selected batches.

In flow-through column experiments, the leachate quality was measured in addition to the gas compositions and volume. To examine the chemical attributes of the leachate, six analytical tests were conducted; dissolved organic carbon (DOC), biochemical oxygen

demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), ammonia nitrogen (NH_3-N), and total phosphorous (TP). pH of leachate samples was also measured. In addition, MnP enzyme activity in selected leachate samples was measured.

The equipment and the test methods used in this research are described in detail in the following sub-sections.

3.3.1 Equipment

The percentage of methane, carbon dioxide, oxygen, and nitrogen in gas was measured using VARIAN CP4900 Micro Gas Chromatograph (VARIAN Canada Inc.). The GC was equipped with a sample injector, capillary column, and a thermal conductivity detector (TCD). Helium was used as a carrier gas. Gas compositions were determined using a GALAXIE software package in communication with the micro GC.

Dissolved organic carbon (DOC) in leachate samples was measured using TELEDYNE TEKMAR Apollo 9000 combustion TOC analyzer.

HACH DR 2800 spectrophotometer was used to measure leachate quality in terms of COD, TN, NH_3-N, and TP. HACH test methods used were adapted from APHA standard methods for the examination of water and wastewater.

3.3.2 Moisture content (MC), dry solids (DS), and volatile solids (VS) in waste

The Moisture contents (MC) of waste samples were determined by drying 10 g of waste sample in an oven (model 1510 E, Sheldon Manufacturing Inc.) at 105 ^{0}C for ~12 h until a constant weight was obtained. The weight of the sample before and after drying was measured (scale model: METTLER AT 250, Fisher Scientific). The weight lost during drying was the MC and the weight remained was the DS content.

The oven dried sample was ignited at 550 ^{0}C for 12 h in an Isotemp Muffle Furnace (Model 550-58, Fisher Scientific) to determine the VS and the ash content of the waste

samples. The weight of the sample after ignition was measured. The weight lost on ignition was the VS and the remaining was the ash.

3.3.3 Field capacity (FC)

Funnel experiments were conducted to determine the field capacity (FC) of partly degraded solid waste materials (Pokhrel, 2006). First, the materials were oven-dried at 105 ^{0}C for 2 h. A filter paper was wetted, drained and fitted into a 500 mL funnel. The funnel was filled with the waste samples and bottom tip of the funnel was closed. Known volume of water was added slowly to the waste material until the waste materials became saturated. A beaker was placed at the bottom of the funnel, the bottom tip was opened, and the drained water was collected. The water was allowed to drain for ~1 h, until no water drained into the beaker. The field capacity was calculated using the difference between the added water and the collected water volume.

3.3.4 Total organic carbon in solid waste (TOC)

To measure the TOC in solid waste samples, modified Mebius procedure by Yeomans & Bremner (1988) was used. The method involves the routine procedure for determination of organic carbon in soil. 500 mg of waste sample was digested with 5 mL of 1 N $K_2Cr_2O_7$ and 7.5 mL of concentrated H_2SO_4 acid for 30 min at 170 ^{0}C. The un-reacted dichromate was determined by titration of cooled digested sample with 0.2 N ferrous ammonium sulfate (FAS) with use of 0.3 mL of phenolphthalein as an indicator. The color change at the end point was from violet to bright green. One boiled control (BC) and one un-boiled control (UC) with same reagents were run parallel to each sample (S) to incorporate the amount of dichromate lost by boiling in the absence of sample in the calculations. Percentage organic carbon was then calculated using the following formula.

$$\%\ Organic\ \ Carbon = \frac{A * 0.2 * 0.003 * 100}{Sample\ \ weight\ (g)} \qquad \textbf{Equation 3-2}$$

where, $A = \frac{(BC - S) * (UC - BC)}{UC} + (BC - S)$

3.3.5 Lignin content in waste

In order to use substrate utilization as an indication of the success of experiments, the lignin content of the waste samples before and after experiments was determined according to ASTM-D1106 standard test method with minor modifications, as described by Lifrieri (2010).

First, the waste samples were grounded to pass a 1 mm sieve and dried at 105 ^{0}C for 2 h. 1 g of prepared sample was added to an extraction thimble, and the test samples were extracted with 1:2 ethanol:benzene solution for 8 h in a Soxlet extraction apparatus. The samples were then transferred into a beaker; 400 mL of water was added and digested in a hot water bath at 100 ^{0}C for 3 h. The digested samples were washed with 100 mL hot water and then dried in the air. The dried samples were transferred to a glass bottle and 15 mL of 72% H_2SO_4 was added slowly while stirring. Once the samples were well mixed with acid, they were kept in a water bath at 20 ^{0}C for 2 h. The acid digested samples were diluted by adding 560 mL of distilled water and boiled for 4 h. After allowing the insoluble materials to settle, the samples were washed again with 500 mL hot water and dried at 105 ^{0}C for 2 h. The weight of the samples were measured and then combusted at 550 ^{0}C for 2 h in a muffle furnace (Isotemp, Fisher Scientific). The weight of the ignited samples was measured. The weight loss on ignition represents lignin.

3.3.6 Dissolved organic carbon (DOC)

The DOCs of leachate samples were measured using the TOC analyzer. First, a test was conducted to assess the presence of inorganic carbon following the method proposed by Nelson & Sommers (1996). 4M HCl was added to the solid waste samples drop-wise allowing sufficient time for inorganic carbon to react (~5 min). As no effervescence was observed, it was assumed a non-significant inorganic carbon presence. The extraction for DOC analysis followed the method outlined by Chefetz *et al.* (1998); 1 g of sample

(oven-dry weight) was placed in 25 mL bottle adding 10 mL of distilled water. The bottles were shaken for 120 min (125 rpm) using a reciprocating shaker (Model 3528CCGM, Lab-Line Instruments Inc.). The suspension was then centrifuged (Model K, International equipment Co.) for 30 min and the supernatant was filtered through a 0.45 μm membrane filter. 5 mL sample of the solution was taken after extraction and DOC was measured immediately using the TOC analyzer.

3.3.7 Biochemical oxygen demand (BOD)

The BOD was measured using APHA standard test method 5210B. The leachate samples were diluted to three different ratios (sample:water ratio of 1:20, 1:30, 1:60) with ultra-pure water which was aerated overnight with phosphate buffer pillow (HACH 14861-66). 300 mL of diluted samples were filled in air-tight glass bottles having a ground-glass stopper until they overfill. One dose of nitrogen inhibitor (HACH 2533-35) was added to each bottle to prevent the interference from nitrogenous demand. Dissolved oxygen (DO) of prepared samples was measured immediately (DO_1) using DO meter (Yellow Spring Instrument Co. Inc. 52 CE). The bottles were closed, sealed with water, and incubated at 20±1 ^{0}C for 5 days in Fisher Scientific low temperature incubator. DO was measured five days later (DO_5) and the BOD was calculated from the difference between initial and final DO as follows. A blank sample without leachate was run each time to check the accuracy.

$$BOD_5\ (mg/L) = \frac{DO_1 - DO_5}{(Leachate\ \ volume/300)} \qquad \textbf{Equation 3-3}$$

3.3.8 Chemical oxygen demand (COD)

The COD was measured using HACH test and tube 10212 (TNT 823) method (equivalent to APHA 5220D standard test method). The test vials included all the reagents in appropriate quantities for 2 mL sample of leachate. The COD reagent included the strong oxidizing agent (potassium dichromate, $K_2Cr_2O_7$), silver catalyst, and mercury ions. 2 mL of leachate sample was added to each test vial and the vials were heated at 150 ^{0}C for 2 h

in DRB200 reactor. When the sample is heated with $K_2Cr_2O_7$ in acidic media with the aid of catalyst, oxidizable organic compounds react, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). Once the heated samples were cooled for 20 min, the amount of green color produced was measured in HACH spectrophotometer at 620 nm and it is directly proportional to the amount of COD present. Accuracy of each test was checked using the standard quality control solution of 1000 mg/L COD.

3.3.9 Total Kjeldahl nitrogen (TKN)

The HACH method 8075 (equivalent to APHA 4500-N_{org} B&C standard test methods) was used to measure TKN in leachate samples. Samples of leachate were first digested with concentrated H_2SO_4 acid in TKN digestion apparatus. Different dilutions of samples, varied from 1 mL to 20 mL of leachate with 3 mL to 10 mL of H_2SO_4, were tested. The samples were digested at 440 ^{0}C for about 4 min until all water vapor was evaporated. The samples were then neutralized using 10 to 15 mL of 50% H_2O_2. After excess H_2O_2 was boiled off by heating 1 more minute, the samples were allowed to cool. Organically bound N was converted to ammonium salts during digestion. Ammonia was then analyzed using HACH 8075 Nessler method. 1 drop of TKN indicator, 1 drop of 1 N KOH, 3 drops of mineral stabilizer, and 3 drops of polyvinyl alcohol dispersing agent were added to 5 mL of diluted sample. The test sample was then diluted up to 25 mL and 1 mL of Nessler reagent was added. After 2 min reaction period TKN test results were read at 460 nm. A sample blank was run simultaneously with an equal amount of deionized water to check the accuracy.

3.3.10 Total nitrogen (TN)

To determine the TN in leachate, the HACH test and tube 10208 (TNT 828) method was used (equivalent to APHA 4500-N.C standard test method). The test reagent included peroxidisulphate, 2, 6-dimethylphenol, sulphuric and phosphoric acid. 0.2 mL of leachate sample was added to the test vial and heated at 100 ^{0}C for 1 h in DRB 200 reactor. Inorganically and organically bonded nitrogen is oxidized to nitrate by digestion with peroxidisulphate. The nitrate ion react with 2,6-dimethylphenol in a solution of sulphuric

acid and phosphoric acid to form nitophenol. Test results were measured at 345 nm. A reagent blank was run each time with nitrogen-free deionized water, and the accuracy was checked with 50 mg/L TN standard solution.

3.3.11 Ammonia nitrogen (NH_3-N)

The HACH 8038 method was used (equivalent to APHA 4500-NH_3. B&C standard test methods) to measure NH_3-N in leachate. 25 mL leachate samples were used. As per the procedure, 3 drops of mineral stabilizer and polyvinyl alcohol dispersing agent were added to the test samples. The test tubes were inverted several times to mix; 1 mL of Nessler reagent was added to each sample. The mineral stabilizer prevents the interference with hardness in the sample. The polyvinyl alcohol dispersing agent aids the color formation in the reaction of Nessler reagent with ammonium ion. A yellow colour formed after 1 min reaction period was measured at 425 nm, and the colour intensity is proportional to the ammonia concentration. A blank sample with deionized water was run each time. The accuracy was checked with 1 mg/L NH_3-N standard solution.

3.3.12 Total Phosphorus (TP)

The TP of leachate was measured using HACH test and tube 10127 (TNT 72) method (equivalent to APHA 4500-P. B&E standard test methods). 5 mL of leachate sample and 1 pack of potassium persulfate powder pillow were added to the test vial, and heated at 150 ^{0}C in DRB 200 reactor for 30 min. Once it was cooled to room temperature, 2 mL of sodium hydroxide and 0.5 mL of molybdovanadate reagent were added to each test vial. After 7 min reaction period, the color intensity was measured at 420 nm. In this method, phosphate present in organic and inorganic forms is converted to reactive orthophosphate when the sample is heated with acid and persulfate. The orthophosphate reacts with molybdate in an acid medium in the presence of vanadium, and gives a yellow color. The intensity of yellow color is proportional to the phosphate concentration. A blank sample and 50 mg/L phosphate standard sample were run parallel to check the accuracy.

3.3.13 pH

The pH of each leachate sample was measured using Accumet AP62 pH meter. The pH probe was standardized to pH 7 and pH 4 before each use.

3.3.14 MnP enzyme activity test

Procedure described by Saetang & Babel (2010) was used to measure MnP enzyme activity in leachate. All the reagents were purchased from Sigma Aldrich (Canada). Phenol red was used as a substrate of the enzyme reaction. Reaction mixture was prepared with 1 mL sodium succinate buffer (50 mM, pH 4.5), 0.4 mL $MnSO_4$ (0.1 mM), 0.7 mL phenol red (0.1 mM), 1 mg/mL gelatin, and 0.5 mL of enzyme added leachate. Reaction was initiated by adding 0.4 mL H_2O_2 (50 μM). 40 μL of NaOH (5 N) was added to 1 mL reaction mixture and absorbance was measured at 610 nm in spectrophotometer (HACH DR/4000 U). However, the readings obtained were not conclusive and it was concluded (will be discussed in Chapter 4) that there were some interferences in the method when the enzyme concentration in leachate are relatively low.

Another attempt was made to measure the enzyme activity using 3-methyl-2-benzothiazolinone hydrazone (MBTH) as a substrate (Castillo *et al*., 1997). The reaction mixture contained 0.066 mL of 0.07 mM MBTH, 0.113 mL of 0.99 mM DMAB, (3-(dimethylamino) benzoic acid), 0.033 mL of 0.3 mM $MnSO_4$, 1.42 mL of 100 mM succinic acid buffer (pH 4.5) and 0.033 mL of enzyme added leachate. The reaction was initiated by adding 0.333 mL of 0.05 mM H_2O_2 and the absorbance was measured at 590 nm.

3.3.15 Most probable number (MPN)

To quantify the microorganisms involved in the process, MPN technique was used (Madigan & Martinko, 2006). Ten-fold serial dilutions of leachate in culture medium were used as shown in Figure 3-3. The culture medium consisted of 10 mL mineral solution (Pfennig I and Pfennig II, 5 mL each), 1 mL wolin metals, 1 mL balch vitamins, 0.1 mL resazurin (0.1% solution), and 0.35 g $NaHCO_3$ (McInerney *et al.*, 1979). Nine mL

of culture medium solution was added to each test tube and 1 mL of leachate was added to the first test tube and serial dilutions thereafter as shown in Figure 3-3. The test tubes were purged with a gas mixture of 80% N_2 and 20% CO_2 and sealed. Headspace gas composition was measured every week until it showed a stable gas production.

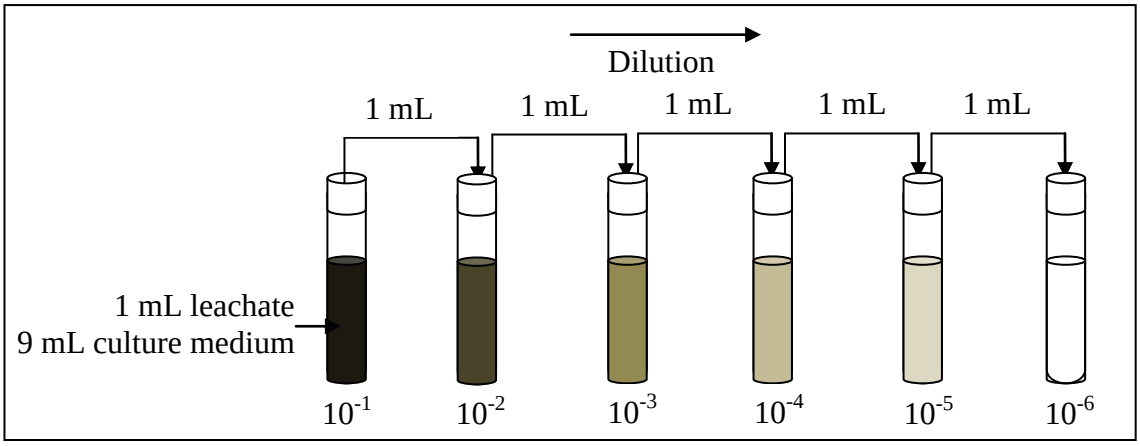


Figure 3-3: MPN analysis

3.3.16 Characterizing the microbial population

Identifying the group of microorganisms that dominates the methanogenesis is important to understand the microbial community involve in the waste degradation process. Acetate and CO_2/H_2 are two common substrates for methanogens (Madigan & Martinko, 2006). 9 test tubes were run simultaneously under anaerobic conditions, 3 only with leachate, 3 with leachate and 0.5 mL of 100 mM acetate solution, the rest with leachate and 10 mL of a gas mixture (20% CO_2 and 80% H_2). Head space gas composition was measured until the gas production became stabilized. In addition, a similar set of experiment was conducted when leachate was augmented with MnP enzyme.

Chapter Four: Laboratory Batch Experiments

This chapter includes the objectives, experimental methodology and design, and the results obtained from laboratory batch experiments.

4.1 Objectives of Batch Experiments

The objectives of conducting batch experiments were to;

1. Determine experimentally whether the enzyme augmentation process could enhance the anaerobic waste degradation and the gas production.
2. Identify the best suitable enzyme among the selected enzymes (LiP, MnP, and SbP) for anaerobic waste degradation at later stages of landfill operation.
3. Identify the optimum amount of H_2O_2 to be used to activate the enzyme (ratio of E:H_2O_2) for maximum gas production.
4. Identify the optimum enzyme dose for a given quantity of waste to obtain the highest gas production rate.
5. Determine the effect of temperature and hence estimate the activation energy of the enzyme-enhanced waste degradation process.
6. Understand the mechanism of the process (to know how enzyme could enhance the waste degradation), and to understand the microorganisms involve in the process to some extent.
7. Obtain the reaction kinetic data, such as maximum reaction rate and kinetic constants.

4.2 Experimental Methodology

4.2.1 Batch experimental procedure

The batch experiments were conducted in 125 mL glass bottles. The bottles were made from clear glass; they had narrow mouth with an open top cap and septa. The bottles were purchased from VWR Scientific, Canada. The quantity of dry waste used for each batch experiment was 2 g.

2 g of dry waste, the corresponding amount of water, enzyme, and H_2O_2 were added to the bottles. The amount of water added to 2 g of waste was ~3 mL, which was 60% more than the FC of waste (48%) due to the fact that the optimum moisture content for the highest waste degradation is about ~60% to 65% (Khanal, 2008). All the weight measurements were obtained using a METTLER AT250 (Fisher Scientific Canada) analytical scale. A micropipette (100 µL) was used to measure small quantity of liquid volumes and volumetric measuring cylinder was used to measure relatively high volumes.

One batch reactor was kept as a control reactor, in which enzyme and H_2O_2 were not added. Once the samples were mixed, the bottles were closed, and sealed using a silicon sealant to prevent any potential air leakage and ensure anaerobic conditions. To make an immediate anaerobic environment inside the bottles, they were purged with ultra-high pure nitrogen gas for approximately 20 min, until the headspace gas composition showed zero oxygen. As shown in Figure 4-1, two needles were used to purge N_2 to minimize the air leakage during purging.

All of the bottles were then kept in a G24 Environmental incubator (New Brunswick Scientific) operating at a temperature of 35±1 ^{0}C (Figure 4-2). The shaking speed of the incubator was adjusted to 'low'. Head space gas composition of each reactor was measured by collecting 0.5 mL of head-space gas using a Gas-tight-sample-lock 2.5 mL syringe (Hamilton # 1002) fitted to a needle. The gas samples were injected into the GC (VARIAN 4900 Micro GC) to measure the gas compositions in terms of CH_4, CO_2, O_2, and N_2. The gas compositions were measured until the stable conditions were reached, characterized by a constant gas production.

Figure 4-1: Nitrogen purging into batch reactors

Figure 4-2: Environmental incubator

The volume of CH_4 produced was calculated using Equation 4-1 as described by Soto *et al*. (1993) (The derivation of Equation 4-1 is included in Appendix 1.1). This is the best method to calculate gas volume for small reactors up to 125 mL (Soto *et al.*, 1993). In Equation 4-1, the net production of N_2 was considered negligible in relation to CH_4 production.

$$v_{CH_4} = \frac{v\, x^{o}_{N_2} x_{CH_4}}{(1 - x_{CH_4} + x_{CO_2})} \qquad \textbf{Equation 4-1}$$

where,

v_{CH_4} = Volume of CH_4 (mL)

v = Volume of gas phase (mL)

$x^{o}_{N_2}$ = Initial mole fraction of N_2 gas

x_{CH_4}, x_{CO_2} = Mole fractions of CH_4 and CO_2 at time t.

Each set of batch experiments was repeated twice or three times (whenever it was needed) to test the repeatability and average values were used for the calculations.

4.2.2 Design of batch experiments

Different sets of batch experiments were conducted to establish the enzymatic augmented waste degradation process.

The first set included preliminary batch experiments. The preliminary experiments were conducted using 3 types of enzymes (MnP, LiP, and SbP) at a fixed value of enzyme dose, 0.25 mg, and E:H_2O_2 ratio of 0.0027. A control sample, without enzymes and H_2O_2, was run simultaneously to evaluate the effect of enzyme addition on anaerobic waste degradation of lignin-rich waste. Some batch reactors were operated by adding only H_2O_2 to monitor the effect of H_2O_2 alone when there was no enzymes.

The second set of batch experiments was designed as a mixed level factorial design (3x5x5). Factors and treatment levels considered in the factorial design are shown in Table 4-1 and Figure 4-3. The experimental factors were; enzyme type, enzyme dose and ratio of E:H_2O_2. Three enzyme types, LiP, MnP, and SbP, were tested at five levels of enzyme dose and five levels of E:H_2O_2 ratio. The five levels of enzyme dose studied were 0.1, 0.2, 0.3, 0.4, and 0.5 mg per 2 g of waste. For each level of enzyme dose, five different levels of E:H_2O_2 ratio, 0.0023, 0.0027, 0.0034, 0.0046, and 0.0068 g/g, were tested.

A control experiment without enzyme and H_2O_2 was carried out simultaneously. The type and the amount of waste (2 g), moisture content (60%), temperature (35 ^{0}C), stirring speed (low), and other environmental conditions were kept constant for all the batch reactors.

Table 4-1: Experimental factors and treatment levels

Factors		Treatment Levels				
		-2	-1	0	1	2
A	Enzyme Type		LiP	MnP	SbP	
B	Enzyme Dose (mg)	0.1	0.2	0.3	0.4	0.5
C	E:H_2O_2 ratio (g/g)	0.0023	0.0027	0.0034	0.0046	0.0068

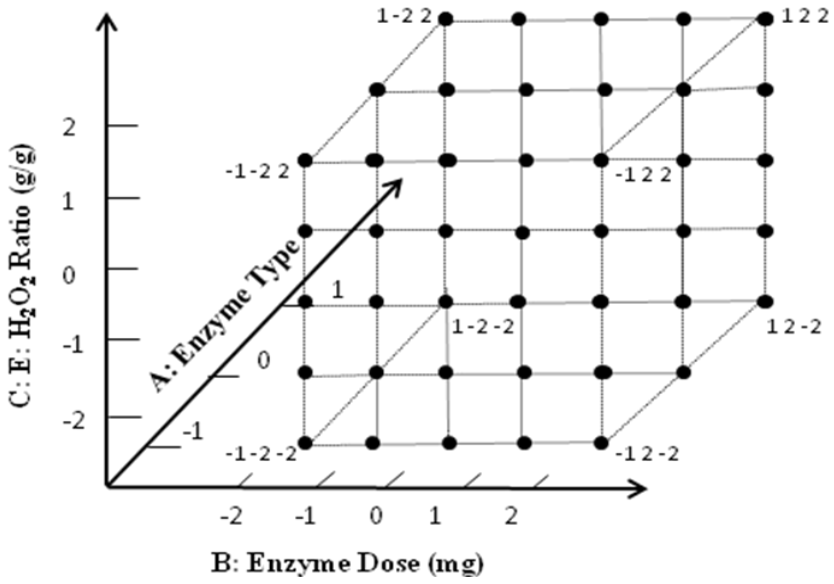


Figure 4-3: Factorial experimental design

Figure 4-4 shows a photograph of laboratory experimental setup.

Figure 4-4: Laboratory experimental setup

Based on the methane production, the best combination of enzyme, E:H_2O_2 ratio, and enzyme dose was identified. A third set of batch experiments was conducted with that best combination to evaluate the effect of temperature on waste degradation and to

estimate the activation energy of the reaction. Previous experiments were conducted at 35 ^{0}C, therefore some of the batches of the third set were conducted at 45 ^{0}C. Furthermore, in previous experiments, enzyme was added at day 0. However, when it comes to the field-scale, the enzyme will be added at the active gas generation phase. Therefore, some experiments were conducted by adding enzyme at day 6 (after the lag phase) to better represent the field-scale scenario.

The last set of batch experiments was designed to obtain kinetic information of the best-performed combination. In addition to the methane production, substrate utilization was also measured over time. Substrate utilization was measured in terms of DOC in leachate, and TOC in solid waste on a regular basis. Since these were batch experiments, one batch reactor had to be opened every sampling day to collect waste sample for testing. The opened batch reactor was discontinued once the sample was obtained. For this reason, 20 identical batch reactors were operated over a period of 40 days.

4.3 Results and Discussion

The cumulative methane yield and the substrate utilization were used as indicators of the effect of enzyme amendment on waste degradation. As described earlier, methane yield was calculated from headspace gas compositions and substrate utilization was determined from the percentage reduction in lignin mass, TOC in waste, and DOC in leachate.

4.3.1 Time dependent methane yield with enzyme enhancement - Preliminary batch experiments

The cumulative methane yield in preliminary experiments is shown in Figure 4-5.

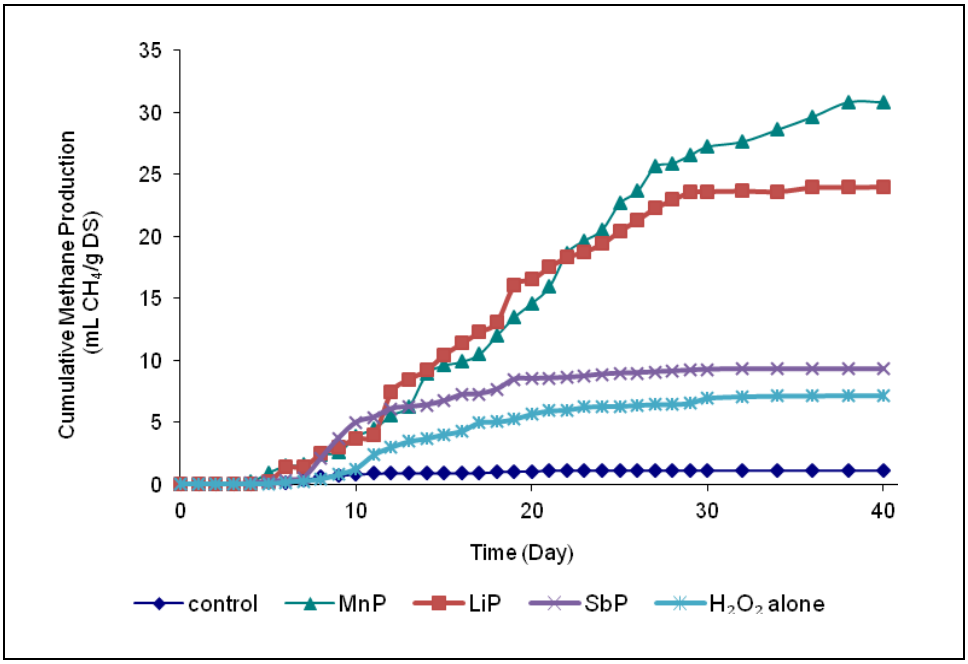


Figure 4-5: Preliminary results - 3 different enzymes (0.25 mg enzyme dose and 0.0027 E:H_2O_2 ratio), control, and H_2O_2 alone.

A significant increase in methane yield was observed in enzyme amended batch reactors compared to the H_2O_2 added reactor and the control reactor. These results confirmed that the addition of peroxidase enzymes positively influences the methane production under anaerobic conditions. H_2O_2 alone can also increase the methane yield compared to the control reactor, but the enhancement in terms of methane production was not as high as when enzymes were activated by H_2O_2. H_2O_2 is a strong oxidant; its applicability in the treatment of various organic and inorganic pollutants from wastewater is well established (Neyans & Baeyens, 2003). However, it is evident from our results that H_2O_2 alone is not sufficient in degrading lignin-rich waste.

4.3.2 Time dependent methane yield with enzyme enhancement - Factorial batch experiments

The variation of cumulative methane yield over time for MnP enzyme at different E:H_2O_2 ratio at 0.3 mg enzyme dose is shown in Figure 4-6.

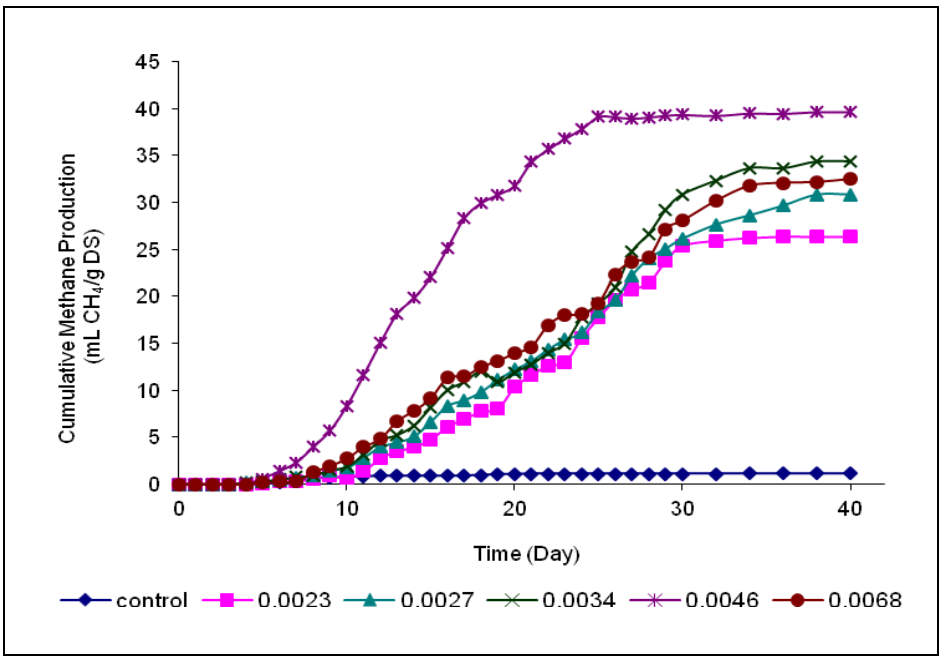


Figure 4-6: Cumulative methane production for MnP at different E:H_2O_2 ratio at 0.3 mg enzyme dose

A similar trend of cumulative methane production over time was observed in each of the three enzymes amended reactors (see Figure 4-7 and Figure 4-8 for 0.3 mg enzyme dose and Appendix 1.2 for other studied enzyme doses, 0.1, 0.2, 0.4, 0.5 mg).

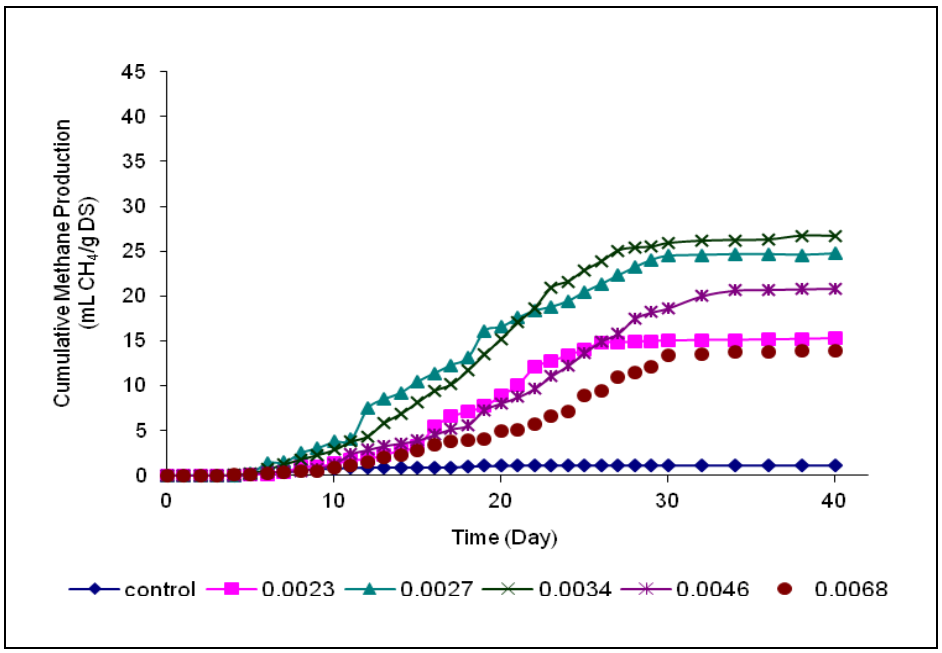


Figure 4-7: Cumulative methane production for LiP at different E:H_2O_2 ratio at 0.3 mg enzyme dose

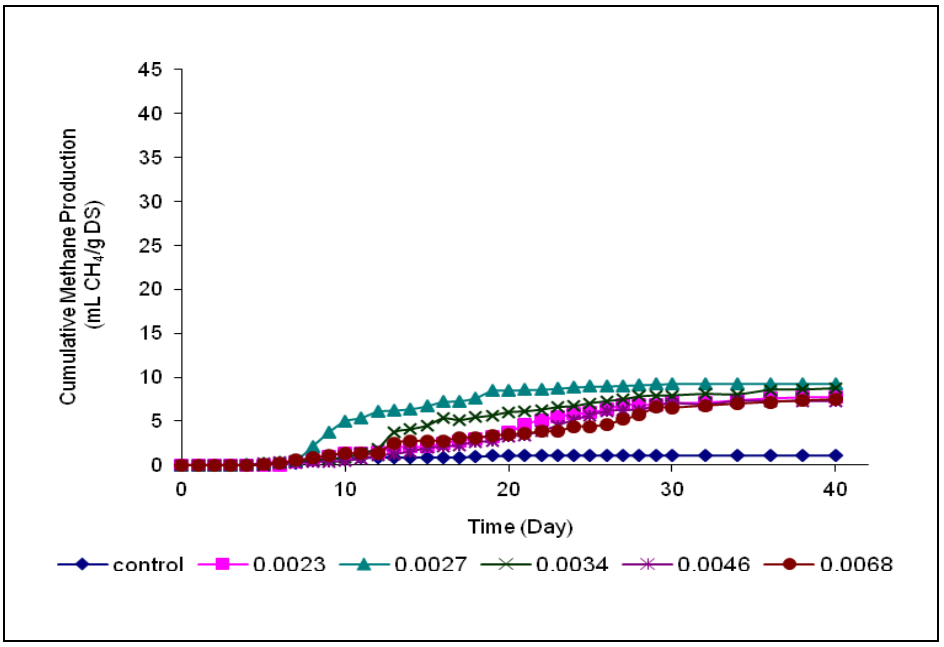


Figure 4-8: Cumulative methane production for SbP at different E:H_2O_2 ratio at 0.3 mg enzyme dose

At the beginning of the experiments, an initial lag phase was observed with negligible methane yield in all enzyme amended reactors and in control reactor. In average, the lag phase was approximately 6~8 days in each combination. The lag phase was assumed to be an acclimation period before enzyme can act on waste to make it accessible to microorganisms. The lag phase was followed by a sharp increase of methane production before the methane yield reached its maximum value as microorganisms convert dissolved organic matter to gas. The final stagewas gas stabilization; available substrates and nutrients become limiting, and the biological activity was inhibited. However, the assumptions made here when explaining the gas production trends were verified experimentally and the results will be discussed in sections 4.3.10.2 and 4.3.12.

Similar trends of methane production were observed by others studying enzyme amendment effects under different conditions (Lagerkvist & Chen, 1993; Cirne *et al*., 2008). However, a direct comparison of our results to the results reported in literature may not be possible because of the significant differences in the substrates, leachate augmentation methods, and the experimental conditions.

During 40 days of batch reactor operation, the methane yield ranged between 4-40 mL/g DS when supplemented with enzymes. The methane yield depended on the type of enzyme, retention time, dose of enzyme, and E:H_2O_2 ratio. In comparison, the control reactor containing a similar amount of waste produced less than 1.5 mL CH_4/g DS over 40 days of batch reactor operation.

The primary reason for higher methane production in enzyme amended samples compared to the control sample is that peroxidase enzymes catalyze the initial depolymerization of lignin by generating highly reactive free radicals. These free radicals are capable of breaking down lignin into smaller molecules. The smaller molecules are easily hydrolyzed compared to the lignin molecule. Lignin depolymerization thus increases the rate of hydrolysis *i.e.,* the rate limiting step in anaerobic waste degradation. On the other hand, methanogens, the microbe responsible for producing methane, have

the ability to utilize only limited number of simple organic compounds as their carbon and energy source (Barber, 2007). The inability of methanogens to utilize complex organic material, such as lignin, could be one of the reasons for higher resistance to waste degradation and methane production. The initial depolymerization of substrate by enzymes would help methanogens to easily utilize the substrate. Furthermore, the initial degradation of lignin will allow microorganisms to easily access un-degraded cellulose and hemicellulose in lignocellulosic substrate. This could also play an important role in enhanced hydrolysis and methane production.

4.3.3 Effect of enzyme type on methane production

The cumulative methane yields at day 40 (the day all the samples showed a stable methane production) for the three enzymes at five levels of enzyme dose at 0.0046 E:H_2O_2 ratio are presented in Figure 4-9.

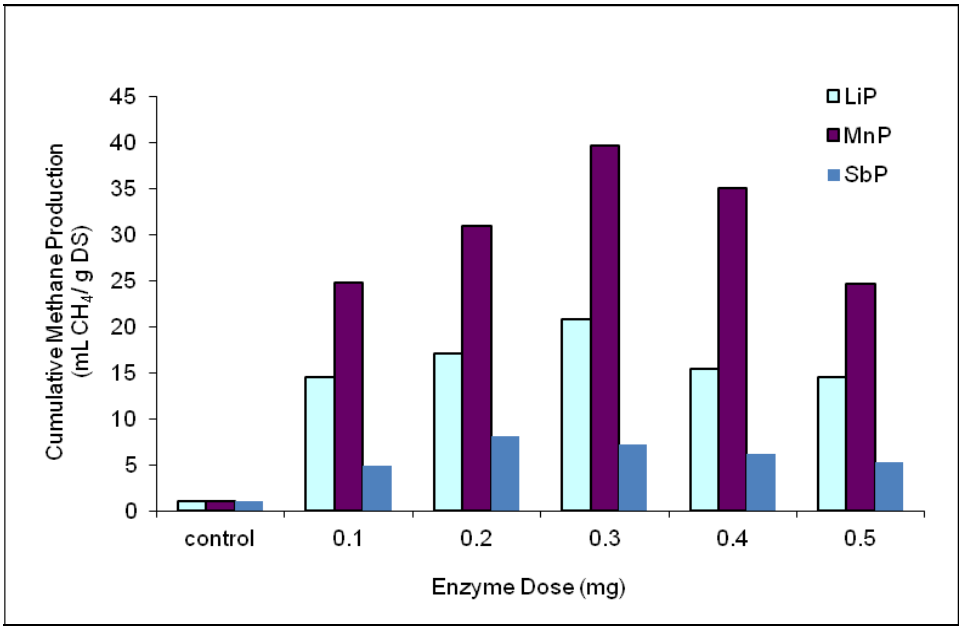


Figure 4-9: Effect of enzyme type on gas production at different levels of enzyme doses at 0.0046 E:H_2O_2 ratio

As shown in Figure 4-6, Figure 4-7, Figure 4-8, and Figure 4-9, MnP showed the best performance in terms of methane yield at each level of enzyme dose, followed by LiP. The methane yield was lowest in the batch reactor which was augmented with SbP. The maximum methane yield of 39.7 mL CH_4/g DS and 20.8 mL CH_4/g DS for MnP and Lip were observed respectively, at 0.3 mg of enzyme dose over 40 day period. The maximum methane yield for SbP was 8.1 mL CH_4/g DS at 0.2 mg of enzyme dose. In terms of average methane production rates, the corresponding values for MnP, LiP, and SbP were 1.0, 0.5, and 0.2 mL CH_4/ g DS/ day, respectively. The methane production rate of the control reactor was much lower (0.03 mL CH_4/ g DS/ day) than the methane production rates of enzyme amended reactors.

MnP and LiP are true lignin degraders (Martinez *et al*., 2005). Their lignin degradation capabilities are better than that of other peroxidases because of their high redox potential. Related to the mechanisms of enzymatic degradation of lignin, LiP oxidizes non-phenolic lignin units by one-electron transfer mechanism resulting in cation radicals. These cation radicals are responsible for the C-C bond cleavage, aromatic ring opening in lignin and other reactions (Gronqvist *et al*., 2003; Martinez *et al*., 2005). In contrast to LiP, MnP generates Mn^{+3}, which is capable of oxidizing both phenolic and non-phenolic lignin units. Free radicals formed by this oxidation reaction are highly reactive and are capable of continuing the depolymerization of lignin (Gronqvist *et al*., 2003). This could be one of the reasons for a higher rate of methane production with MnP enzyme, compared to the other two enzymes.

Furthermore, SbP is made from soybean seed whereas LiP and MnP are made from white rot fungi which are capable of degrading lignin. SbP showed the least performance in this study and therefore not recommended for enhancing the degradation of lignin-rich waste materials.

4.3.4 Effect of enzyme dose

As shown in Figure 4-9, the effect of enzyme dose on methane yields at 0.0046 E:H_2O_2 ratio was highly significant. The methane production trends were similar at all five E:H_2O_2 ratio (results are included in Appendix 1.3). The amount of methane produced was increased with an increasing enzyme dose at a given ratio of E:H_2O_2, until it reached a maximum value. Thereafter, it showed a negative effect and methane production was decreased with an increasing enzyme dosage. Several factors could contribute to this behaviour, including toxicity and inhibitory effects created by an excess enzyme, shifting the enzyme-catalytic reaction pathways in a different direction, and decreasing the amount of electron mediators, such as cation radicals (Barr & Aust, 1994).

However, the results obtained during the substrate utilization experiments in our study (shown in

Figure 4-18) suggest that the substrate limiting behaviour in batch reactor lead this observation. As substrate was used up, the enzyme's active sites may be no longer saturated, decreasing the enzyme activity. In order to justify this fact, a known quantity of enzyme was added after the gas production had stabilized, i.e. at day 40. The results are shown in Figure 4-10. No significant increase in methane production was observed by a second addition of enzyme.

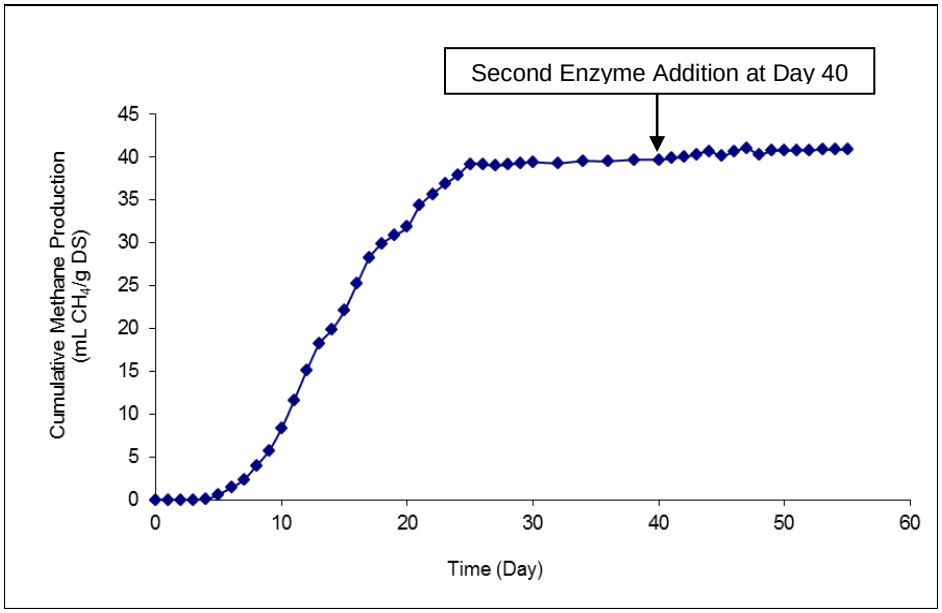


Figure 4-10: Second addition of enzyme (MnP, 0.3 mg, 0.0046 E:H_2O_2)

4.3.5 Effect of enzyme: H_2O_2 ratio

Figure 4-11 shows the cumulative methane yield at day 40 for each enzyme at five E:H_2O_2 ratio at 0.3 mg enzyme dose. A similar trend was observed at all the other levels of enzyme doses (see Appendix 1.4 for results obtained in other enzyme doses).

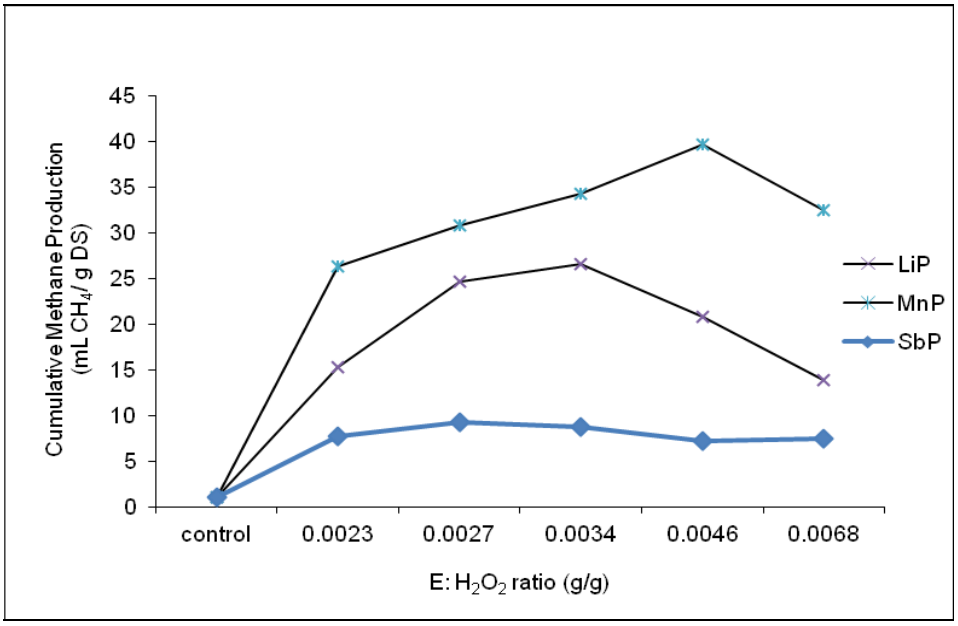


Figure 4-11: Effect of E:H_2O_2 ratio for different enzymes at 0.3 mg enzyme dose

As shown in Figure 4-11, the effect of E:H_2O_2 ratio on methane production was highly significant in MnP and LiP added reactors. The methane production was increased with an increasing E:H_2O_2 ratio at a given enzyme dose to a maximum and then decreased with increasing E:H_2O_2 ratio. As described by Saunders *et al*. (1964), peroxidase enzymes could be inactivated by excess H_2O_2 by the formation of enzymically-inactive compounds. This is a potential cause of inhibition of waste degradation process at high H_2O_2 levels. The methane production observed in SbP added reactors did not show a similar trend in the studied range of E:H_2O_2 ratio.

The optimum E:H_2O_2 ratio for LiP, MnP, and SbP amended reactors at 0.3 mg enzyme dose were 0.0046, 0.0034, and 0.0027, respectively.

4.3.6 Analysis of Variance (ANOVA) model

The results obtained from factorial design experiments were further analyzed using the ANOVA model. MINITAB software was used to solve the ANOVA model. Table 4-2

shows the raw (average) data used in this model (cumulative methane production at day 40, R-response) for different combinations of enzyme type (A), enzyme dose (B), and E:H_2O_2 ratio (C).

Table 4-2: Cumulative methane production (mL CH_4/ g DS) results at day 40

	Enzyme:H_2O_2 ratio				
LiP	0.0023	0.0027	0.0034	0.0046	0.0068
Enzyme Dose (mg) 0.1	8.4	9.6	17.2	14.5	11.9
0.2	8.4	13.0	26.0	17.1	13.1
0.3	15.3	24.7	26.7	20.9	13.9
0.4	15.3	18.2	25.2	15.5	15.3
0.5	11.8	14.3	16.6	14.5	12.9

	Enzyme:H_2O_2 ratio				
MnP	0.0023	0.0027	0.0034	0.0046	0.0068
Enzyme Dose (mg) 0.1	11.2	17.7	19.2	24.8	24.9
0.2	13.4	17.8	24.4	30.9	27.5
0.3	26.4	30.8	34.3	39.7	32.5
0.4	16.9	27.0	31.9	35.1	32.0
0.5	15.9	21.8	24.5	24.7	22.1

	Enzyme:H_2O_2 ratio				
SbP	0.0023	0.0027	0.0034	0.0046	0.0068
Enzyme Dose (mg) 0.1	6.3	7.0	5.4	4.9	4.5
0.2	5.5	8.4	11.3	8.1	11.9
0.3	7.8	9.3	8.7	7.2	7.5
0.4	5.5	6.6	6.7	6.2	6.8
0.5	4.6	5.1	4.1	5.3	4.5

Table 4-3 shows the ANOVA results.

Table 4-3: ANOVA results obtained from MINITAB

Source	Degree of Freedom	Sum of Squares	Mean Square	$F_{calculated}$	F_{table}	
A	2	12593.3	6296.6	1843.4	3.1	$F_{0.05,2,150}$
B	4	1812.4	453.1	132.6	2.4	$F_{0.05,4,150}$
C	4	1460.1	365.0	106.9	2.4	$F_{0.05,4,150}$
A*B	8	736.1	92.0	26.9	2.0	$F_{0.05,8,150}$
A*C	8	1322.1	165.3	48.4	2.0	$F_{0.05,8,150}$
B*C	16	341.6	21.3	6.2	1.7	$F_{0.05,16,150}$
A*B*C	32	353.2	11.0	3.2	1.5	$F_{0.05,32,150}$
Error	150	512.3	3.4			
Total	224	19131.1				

The degrees of freedom for each individual factor were calculated by subtracting 1 from its number of levels. The degrees of freedom associated with the interactions between factors were calculated by multiplying the degrees of freedom corresponding to each factor. The sums of squares were calculated by the sum of squared differences between the sample means of each group and the overall mean, weighted by the sample size in each group. The mean squares were obtained by dividing the sum of squares by its appropriate degrees of freedom. The F value for each factor and interaction was calculated by dividing the specific mean square by the error mean square (see appendix 1 for detailed information on calculations and equations). Assuming a 0.05 significance level (i.e. at 95% confidence interval) and with the resultant degrees of freedom, a critical F value was obtained from F distribution tables (attached in Appendix 1.5) for each factor and interaction. Those calculated F values exceeding the related critical F values obtained from the tables were assumed to be significant factors or interactions at the 0.05 significance level.

From Table 4-3, it is evident that each single factor, A (enzyme type), B (enzyme dose), and C (E:H_2O_2 ratio), was significant as were the interactions of AB, AC, BC, and ABC. From these results, it can be stated that methane production rates are not only related to the individual factors studied, but also to the interaction between them.

Plots obtained from MINITAB for main factor effect and interactions between factors are shown in Figure 4-12 and Figure 4-13, respectively. R represents the response, the cumulative methane production at day 40.

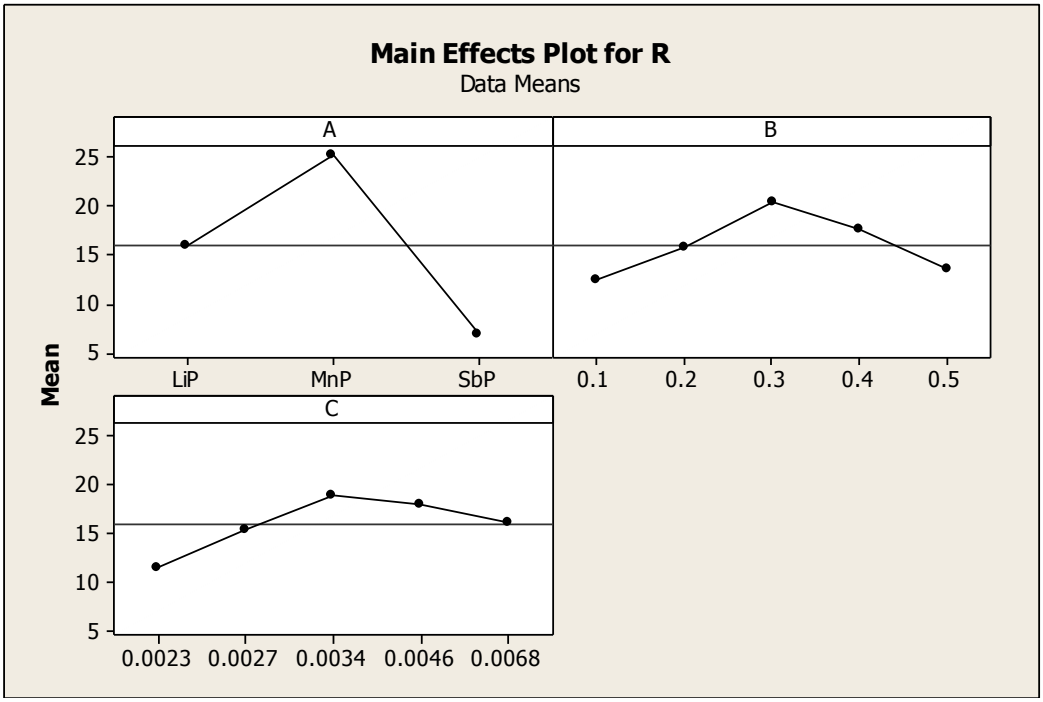


Figure 4-12: Main effect plot obtained from MINITAB

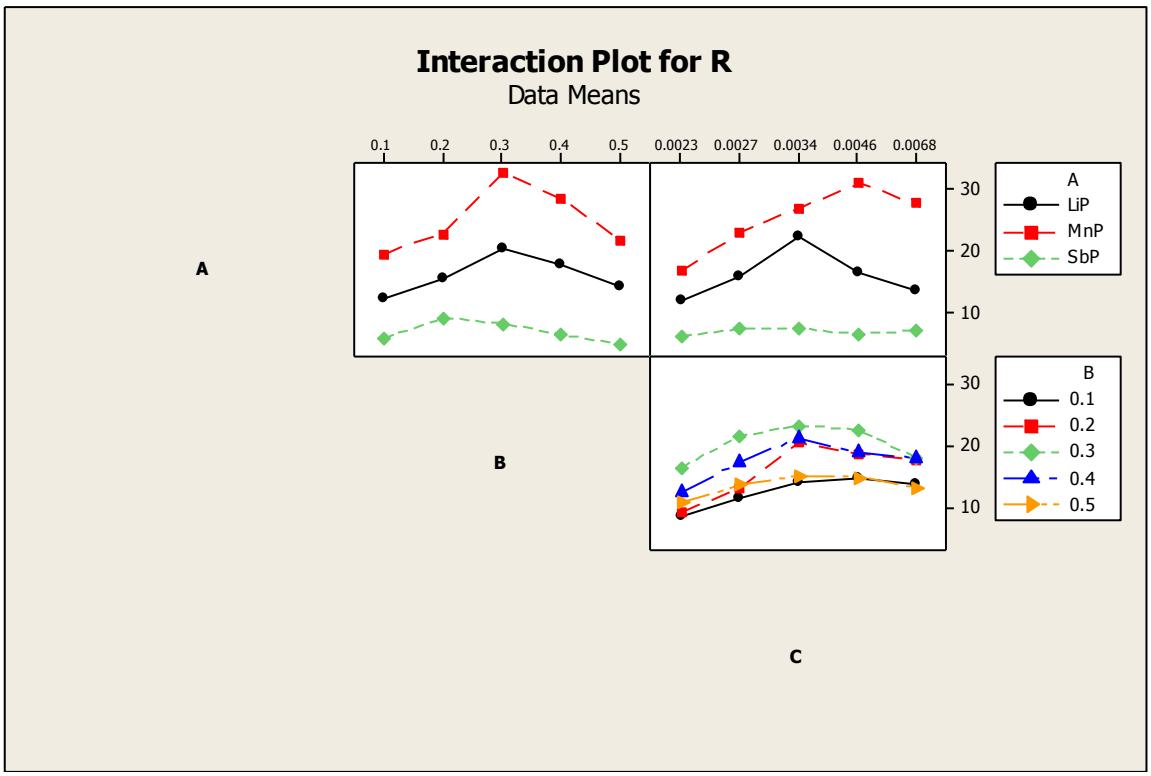


Figure 4-13: Interaction plots obtained from MINITAB

Considering the results obtained through the ANOVA model and previously described plots, the enzyme MnP was identified as the most suitable enzyme among the three potential enzymes tested *i.e.*, LiP, MnP, and SbP. The best combination of enzyme dosage and E:H_2O_2 ratio for MnP enzyme was 0.3 mg and 0.0046, respectively. This best combination was then used for further experiments.

4.3.7 Biogas concentration profiles

A significant difference in methane composition in biogas was observed in the enzyme treated samples and the control sample. The trends are shown in Figure 4-14. For the enzyme treated samples, the methane composition remained between 10-50% after the lag phase, depending on the type of enzyme, dose of enzyme, and E:H_2O_2 ratio. The methane composition of the control reactor was very low, *i.e.* ~2%. The highest methane

composition observed was 48% at the combination of MnP augmented waste at its optimum enzyme dose and E:H_2O_2 ratio.

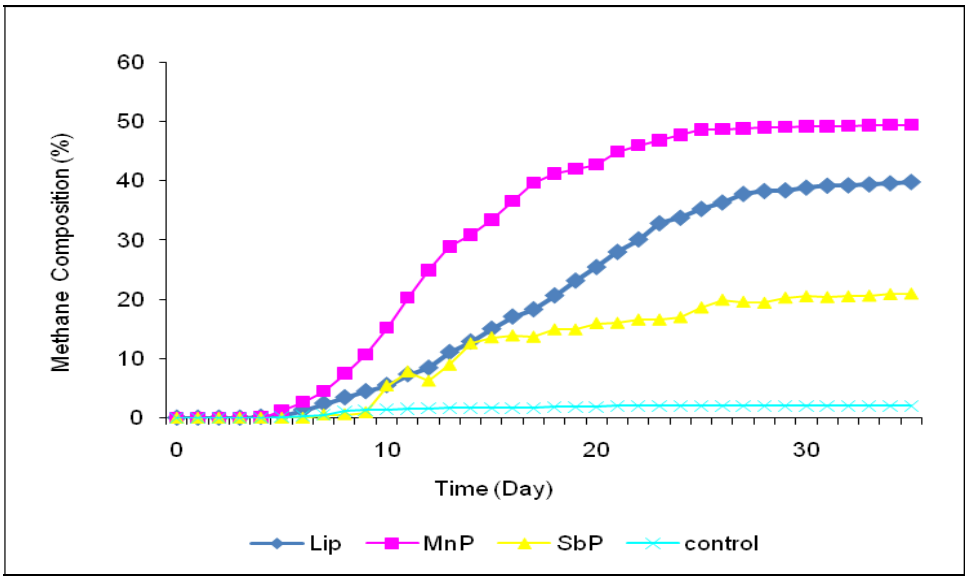
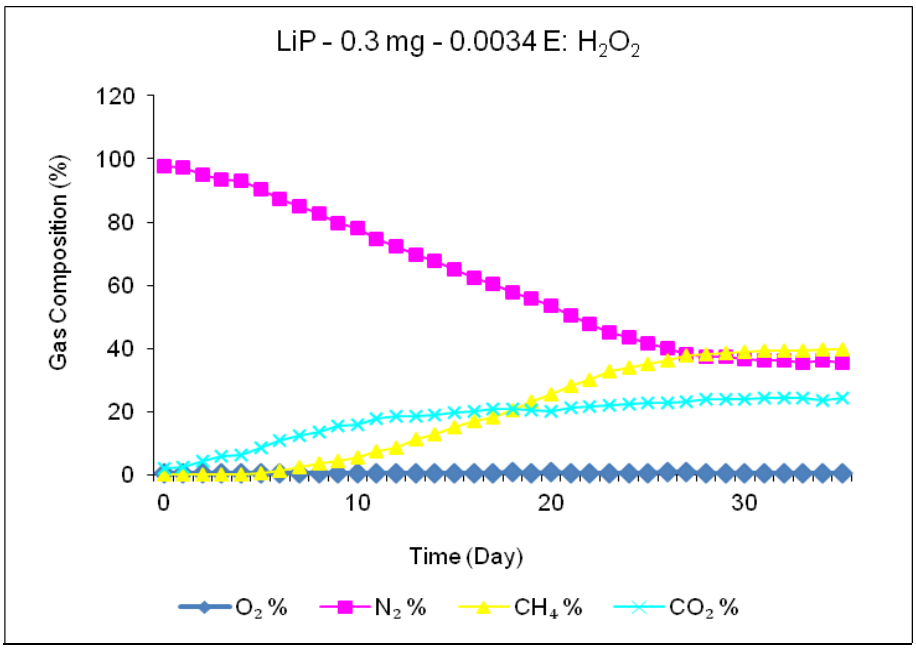
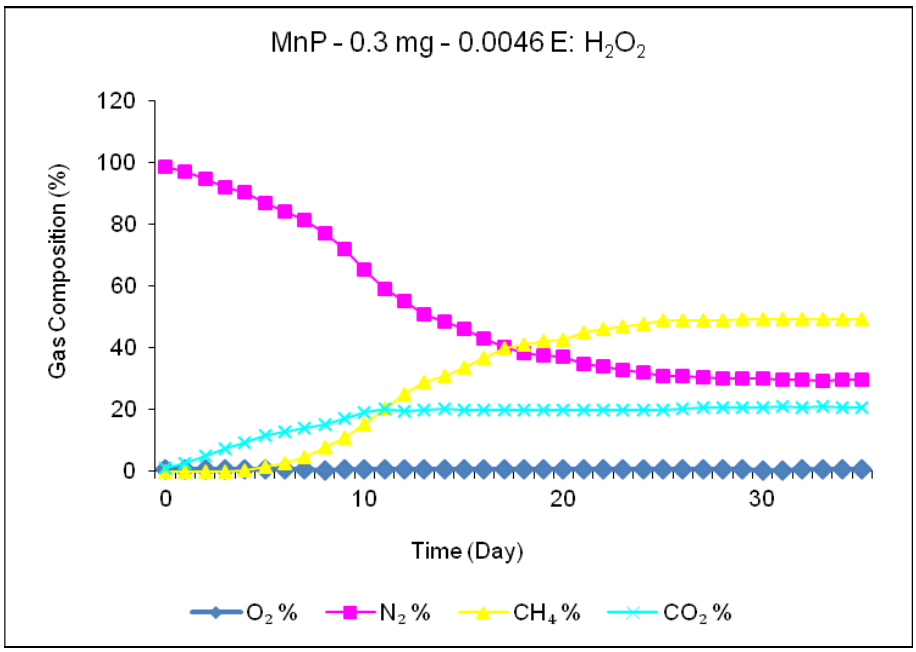


Figure 4-14: Methane composition in biogas

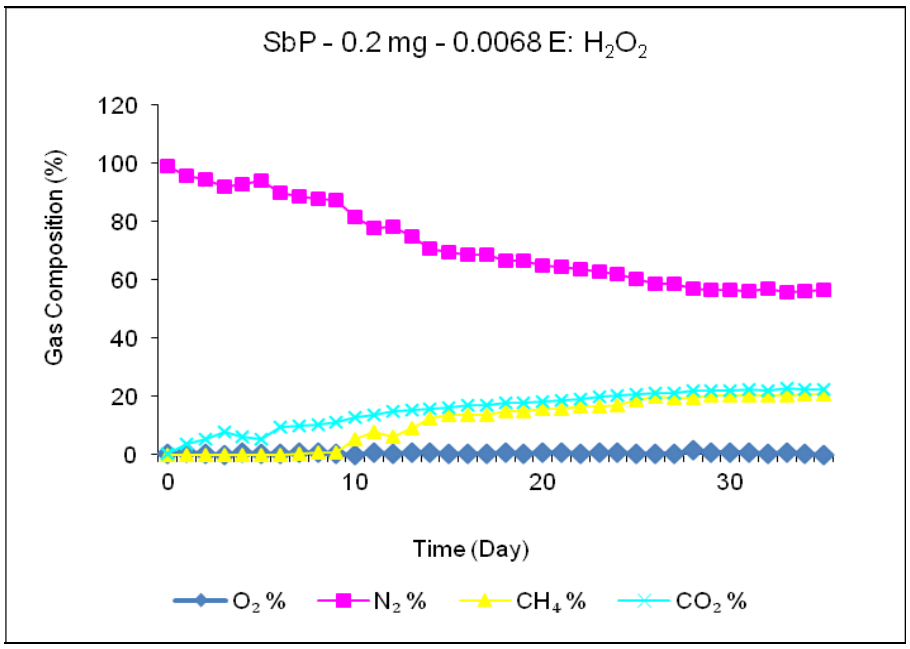
The remaining content of the biogas were N_2, CO_2, and O_2. The gas composition depended on the retention time, enzyme type, E:H_2O_2 ratio, and enzyme dose in each sample. Gas compositions in each enzyme at their best combination of enzyme dose and E:H_2O_2 ratio are shown in Figure 4-15.


LiP - 0.3 mg - 0.0034 E: H_2O_2
Gas Composition (%)
120
100
80
60
40
20
0
0
10
20
30
Time (Day)
O_2 %
N_2 %
CH_4 %
CO_2 %

(a)


MnP - 0.3 mg - 0.0046 E: H_2O_2
Gas Composition (%)
120
100
80
60
40
20
0
0
10
20
30
Time (Day)
O_2 %
N_2 %
CH_4 %
CO_2 %

(b)



(c)

Figure 4-15: Gas composition in biogas (a) Lip (b) MnP (c) SbP

As evident from Figure 4-15, each reactor was operated under anaerobic condition with negligible amount of oxygen during the experimental period. Initially each reactor had nearly 100% N_2. CO_2 started appearing in day 1 in each reactor. Once the lag phase (6 ~7days) was over, CH_4 production was started, followed by a significant increase. Each reactor showed a similar trend, the reactor with MnP showed the highest CH_4 composition among the 3 of the enzymes with 48% CH_4.

4.3.8 Effect of temperature

As mentioned previously, batch experiments were conducted at two different temperatures, 35 ^{0}C and 45 ^{0}C, to estimate the activation energy. Figure 4-16 shows the variation of methane production in enzyme added and control reactors at the two temperatures.

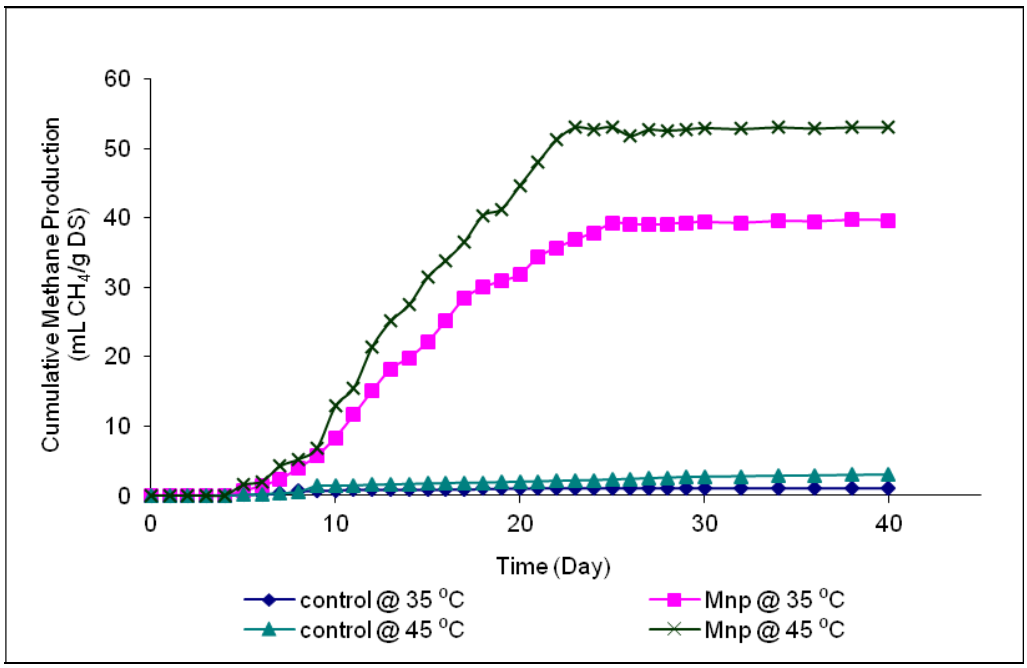


Figure 4-16: Effect of temperature

In enzyme added reactors, cumulative CH_4 yield was 1.3 times higher at higher temperature (45 ^{0}C) than at lower temperature (35 ^{0}C). The 7-fold increase in cumulative CH_4 yield was observed in control reactors at higher temperature. Anaerobic degradation can be carried out under mesophilic (~25-40 ^{0}C) and themophilic conditions (~45-60 ^{0}C) (Gracia-Heras, 2003). However, in general, the mesophilic anaerobic degradation is widely used compared to the themophilic degradation, mainly because of low energy requirement at field scale and higher process stability (Ferrer e*t al*., 2008). In the present study, all the experiments were therefore conducted at 35 ^{0}C even though higher temperature showed relatively higher efficiency than the low temperature operation.

To estimate the activation energy associated in enzyme added and control reactions, the kinetic constants at two different temperatures were first derived using the kinetic model, which will be described in Chapter 6. The activation energy was then calculated using Arrhenius equation. The results will be discussed in Chapter 6 together with the kinetic data.

4.3.9 Effect of enzyme addition at day 6

In practice, the enzyme addition in the field would take place at the active gas generation phase. Therefore, a series of experiments were conducted by adding enzyme at its active gas generation phase compared to the enzyme addition at the beginning of the experiments. Figure 4-17 represents the variation of results under two conditions.

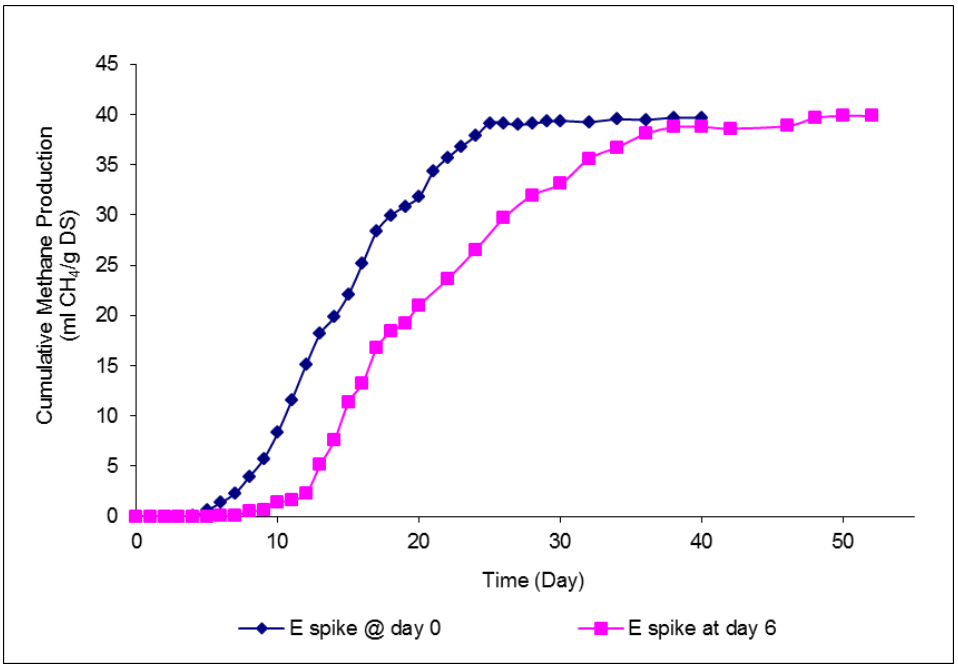


Figure 4-17: Effect of enzyme addition point (MnP, 0.3 mg, 0.0046 E:H_2O_2)

It was noted from the results that the cumulative methane yields under two different conditions, enzyme addition at day 0 and at day 6, were similar at the end of the experimental period. There was a lag phase prior to gas generation in each case with a longer lag phase (9 days lag phase) when the enzyme was added at day 6. This justifies that the enzyme addition could not produce methane instantaneously; methane production is the last step of anaerobic degradation process, it has to go through several intermediate steps. Enzymatic reaction might start immediately after activation that leads to increase the rate of waste hydrolysis. However, before any conclusion can be made on the

enzymatic reaction, further experiments need to be conducted to study the enzyme reaction process in finer details.

4.3.10 Substrate utilization

Substrate utilization in terms of lignin content, solid TOC, and leachate DOC was used as an indication to evaluate the process performance in addition to the cumulative methane yield.

4.3.10.1 Lignin content

The lignin content (% of VS) of solid waste was measured before and after batch experiments and the percentage lignin conversion for different enzymes at their best combination of enzyme dose and E:H_2O_2 ratio was calculated. The results are summarized in Table 4-4.

Table 4-4: Lignin conversion by different enzymes

Enzyme	% Lignin before experiments	% Lignin after experiments	% Lignin Conversion
control	81.9	76.9	6.2
LiP	81.9	38.6	52.9
MnP	81.9	25.9	68.4
SbP	81.9	52.0	36.5

With a 68.4% lignin reduction, the enzyme MnP showed the best performance among the three enzymes tested. This result is compatible with the methane production results where the enzyme MnP showed the best performance in terms of maximum methane yield. The results verified that the lignin in waste was actually degraded by enzyme augmentation.

4.3.10.2 TOC in waste

Figure 4-18 shows the variation of TOC in waste in MnP enzyme added reactor (at their best combination) and control reactor. The rate of TOC utilization in enzyme added reactor was significantly higher than the control reactor. The substrate was consumed by nearly 97% at the end of the experimental period when the waste was augmented with enzyme. The substrate consumption in terms of TOC in control reactor was only 26%.

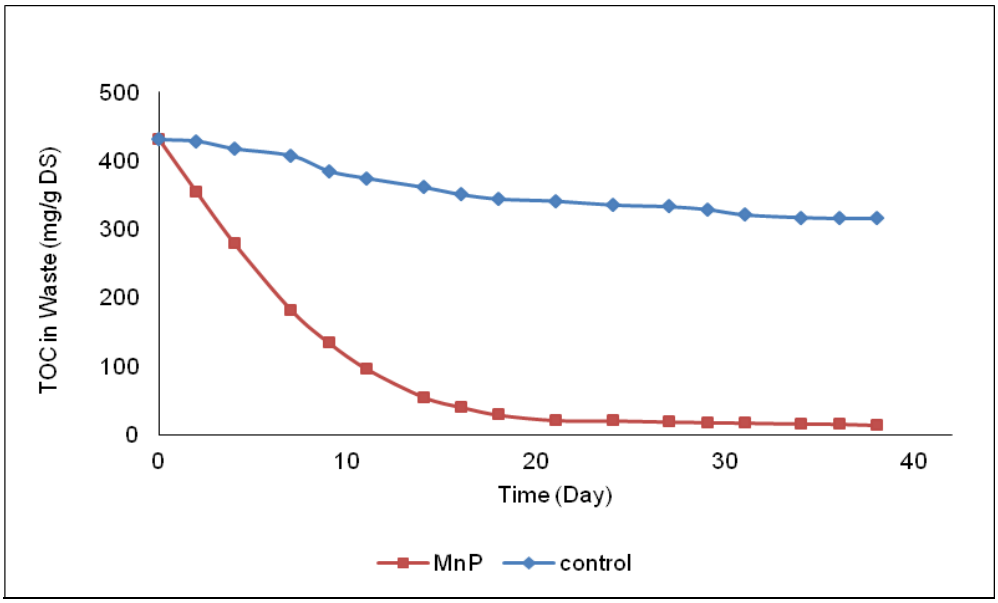


Figure 4-18: TOC in waste

Two main conclusions can be made from the TOC results; enzyme addition actually increased the consumption of TOC in waste compared to the control reactor and the reaction was substrate limited at the end of the experimental period, the reason for not increasing the gas production by a second addition of enzyme or the excess enzymes.

The TOC results were used to evaluate and validate the kinetic model which will be discussed in Chapter 6.

4.3.10.3 DOC in leachate

As shown in

Figure 4-19, the DOC of leachate in enzyme added reactor was increased at a higher rate. After a lag period of about 6 days, the increase of DOC concentration in leachate from the test cell was sharp; DOC reached a peak value of 3286 mg/L, while the DOC concentration in the leachate from the control reactor reached a peak value of only 1093 mg/L at a slower rate.

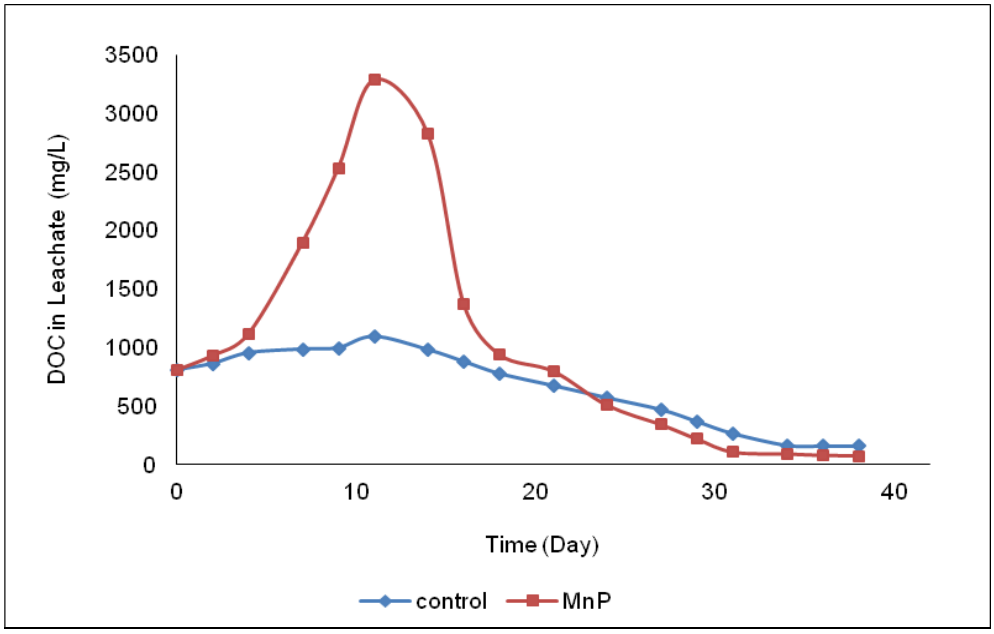


Figure 4-19: DOC in leachate over time

The DOC in leachate and TOC in waste can be correlated. The highest DOC in leachate was observed in around day 12, while the rate of TOC utilization was dramatic during 0-12 days. These results justified the assumption of ‘enzyme addition could enhance the waste hydrolysis'. The increasing DOC in leachate and decreasing TOC in waste were indications of waste hydrolysis.

4.3.10.4 pH variation in leachate

Variation of leachate pH over time in the control and the MnP enzyme added batch reactor are shown in

Figure **4-20**. During the initial lag phase, pH of the leachate was nearly constant at 6.3. When the gas production started at around day 6, the pH of enzyme added sample was decreased up to 5.2, because acidogenic conditions in the reactor became more intense. During the initial gas production stage, fermentative bacteria hydrolyze the solid organics into primarily volatile fatty acids and alcohols which leads to a lower pH (Warith *et al.*, 1999). As the methane production rate increases, methanogens utilize volatile fatty acids and other intermediate compounds. This conversion causes pH in leachate to increase and thereafter reaching constant level of pH as methane production stabilizes. pH in the control reactor did not show any significant change as in the test reactor indicating the less waste conversion process as indicated by less methane production.

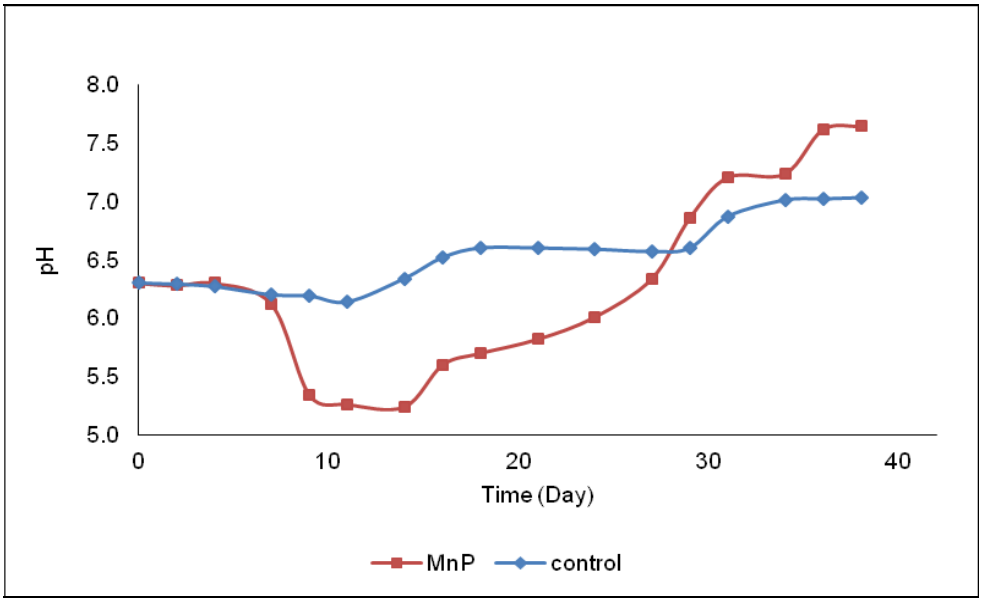


Figure 4-20: Variation of pH

4.3.11 Explanation of mechanism using chemical characterization

The results discussed in section 4.3.10.2 to section 4.3.10.4 are highly correlated and the mechanism involved in the enzymatic enhanced anaerobic waste degradation can be explained (or justified experimentally) to some extent. The role of enzyme was to enhance the conversion of TOC in waste to DOC in leachate. In other words, enzymes increased the rate of waste hydrolysis, the rate-limiting step in anaerobic waste degradation. Once the rate-limiting step was accelerated, the microganisms could have a better accessibility to the substrate which leaded to an increase in methane production through several intermediate stages.

4.3.12 Explanation of mechanism using microbial characterization

To understand the waste degradation mechanism in finer details, microbial characterization tests were conducted. The first attempt was to estimate the microbial community quantitatively using MPN technique. The results obtained were not repetitive and any specific trends could not be observed, hence they were not conclusive and also not included in the thesis.

The second attempt was to develop the methane growth curves when the leachate was supplemented with two different substrates (CO_2+H_2, and acetate) to understand the main group of methanogens who dominate the methanogenesis process. The methane yield when leachate was supplemented with substrates and when leachate and enzyme solution was supplemented with substrates are shown in Figure 4-21 and Figure 4-22, respectively.

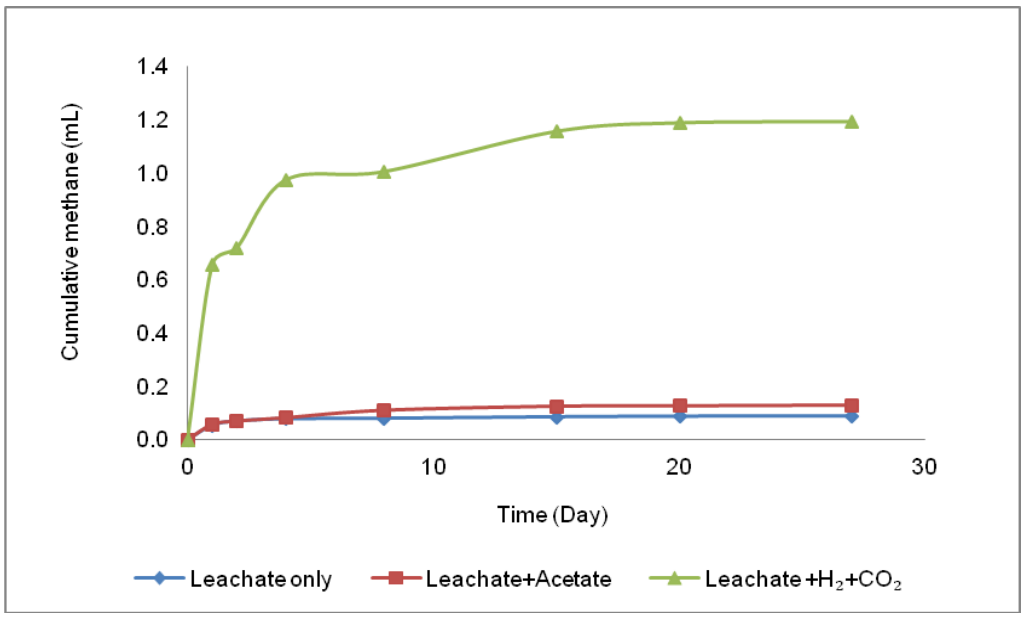


Figure 4-21: Methane production curve (only leachate and substrate)

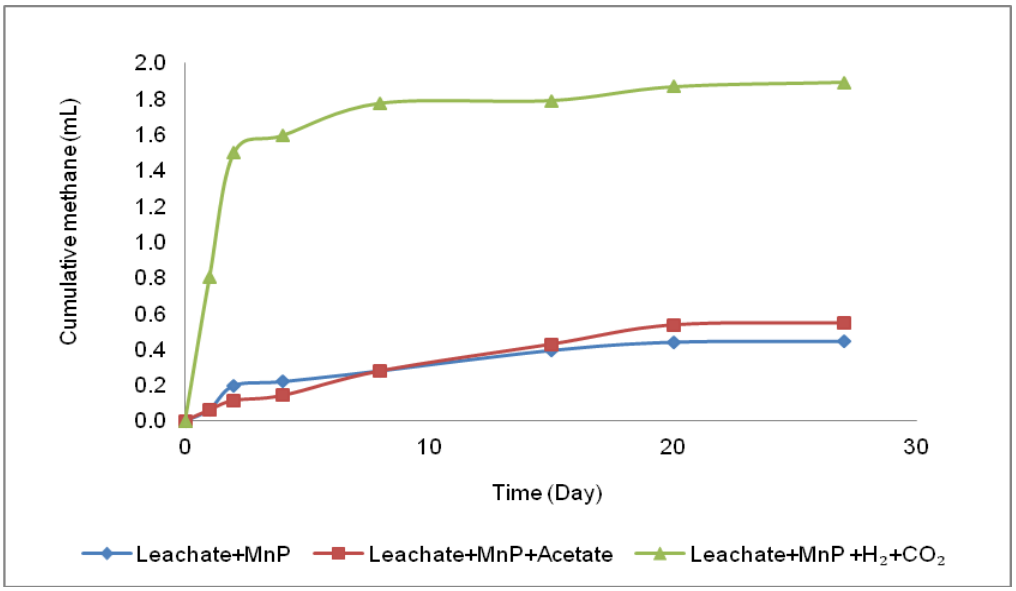


Figure 4-22: Methane production curve (leachate, enzyme, and substrate)

The samples with CO_2+H_2 produce more methane compared to the samples with acetate and control (no substrate) in both cases. In leachate only samples, methane yield when supplemented with CO_2+H_2 was nearly 9 times higher than in acetate added samples, and

13 times higher than in control samples. Similarly, in leachate and enzyme samples, CO_2+H_2 added samples showed 3 fold increase in methane yield than in acetate added samples, and 4 fold increase than control samples. It can be therefore concluded the methanogenesis of anaerobic waste degradation process in this study was dominated by hydrogenotrophic methanogens even though acetotrophic methanogens also involved in the process.

When enzyme was added to the leachate, a higher methane yield was observed, because leachate constituents of organic materials as well. The methane production ratio of two different substrates in two cases was significantly different as described. However, further experiments need to be conducted before comparing the ratio or made any conclusions of the bacterial community involve in two different scenarios.

4.3.13 Enzyme activity

Enzyme activity was measured in terms of enzyme concentrations (U/L). Results obtained up to day 7 are presented in Figure 4-23. The results obtained after day 7 were not reliable in terms of the repeatability and the absorbance readings and hence not included in Figure 4-23.

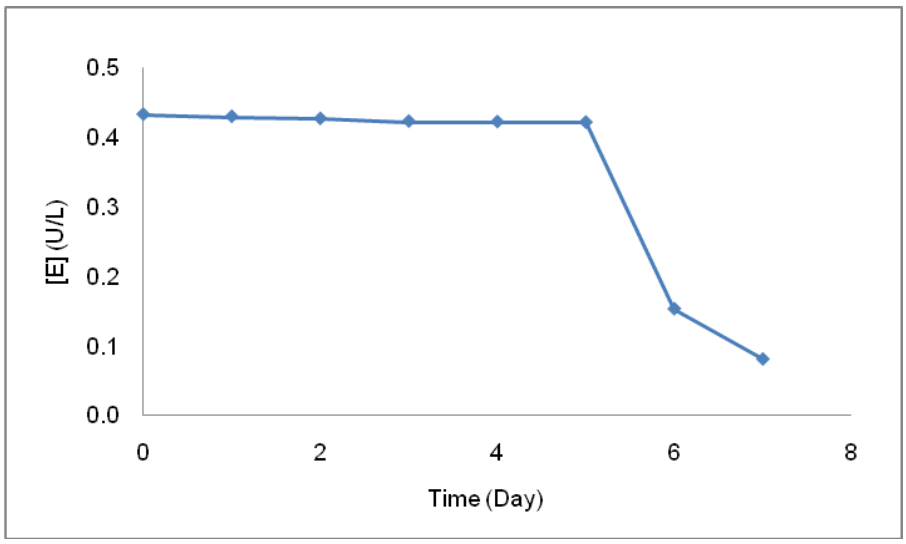


Figure 4-23: Variation of enzyme activity in batch reactor

The enzyme activity declined rapidly after day 5, up to ~81% by day 7. Two reasons could explain this behavior. The enzyme could be actually deactivated by day 7 either due to substrate limiting or product inhibition. However, substrate utilization trend shown in

Figure 4-18 shows that the system had enough substrate (~182 mg TOC/g DS) by day 7. The potential reason for lower enzyme activity therefore could be the product inhibition, especially the different enzymes produced during waste degradation process such as protease (Jones & Grainger, 1983). Jones & Grainger (1983) measured cellulase activity to study the microbiological behavior of the refuse ecosystem. Cellulase activity decreased rapidly after the first 10 day of their experiment, and they suggested that cellulase enzyme deactivation by protease, a resultant product of protein hydrolysis.

To understand product inhibition during the waste degradation process, it may be necessary to conduct some experiments with sterilized waste. It would help to demonstrate the life of enzyme when there was no microbial growth, no secreted enzymes, and no degradation products that might interfere with the assay itself.

The enzyme activity results presented here was used to find the enzyme decay constant which will be discussed in Chapter 6.

Considering the results obtained in microbial characterization test and enzyme activity tests, further experiments are recommended. Even though several potential causes to the observed behaviours were discusses here, sufficient experiments were not conducted to support the facts.

As mentioned previously, some of the apparent trends in microbial related experiments in this study were not conclusive. Similarly, some in-conclusive results were observed by Lagerkist & Chen (1993) on their attempt to enhance waste degradation by adding cellulose enzymes. They suggested that this may be due to a lack of homogeneous distribution of properties within the waste matrix, there can be pockets where methanogens and other microorganisms can develop in spite of the macro environment which is reflected by the leachate. On the other hand, the difficulties observed in leachate biological characterization could arrive when the standard analytical methods for water and wastewater are used for leachate analysis. Therefore the analytical procedures may need revisions before conducting further experiments in this area.

For example, as mentioned in the Manual of Clinical Enzyme Measurements (1972), the enzyme assay must be designed in a way that the observed activity is proportional to the amount of enzyme present for enzyme concentration to be the only limiting factor. It is satisfied only when the reaction is zero order. Therefore, to measure enzyme activity ideally, the measurement must be made in the portion where the reaction is zero order. A reaction of substrate degradation is most likely to be zero order initially since substrate concentration is higher. As substrate is used up, the enzyme's active sites are no longer saturated, substrate concentration becomes rate-limiting, and the reaction becomes first order. In this case, the enzyme activity and amount present is not directly proportional, which could lead to an in-conclusive behavior.

4.4 Results Variability

Each batch experiment was carried out in duplicate or triplicate to assess the variability in experimental measurements.

The variability refers to the distribution of the measured values of a repeated experiment. There are several ways to make a reasonable estimate of the variability of measurements or the random error in a particular measurement (Laboratory Educators in Natural Sciences and Engineering, 2003).

1) Standard deviation (SD) of a series of measurements

SD determines how much variation of an individual data point exists from its mean. SD is mathematically represented by,

$$SD = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}{\left(n-1\right)}} \qquad \textbf{Equation 4-2}$$

where, x_i , $\bar{x}$ are measured value at specific point, and the mean of the measured values, respectively. n is the number of repetitions.

The standard deviation obtained from each replicate sample (for data which has significant digits) in this study at each data point was less than 8% (Refer Table 4-5 for the best combination). A relatively low standard deviation indicated that the data points tend to be close to the average, they were not spread out over a large range of values. Even though SD is a good way to represent the error, SD becomes an accurate representation of the true value only if there are an infinite number of measurements (Harrison, 2001).

2) The standard error in the mean (SEM)

SEM is used to find the error or the uncertainty in the estimated value of the mean; it assesses the precision of the study. The SD tells us the distribution of individual data points around the mean, and the SEM informs us how precise our estimate of the mean is (Nagele, 2001). Mathematically, the standard error of the mean is represented by,

$$SEM = \frac{SD}{\sqrt{n}} \qquad \textbf{Equation 4-3}$$

where, SEM is the standard error of the mean, SD is the standard deviation, and n is the number of times the measurement was repeated.

In this study, taking SD at its highest as 8.0, the highest possible SEM was estimated as 4.6. A relatively low value of SEM represents that the effect of random errors or uncertainties during the experiments are less significant. A lower SEM compared to SD indicates that there is less precision error in a sample mean than in the individual measurements (Mills & Chang, 2004). Therefore, the average values of the measured data were used for the calculations throughout this thesis.

3) Confidence level (CL) and confidence interval (CI)

The use of confidence level in error analysis is relatively a comprehensive way to represent the error. An interval within which the mean of the sample lies with a specified confidence level is referred to as the confidence interval. This interval has fixed end points, where the mean might be in between or not. The following equation can be used to estimate the lower and upper end points at 95% confidence level by assuming that the sample is normally distributed,

$$\left(\overline{X} - 1.96\,SEM\right) \le \mu \le \left(\overline{X} + 1.96\,SEM\right) \qquad \textbf{Equation 4-4}$$

where, $\overline{X}$ is the sample mean, *SEM* is the standard error of the mean, and μ is the mean to be estimated within the end points.

For example, in this study, at day 40, MnP enzyme at its best combination showed the average cumulative methane production of 39.7 mL (Refer Table 4-5). The calculated SEM at this data point was 0.9. Therefore, the confidence interval for this specific data point can be calculated as $(38.0) \le \mu \le (41.5)$. This result indicates that, in 95% of the cases, mean methane production will be between 38.0 and 41.5 mL, but in 5% of the cases it will not be.

Table 4-5 summarizes the results of error analysis conducted based on Equations 4-2 to 4-5, for MnP enzyme at its best combination of enzyme dose and E:H_2O_2 ratio.

Table 4-5: Error analysis for MnP at 0.3 mg enzyme dose and 0.0046 E:H_2O_2 ratio

Time (day)	Cumulative methane production (mL CH_4/g DS)				Error Analysis			
	Run 1	Run 2	Run 3	Mean	*SD*	*SEM*	CI based on 95% CL	
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
5	0.2	1.4	0.3	0.6	0.7	0.4	0.2	1.4
6	0.4	2.0	1.9	1.4	0.9	0.5	0.4	2.4
7	1.2	3.5	2.4	2.4	1.1	0.6	1.1	3.6
8	2.7	4.3	5.0	4.0	1.2	0.7	2.7	5.4
9	4.9	6.0	6.3	5.7	0.8	0.4	4.9	6.6
10	9.2	6.3	9.6	8.4	1.8	1.0	6.3	10.4
11	15.1	8.4	11.7	11.7	3.4	2.0	7.9	15.6
12	21.1	12.6	12.3	15.3	5.0	2.9	9.6	21.0
13	26.5	14.8	14.6	18.6	6.8	3.9	10.9	26.3
14	28.2	16.6	16.0	20.2	6.9	4.0	12.5	28.0
15	30.3	20.1	17.2	22.5	6.9	4.0	14.7	30.3
16	31.7	24.7	20.1	25.5	5.8	3.4	18.9	32.1
17	32.5	28.3	24.5	28.4	4.0	2.3	23.9	32.9
18	33.0	28.6	28.4	30.0	2.6	1.5	27.0	32.9
19	33.3	29.9	29.6	30.9	2.0	1.2	28.6	33.2
20	33.6	30.9	31.0	31.9	1.6	0.9	30.1	33.6
21	36.3	32.2	34.6	34.4	2.1	1.2	32.0	36.7
22	38.0	33.4	35.9	35.7	2.3	1.3	33.1	38.4
23	39.6	34.6	36.5	36.9	2.5	1.5	34.1	39.7
24	39.6	37.3	36.7	37.9	1.5	0.9	36.1	39.6
25	41.0	38.1	37.0	38.7	2.1	1.2	36.3	41.1
26	41.0	39.0	37.1	39.0	1.9	1.1	36.9	41.2
28	41.3	39.4	37.4	39.4	2.0	1.2	37.1	41.7
30	41.5	39.4	38.0	39.6	1.6	1.0	37.8	41.5
32	41.5	39.4	38.1	39.7	1.7	1.0	37.7	41.6
34	41.6	39.4	38.1	39.7	1.6	0.9	37.9	41.5
36	41.6	39.5	38.1	39.7	1.7	1.0	37.8	41.7
38	41.6	39.5	38.1	39.7	1.4	0.8	38.1	41.3
40	41.6	39.5	38.1	39.7	1.5	0.9	38.0	41.5

Overall, one of the important questions that arises when repeating an experiment is that 'how many times an experiment or the measurement should be repeated?'. It can be seen from Equation 4-4 that the SEM decreases as number of repetitions increases. For example, if the measurement is repeated 4 times, the error can be reduced by a factor of two. Repeating the measurement 9 times reduces the error by a factor of three. The larger the number of measurements, the smaller the standard error, and the closer the sample mean approximates the population mean. Therefore the best answer to the above question is 'as many times as possible' however, the data collection effort would need a lot more time, energy, and money.

In this study, the experiments were repeated only 2 or 3 times, which also leads to an uncertainty in undertaking an accurate error analysis. Therefore, many repetitions in experiments are recommended before a comprehensive and more accurate error analysis could be conducted. It will help to minimize the uncertainties associated in the process.

4.5 Conclusions and Recommendations

The results obtained from the batch experiments in this study showed the feasibility of leachate augmentation in enhancing waste degradation and methane production. In this study, the gas production, gas composition, leachate quality, and substrate utilization results were interrelated to the waste degradation behaviour; because of the uncertainties observed in the efforts to understand the microbiological behaviour in a finer detail. As also concluded in the review conducted by Barlaz *et al*. (1990) on microbial dynamics of methane producing ecosystems, this could be one of the best available methods to represent the refuse ecosystems.

Optimum process parameters were identified through series of batch experiments. In every aspects studied, MnP enzyme showed the best performance. The best combination for MnP was 0.3 mg enzyme dose and 0.0046 g/g E:H_2O_2 ratio; all the other combinations studied for MnP were better than the combinations in other enzymes. LiP was shown to be the second best enzyme with 0.3 mg enzyme dose and 0.0034 g/g

E:H_2O_2 ratio. SbP showed the worst performance in this study, which implies that SbP is not a feasible enzyme for enhancing the degradation of lignin-rich waste.

Even though the batch experimental results provide an effective method for selecting the best options, the results could not be directly applied to field conditions, especially due to the difference between two processes, such as leachate recirculation. Therefore, the process needs to be scaled-up through an intermediate step, involving flow-through column experiments, before proceeding to apply it at a field-scale level.

Chapter Five: Laboratory Flow-Through Column Experiments

This chapter discusses the objectives, experimental setup, procedure and design, scale up approach, and the results associated with laboratory flow-through column experiments.

5.1 Objectives of Flow-Through Column Experiments

As described in Chapter 4, the batch experimental study was designed to test various parameters in a simplified system, but the results could not be directly applied to enhancing gas production in field-scale applications. The simplified batch system lacked both the necessary scale and processes such as leachate recirculation to reflect actual field conditions. Therefore, the process was scaled up to a set of flow-though column experiments to mirror landfill conditions for testing the enzyme augmentation concept in a cost-effective and controlled manner prior to field-scale application.

The objectives of conducting flow-through column experiments were to,

1. Assist the scale up process.
2. Determine the impact of enzyme added leachate recirculation in lignin rich waste degradation.
3. Optimize the quantity of enzyme by changing operating conditions, especially leachate recirculation parameters, such as leachate recirculation frequency.

5.2 Scale Up and Scale Down Approach

The objective of scale up was to obtain similar yields with the same product distribution in flow-through columns and in field-scale as obtained in batch reactors. In this research, the similarity theory was used to scale up the process as described by Sola & Godia (1995).

The Calgary biocell (discussed in detail in Chapter 7) was used as the base line to obtain the flow-through column variables, especially the transport parameters. The biocell was scaled down based on the flow dynamic similarity, as it is an existing research facility.

Reynolds number becomes an important dimensionless number in scale down processes as it characterizes the similar flow patterns (Sola & Godia, 1995). Similar leachate recirculation rates were then applied in laboratory flow-through columns as in the field-scale operation. Reynolds number (Re) for packed bed can be written as,

$$\mathrm{Re} = \frac{\rho d V}{\mu(1-\varepsilon)} \qquad \textbf{Equation 5-1}$$

where,

ρ = density of leachate (kg/m^3)

d = average diameter of waste particles (m)

V = superficial velocity of leachate flow (m/s)

μ = viscosity of leachate (kg/m.s)

ε = void fraction in packed waste

For flow dynamic similarity, Re in flow-through column and Calgary biocell needed to be equal.

$$\mathrm{Re}_{column} = \mathrm{Re}_{biocell} \qquad \textbf{Equation 5-2}$$

Substituting Equation 5-1 in Equation 5-2,

$$\left(\frac{\rho_c d_c V_c}{\mu_c(1-\varepsilon_c)}\right) = \left(\frac{\rho_b d_b V_b}{\mu_b(1-\varepsilon_b)}\right) \qquad \textbf{Equation 5-3}$$

subscripts *c* and *b* represent the column and biocell, respectively.

Under the condition that, $\mu_c = \mu_b$ and $\rho_c = \rho_b$, the following relationship was derived.

$$V_c = \frac{(1-\varepsilon_c)d_b V_b}{(1-\varepsilon_b)d_c} \qquad \textbf{Equation 5-4}$$

Equation 5-4 can be further simplified by using the relationship shown below for superficial velocity of leachate flow.

$$V = \frac{Q}{A} \qquad \textbf{Equation 5-5}$$

where,

Q = leachate flow rate (m^3/s)

A =cross sectional area (m^2)

Substituting Equation 5-5 in Equation 5-4, leachate flow rate in columns can be written as,

$$Q_c = \frac{(1-\varepsilon_c)d_b Q_b A_c}{(1-\varepsilon_b)d_c A_b} \qquad \textbf{Equation 5-6}$$

Equation 5-6 was then used to estimate the leachate recirculation flow rate in flow-through column experiments. Input parameters are shown in Table 5-1.

Table 5-1: Input parameters of the scale down approach

Parameter	Value
d_b (m)	0.1
d_c (m)	0.005
Q_b (m^3/s)	0.003
A_b (m^2)	7225
A_c (m^2)	0.015
ε_b	0.56
ε_c	0.4

With the input parameters shown in Table 5-1, Q_c was estimated to be about 10 mL/min, which was used as leachate recirculation flow rate in flow-through column experiments.

5.3 Experimental Methodology

5.3.1 Column experimental setup

Two columns were operated simultaneously. Both columns were designed to reproduce landfill bioreactor conditions as much as possible. Figure 5-1 and Figure 5-2 show a schematic diagram of column and a photograph of the laboratory experimental setup, respectively.

The height and the diameter of the columns were 1 m and 14 cm, respectively. The columns were made from plexiglas. Each column consisted of leachate collection, sampling and recirculation, and gas collection and sampling. A layer of gravel, 20 cm thick, and a screen were placed at the bottom of each column to prevent clogging of leachate outlet and pipes from fine particles. A gas measuring device, comprising of an inverted graduated cylinder submerged in a solution of 20% $NaSO_4$ and 5% H_2SO_4 (by

mass) (Suna Erses & Onay, 2003), was connected to each column, with control valves. A leachate collection tank was connected to each column to collect and store the leachate before recirculation. Two variable flow peristaltic pumps (VWR 57951-016) were used to recirculate leachate from the leachate collection tank to the top of the column. An inverted funnel which had a base with evenly punched holes was fixed to the leachate distribution point in the column to improve the distribution of leachate in the waste. All the piping in the system included 1/4" stainless steel pipes.

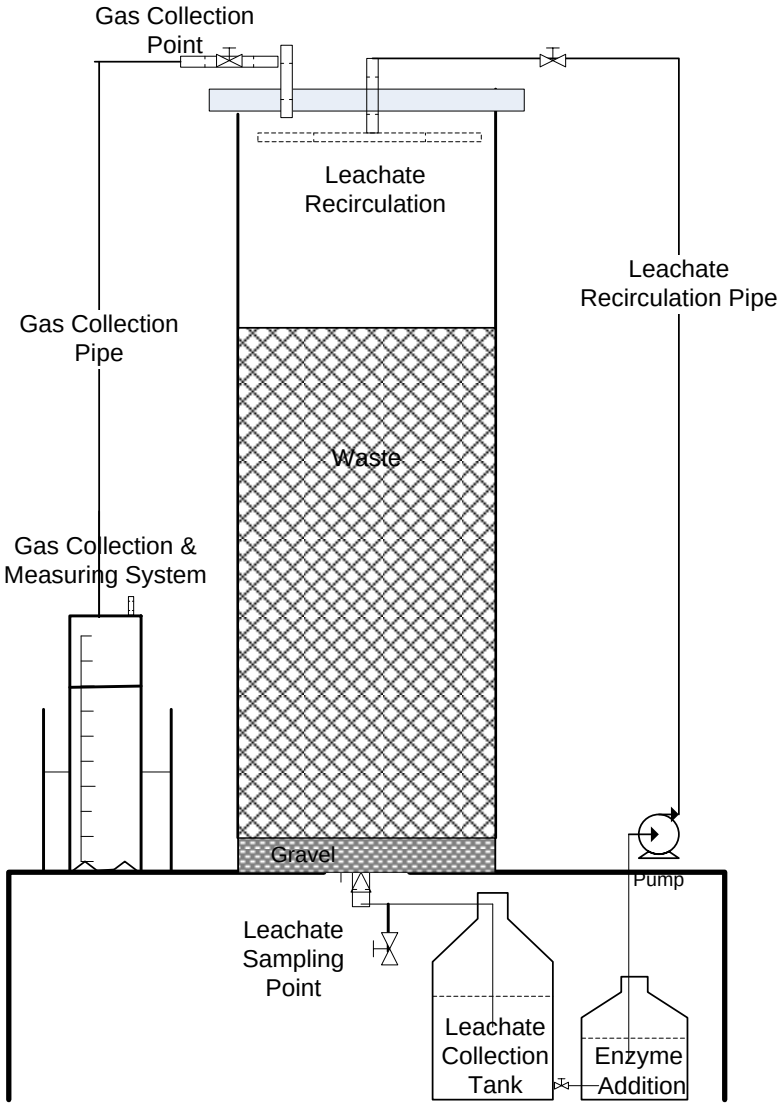


Figure 5-1: Schematic diagram of a flow-through column

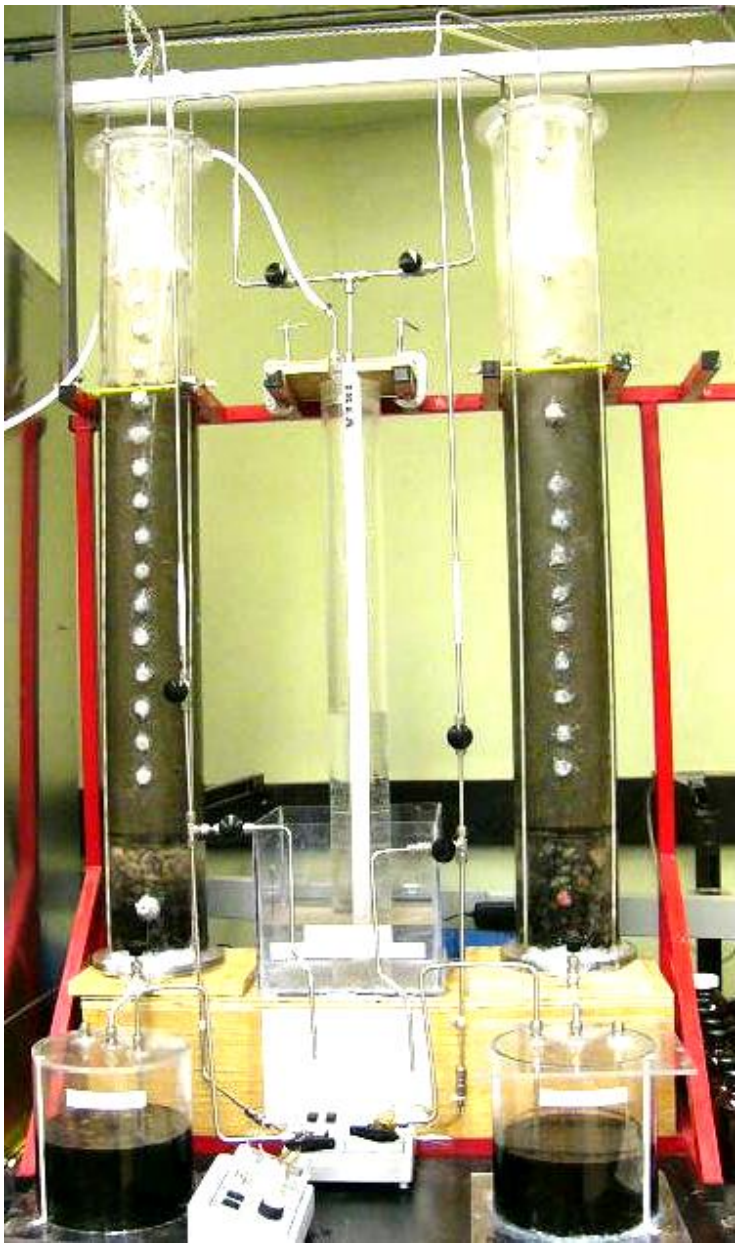


Figure 5-2: Laboratory experimental setup

5.3.2 Column experimental procedure

Each column was filled with 9 kg of uniformly mixed representative MSW. The waste used was the same as in the batch experiments. In order to accelerate waste degradation and to facilitate compaction, waste was shredded to a diameter of 5 mm. 9 kg of wet waste was filled up to a height of 66 cm in each column. After waste placement, the

columns were purged with N_2 gas to replace O_2 and to create an anaerobic environment. The columns were then sealed to prevent air entry.

Flow-through column experiments consisted of three stages, initial adjustment (acclimatization) phase, operational phase, and stabilization phase.

5.3.2.1 Initial adjustment phase

In the initial acclimatization phase, the moisture content of the waste was brought to the field capacity of 48% (by weight) by adding tap water to the columns daily, until the amount collected from the column showed the same amount of water as that added the previous day (Warith *et al.*, 1999). The saturation of waste in the columns was necessary because it allowed waste degradation to be initiated quickly, by the intrusion of humidity and microbial seeding (Francois *et al.*, 2007). The produced leachate was collected and stored in the leachate collection tank.

5.3.2.2 Operational phase

The operational phase included the enzyme addition to the test column, leachate recirculation, monitoring gas production, and measuring leachate quality. Once the initial adjustment phase was over, activated enzyme, MnP, as identified from batch experiments, was added to the leachate tank of the test column. The leachate was then recirculated.

The quantity of produced gas was measured once in 2-3 days by opening the valve connected to the gas measuring unit. When the gas production was high, the gas volume was measured every day. Compositions of CH_4 and CO_2 in biogas were determined by injecting 0.5 mL of headspace gas to the VARIAN 4900 Micro GC. The columns were kept at room temperature (25±2 ^{o}C) and the gas measurements were taken at the room temperature and room pressure (1 atm). Leachate samples were tested on a regular basis (~once a week) to determine the degree of waste stabilization in columns. Leachate

samples were analyzed for COD, BOD, DOC, pH, NH_3-N, TN, and TP using the analytical test methods described in Chapter 3.

5.3.2.3 Stabilization phase

Over time, the gas production declined, and eventually stopped. The gas volume, compositions, and leachate quality were measured until the columns reached this stabilization phase. Once the gas production stabilized, the columns were dismantled to obtain the waste samples to measure the TOC in degraded solid waste.

5.3.3 Design of column experiments

Two sets of flow-through column experiments were conducted.

5.3.3.1 First set of flow through column experiment

The first set was conducted to study the impact of enzyme augmentation and recirculation. Two columns filled with partly degraded MSW were used simultaneously; a column undergoing enzyme addition and leachate recirculation and the other only with leachate recirculation (control, without enzyme). Based on the results from batch experiments (refer Chapter 4) and a scale up model (refer Chapter 6), 12 mg of activated MnP enzyme (for 9 kg of wet waste) was added to the leachate tank in the test column. The leachate was then recirculated in both columns at a rate of 10 mL/min for 20 min, once in two days. As described previously, the leachate recirculation rate was determined from Equation 5-6. The waste characteristics, compacted density, leachate recirculation rates and other operating parameters and environmental conditions were similar in both columns. Augmentation of leachate with MnP enzyme was the only manipulated variable.

The results from the first set of experiments showed that the columns were limited in nutrients, in terms of phosphorous (discussed in section 5.4). But, the nutrients were not added during the experimental period, as the objective was to check the sole effect of enzyme addition. Once the gas production was stabilized, nutrients were added prior to

starting the second set. After the nutrients addition, the columns were dismantled, and the nutrient-enriched waste was used in the second set.

A mixture of two separate fertilizers, 4-12-4 liquid and 30-10-10 solid (purchased from the Home Depot, Calgary), was used to achieve the ideal nutrient ratio. COD:N:P ratio at its higher range of 100:4:1 was used to enrich the waste with nutrients (Mata-Alvarez, 2003). In order to form a 4:1 ratio of N:P for the existing COD in two columns, 82 mg/L of N and 21 mg/L of P were added to the leachate prior to recirculation. The nutrient-added leachate was then recirculated at a rate of 10 mL/min for 20 min, once in two days, until gas production stabilized again. The gas production and leachate quality in terms of COD, TN, and TP were measured.

5.3.3.2 Second set of flow through column experiments

The objective of conducting a second set of column experiments was to optimize the quantity of enzyme required to obtain a certain conversion of waste-to-gas by increasing the number of leachate recirculations. In other words, the second set was intended to support empirically the scaling up process.

In practice, knowing the un-bounded quantity of enzymes after each recirculation was important. If there was any un-bounded enzyme after one recirculation, the leachate can be recirculated many times until all the enzymes were bounded to the waste matrix. On the other hand, if all enzymes were bound to waste in one cycle, the leachate recirculation can be paused until the enzyme was used up, allowing enough time for complete contact. Although measuring the enzyme activity before and after each recirculation was necessary, as described in Chapter 4, our attempts at measuring enzyme activity were not successful. Therefore an alternative method was used to achieve the required information indirectly.

The scale up model was first used to calculate the quantity of enzyme to achieve the waste conversion in one cycle of leachate recirculation. 10 mg of enzymes was required

to obtain 80% of waste conversion under certain requirements (details provided in Chapter 6). The model was then modified to calculate the number of recirculations, if the enzyme quantity was decreased by a certain percentage. This analysis showed that the leachate needed to be recirculated 7 times to obtain 80 % waste conversion with 2 mg of enzymes.

As in the first set, both flow-through columns filled with partly degraded MSW were run simultaneously. The nutrient-enriched waste in the control column and the experimental columns after the first set of experiments were mixed and used in the second set of experiments. In order to increase the porosity to ~0.4, styroform pieces were added to the waste at a 4:1 ratio of waste:styroform. The mixture of waste and styrofoam was filled in both columns up to 60 cm height to a density of approximately 550 kg/m^3. The columns were insulated to prevent the heat losses.

Tap water was added to both columns to reach the field capacity. In order to obtain baseline data, leachate hydrographs were developed. A known quantity of water (ranging from ~15 to115 mL) was added to the waste columns every 1 min during the first 20 min and the volume drained was recorded every 1 min until there was no leachate production. The collected leachate was then recirculated 3 times and the average values were used to plot the hydrographs (volume collected vs time).

The leachate was then recirculated in both columns at a rate of 10 mL/min for 20 min twice a week without adding any enzymes for about a month to better represent the field scale operation prior to enzyme addition. The gas production was monitored, and in the second set, this was considered to be the control operation. Once the stabilized gas production was observed, two different operations were carried out in the two columns.

As predicted from the scale up model (refer Chapter 6), 10 mg of MnP enzyme was added to the column 1 leachate tank and enzyme augmented leachate was recirculated only once at a flow rate of 10 mL/min for 20 min. After the first recirculation, there was

no more leachate recirculation in column 1, only the gas production was monitored during the monitoring period. The quantity of enzyme added in column 2 was decreased by 80% from the amount used in column 1 (*i.e.* 2 mg of MnP); then the leachate was recirculated 7 times at a flow rate of 10 mL/min for 20 min, twice a week. The gas production and leachate quality in terms of COD, DOC, TN, TP, and pH after each recirculation were measured until the stabilization stage.

5.4 Results and Discussion

The cumulative methane production and leachate quality were used as indicators of the effectiveness of the process of enzyme augmentation.

5.4.1 Cumulative methane production and methane composition

Figure 5-3 shows the cumulative methane yields in the enzyme augmented column (experimental) and the control column as observed during the first set of flow-through column experiments.

The experimental column showed about a 5-fold increase of methane production over that in the control column, with a cumulative methane production of 1983 mL versus 398 mL in the control column for 7.4 kg of DS. The CH_4 production trends were similar in both columns, with a 10 day initial lag phase followed by an increase in gas production, and reaching stabilized CH_4 yield after approximately 100 days. Methane production rate in the experimental column was considerably higher than that in the control cloumn.

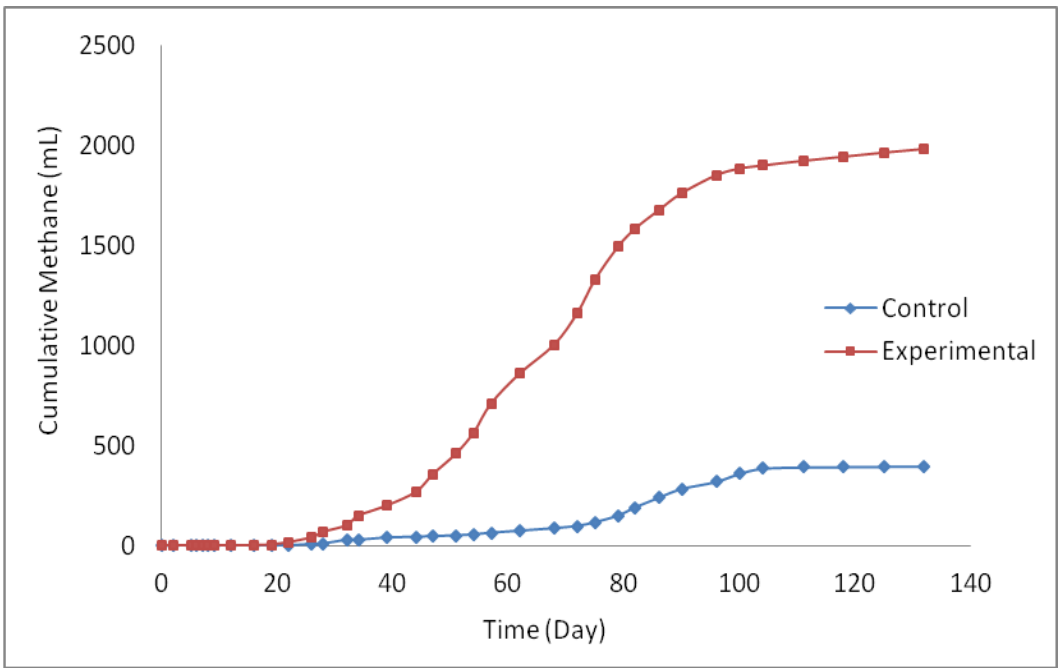


Figure 5-3: Cumulative methane production in control and MnP augmented (experimental) column

As shown in Figure 5-4, the initial CH_4 composition of biogas was very low due to the prevailing acidogenic conditions in the reactor (as validated from pH variation in Figure 5-8). An increase in CH_4 content as a result of the increasing methanogenic activity was then observed during the period of day 10 to approximately day 100. The CH_4 composition in biogas increased continuously to a peak of 32% in the control column towards the end of the monitoring period, with an earlier peak of 45% at day 85 in the MnP enahnced experimental column. The CH_4 concentration in experimental column reached a maximum of 51% by the end of the experimental period, which indicated the success of enzymatically-enhanced leachate recirculation process.

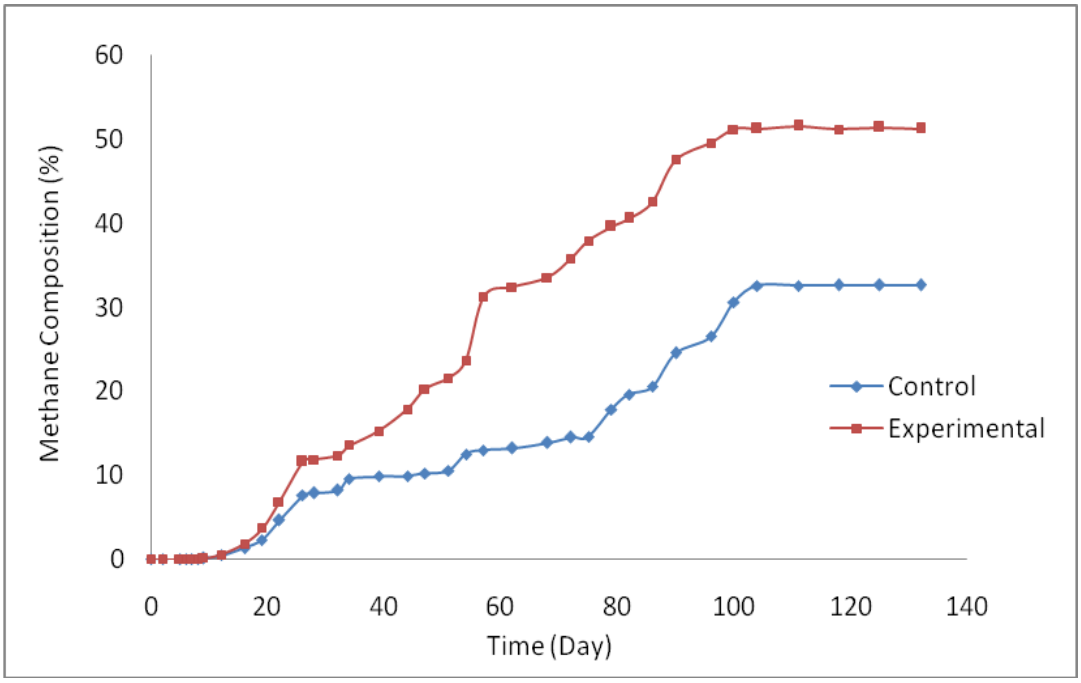


Figure 5-4: CH_4 composition in the control and MnP augmented (experimental) column

The gas production results indicated that the enzymatic enhancement of waste degradation was not only limited to batch reactors, the process could also be successfully scaled up to flow-through column reactors by recirculating enzyme augmented leachate.

5.4.2 Comparison of methane yield in batch and flow-through column experiments

Although the methane yield and the composition trends were similar to the batch reactor experiments (refer Chapter 4), the methane yields (expressed as mL CH_4 produced per g of dry waste) in flow-through column reactors were much lower than the batch reactors. The methane yield in batch reactors augmented with MnP at their best combination was approximately 40 mL CH_4/g DS, while the flow-through column operated with MnP augmented leachate showed only about 0.3 mL CH_4/g DS. A decreased contact between the enzyme and waste in flow-through columns, possibly due to channelling effects, incomplete diffusion, mass transfer resistance, and long term enzyme inactivation were

considered to be the main reasons for a diminished methane production in flow through columns. Furthermore, batch experiments were conducted at 35±1 ^{0}C while the columns were operated at room temperature 25±2 ^{0}C. Also, the batch experimental reactors were stirred throughout the experimental period assuming sufficient mixing of the ingredients inside the reactors. Relatively high temperature and the other controlled conditions provided in the batch experiments could also have contributed to a higher methane yield than in the flow-through column experiments.

5.4.3 Methane production rate VS dissolved organic carbon (DOC) variation

The variation of the daily methane production and DOC in leachate were plotted, as shown in Figure 5-5, to explain the correlation between these two parameters. Consistent with the expected effects of the enzyme treatment, the experimental column demonstrated increased methanogenic activity, as indicated by daily gas production values. The primary reason is that the enzyme increased the hydrolysis rate of waste as shown by the increase in DOC in leachate. The microorganisms were able to degrade DOC rapidly, increasing the methane production rate.

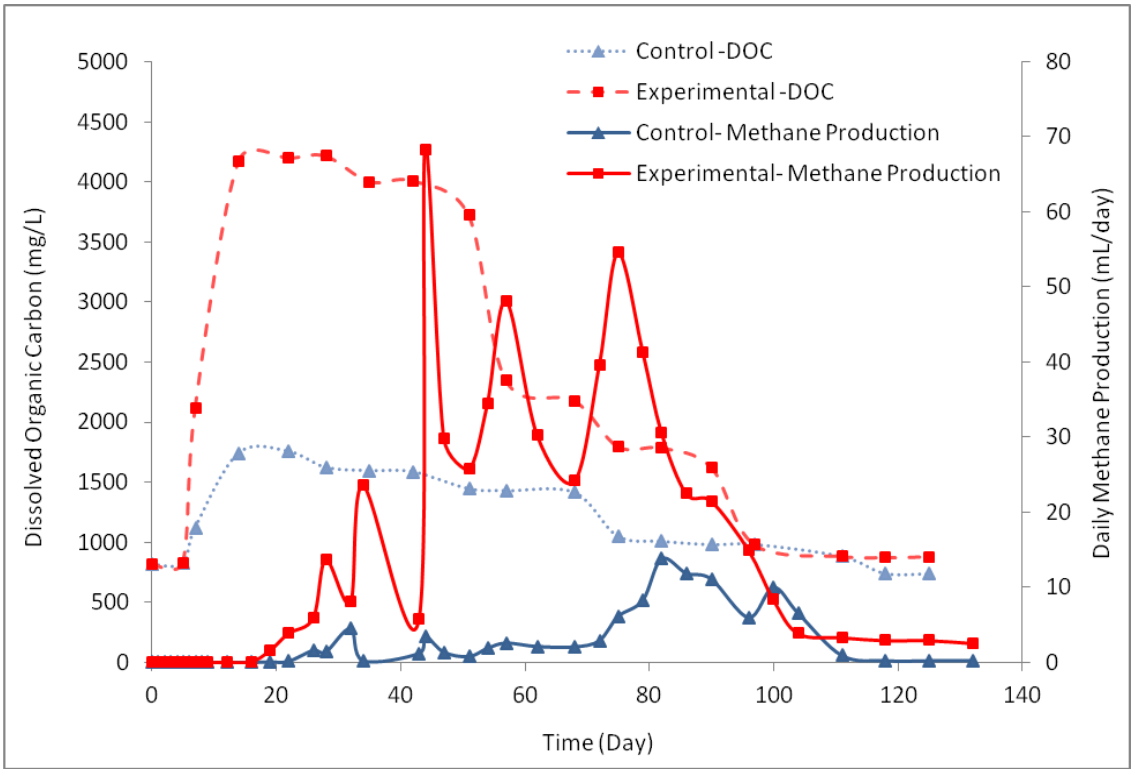
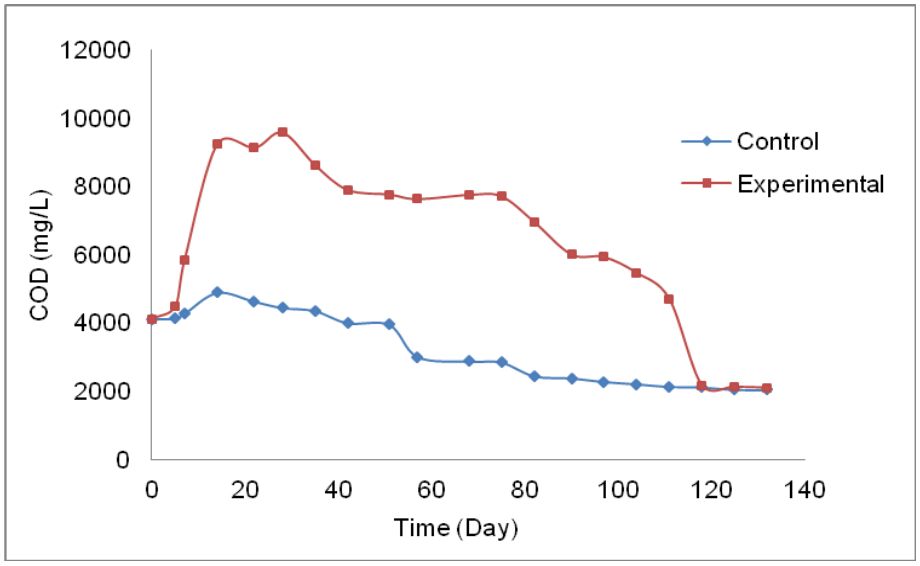


Figure 5-5: Daily CH_4 production rate and DOC variation in control and MnP augmented (experimental) column

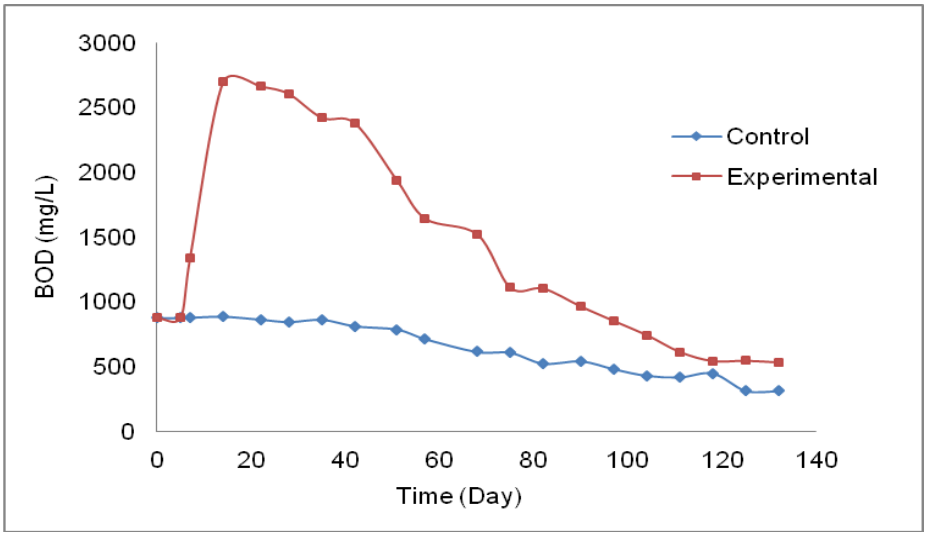
As evident from Figure 5-5, the methane production rate and the DOC variation over time were highly correlated. As noted previously, there was an initial lag phase (~ 10 days) of gas production. With the enzyme addition, the DOC in the experimental column increased from 820 mg/L to 4215 mg/L during this lag period. The DOC in the control column increased from 820 mg/L to 1756 mg/L, possibly due to the moisture adjustments in the system through leachate recirculation. The DOC then decreased in both columns with the increasing gas production, indicating that the anaerobic microorganisms began to consume the DOC while producing methane. The stabilization of methane production rates appears to be related to a decrease in the DOC that began to show a steady decrease only after about day 100. These observations verified that the limiting factor in gas production, and thus methanogenic activity, was the availability of carbon in the system.

5.4.4 COD and BOD variation

The COD and BOD in leachate were measured after each recirculation event to evaluate the effect of enzymatically enhanced leachate recirculation on waste degradation. The COD and BOD results are shown in Figure 5-6 (a) Figure 5-6 (b), respectively. Both COD and BOD showed trends similar to that for the DOC variation with time.



(a)



(b)

Figure 5-6: COD (a) and BOD (b) variation in control and MnP augmented (experimental) column

Both COD and BOD in the experimental column increased at a rapid rate initially during the lag period of gas production due to the rapid release and hydrolysis of organic matter from solid waste into the leachate. The variation of COD and BOD of leachate in the control column was not significant compared to the experimental column. During a lag period of ~10 days, the COD concentration in the experimental column increased from 4120 mg/L to 9245 mg/L, whereas the COD in the control column increased only to a peak of 4896 mg/L. The initial BOD concentration in both columns was 875 mg/L. The BOD in the experimental column reached a peak value of 2697 mg/L, while the BOD in the control column reached 886 mg/L, by the end of the lag phase. The considerable increase in both COD and BOD values in the experimental column clearly showed that the enzymatically-enhanced leachate recirculation positively affected the process by increasing the rate of waste hydrolysis in landfill simulators (flow-through columns) providing more accessibility to the microorganisms. As the gas production was increased, a significant decrease in COD and BOD of the leachate was observed in the experimental column, indicating the utilization of organic matter in leachate by microorganism to produce biogas.

Furthermore, the initial concentrations of DOC, COD, and BOD in both experimental and control columns were found to be approximately similar indicating a uniformity in the waste composition in both reactors at the beginning of the experiments.

The trends shown in Figure 5-6 are comparable to the results presented by other researchers (San & Onay, 2001; Sponza & Agdag, 2004; Bilgili *et al.*, 2007). A direct comparison of our results to the results reported in literature may not be possible because of the significant differences in the substrates, leachate augmentation methods, and the experimental conditions.

The proportion of biodegradable organic carbon in the leachate, indicated by the BOD/COD ratio, was obtained from the COD and BOD results observed in the experimental and the control columns. Plots are shown in Figure 5-7.

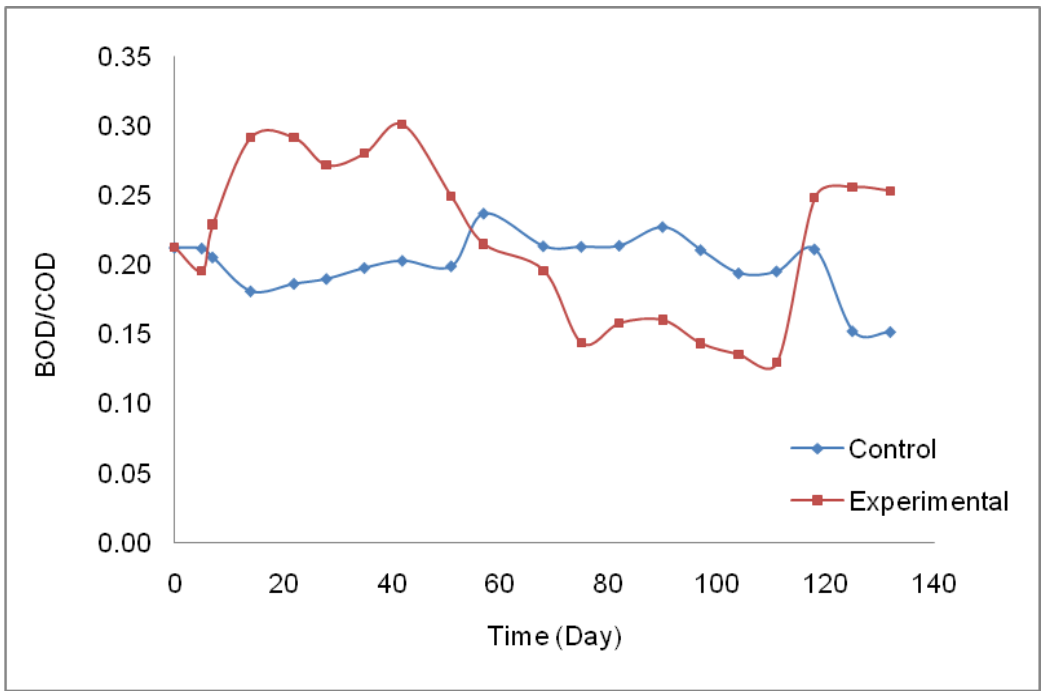


Figure 5-7: Variation of BOD/COD ratio

The BOD/COD ratio in the experimental column was increased from approximately 0.21 to 0.30 during initial 40 days, indicating the increasing biodegradability of organic matter due to solubilisation. It can also be observed that the BOD/COD ratio in experimental column decreased during day 60 to day 110 from 0.30 to 0.13. This suggests that the decrease in biodegradable organic fraction compared to the non-biodegradable fraction of waste. No noticeable variation of BOD/COD ratio in control column was observed during the experiment period of 132 days; the ratio remained below 0.24 during the experimental period. However, towards the end of the experiments, unexpectedly, an increasing trend of BOD/COD ratio in experimental column was observed. Further experiments to monitor the variation of BOD/COD in flow-through columns may be needed in the future to explain this behaviour.

5.4.5 pH variation

Another important parameter that describes the overall path of waste degradation is pH. Figure 5-8 shows the variation of pH in the experimental and the control column during the experimental period. The trends were similar to the trends observed in the batch experiments.

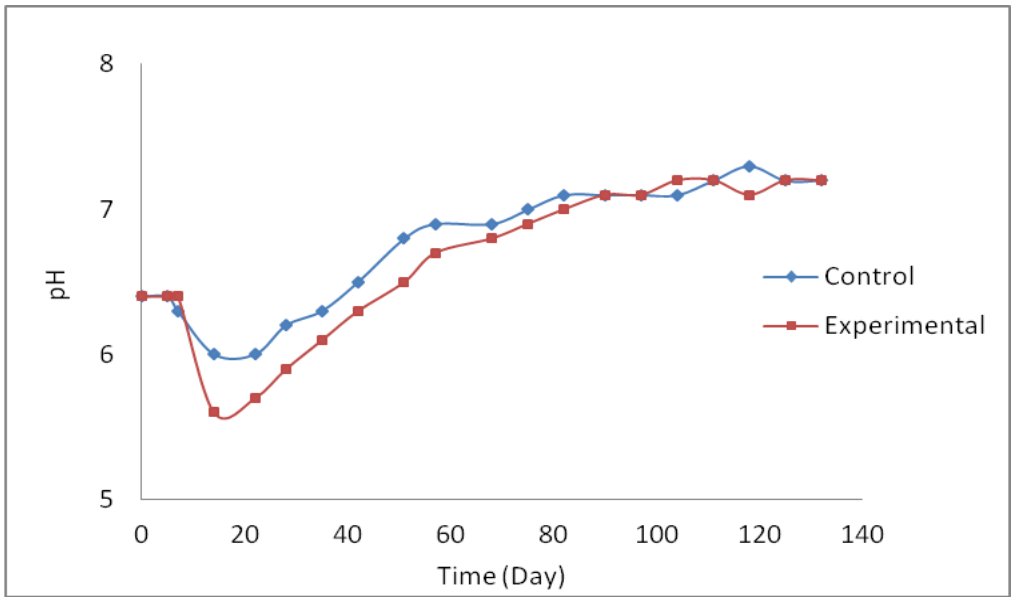


Figure 5-8: Change in pH in leachate of control and MnP augmented (experimental) column

After the initial adjustment phase was completed, the anaerobic decomposition of waste proceeded with a decrease in pH from 6.4 to 5.6 and 6.0 in the experimental and control columns, respectively. As explained in Chapter 4, this is an indication of an increase in acetogenic microbial activity in both the systems, with a greater increase in the total activity following MnP enzyme addition. The pH then increased in both columns gradually up to 7.0, accompanied by an increase in methane production establishing the methanogenic conditions in the system.

5.4.6 Variation of ammonia-nitrogen (NH_3-N)

The variations in the NH_3-N concentration in the experimental and the control columns are shown in Figure 5-9.

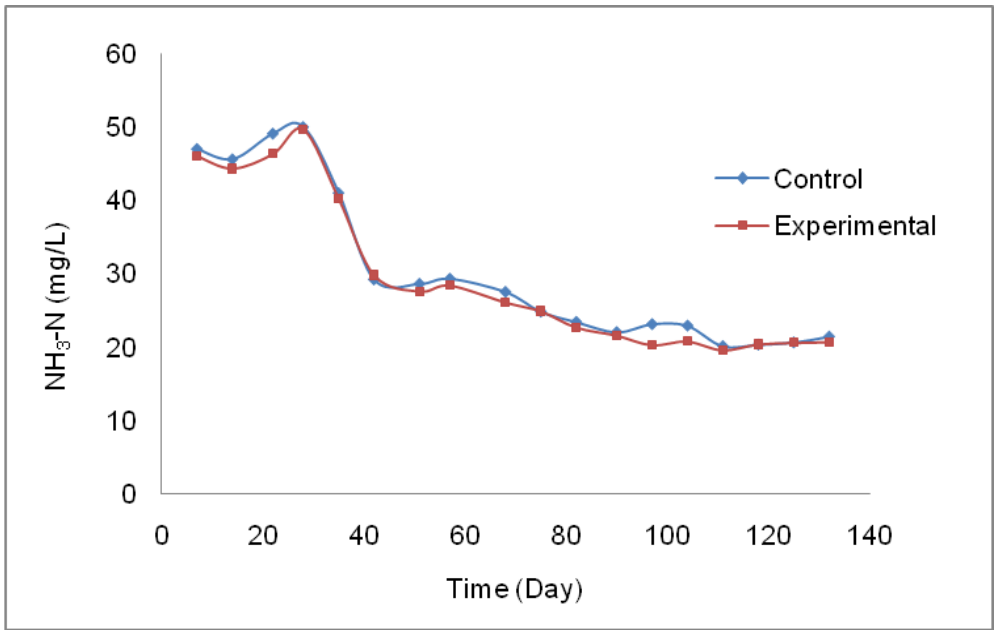


Figure 5-9: Change in NH_3-N in leachate of control and MnP augmented (experimental) column

There were no significant differences in the NH_3-N concentrations between the two reactors. The initial NH_3-N concentration increased from 45 mg/L to ~50 mg/L in both reactors during the initial 7-30 day period. This increase results from the decomposition of organic matter containing nitrogen, protein, and amino acids (San & Oney, 2001, Bilgili *et al.*, 2007). A decreasing trend was then observed during the 30-50 day time period followed by a slight variation during the rest of the experimental period. As described by Sponza & Agdag (2004), the decrease of NH_3-N could be attributed to NH_3 consumption by anaerobic bacteria to develop their cellular components. However, some researchers reported that NH_3-N in the landfill operation with leachate recirculation to be constant after the initial lag phase, due to the fact that recirculation practice could re-

introduce NH_3 to the system leading to NH_3 accumulation (San & Oney, 2001, Bilgili *et al.*, 2007).

Overall, the relatively low level of NH_3-N observed during our experiments suggests that there could not be inhibition in anaerobic bacteria by the accumulation of NH_3-N.

5.4.7 Total phosphorous (TP)

Phosphorous is an essential nutrient in the anaerobic waste degradation process. TP in leachate was measured to check whether there is an adequate amount of nutrient in the system. The variations of TP in experimental and control column are shown in Figure 5-10.

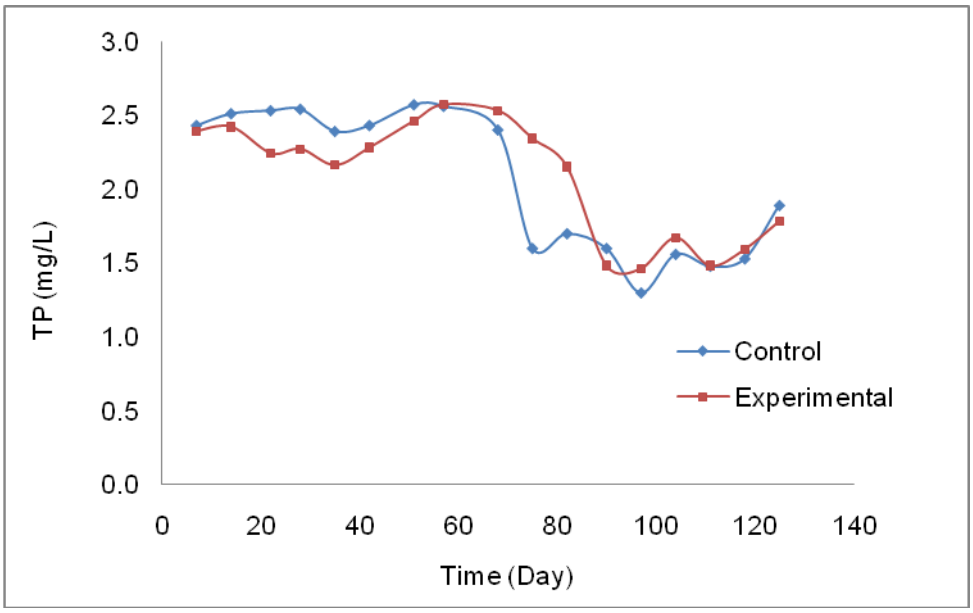


Figure 5-10: Change in TP in leachate of control and MnP augmented (experimental) column

The TP variation in both columns showed a similar trend, a decreasing behaviour of TP was observed in the system as the waste degradation proceeded. This could be due to the use of P by microorganisms for their cellular growth.

5.4.8 Total Kjeldahl nitrogen (TKN)

The other important macro nutrient for the anaerobic bacteria is N. Therefore, TKN in leachate was measured, but the results obtained were inconclusive and not included here. As per the standard methods, organic nitrogen and ammonia nitrogen are determined together and have been referred as 'Kjeldahl nitrogen'. TKN results obtained were always lower than the ammonia nitrogen reading; this indicated an inaccuracy of the results. Even though many trials were done with a series of leachate dilutions, assuming the interferences arrived through inaccurate dilution ratio, reasonable results could not be observed. Therefore, total nitrogen (TN) in leachate samples was measured in the second set of column experiments.

5.4.9 Availability of nutrients

It is important to check the availability of nutrients in a system, as nutrients are necessary to synthesize the cellular matter. Various researchers have suggested different ratios of organic matter (expressed as COD), N and P, based on the biodegradability of the waste (Christensen & Kjeldsen, 1989; Mata-Alvarez, 2003, Khanal, 2008). However, as suggested by Mata-Alvarez (2003), an average ratio of COD:N:P of 500:6:1was recommended for a substrate to be anaerobically degraded. This ratio was used as a criterion in this study to determine the required nutrient levels in terms of TP.

Table 5-2 shows the experimentally measured COD and TP over time and the theoretical levels of P required for the available COD in both the control and the enzyme added columns. The available and required levels of TN in the system could not be compared in the first set of flow-through column experiments.

Table 5-2: Comparison of available and required levels of TP in leachate

Time (Day)	Experimental				Theoretical- Based on COD:N:P ratio of 500:6:1	
	Control		Enzyme Added		Control	Enzyme Added
	COD (mg/L)	TP (mg/L)	COD (mg/L)	TP (mg/L)	TP (mg/L)	TP (mg/L)
7	4278	2.43	5853	2.39	7.1	9.8
14	4896	2.51	9245	2.42	8.2	15.4
22	4632	2.53	9134	2.24	7.7	15.2
28	4452	2.54	9587	2.27	7.4	16.0
35	4361	2.39	8632	2.16	7.3	14.4
42	4004	2.43	7900	2.28	6.7	13.2
51	3952	2.57	7763	2.46	6.6	12.9
57	3010	2.56	7632	2.57	5.0	12.7
68	2876	2.4	7754	2.53	4.8	12.9
75	2852	1.6	7712	2.34	4.8	12.9
82	2449	1.7	6963	2.15	4.1	11.6
90	2384	1.6	6007	1.48	4.0	10.0
97	2281	1.3	5930	1.46	3.8	9.9
104	2213	1.56	5471	1.67	3.7	9.1
111	2136	1.48	4712	1.48	3.6	7.9
118	2119	1.53	2179	1.59	3.5	3.6
125	2047	1.89	2123	1.78	3.4	3.5

As the available carbon (in terms of COD) increased, the required P also needed to be increased. But, the available TP (experimental) in both columns was significantly less than the required (theoretical) levels, indicating that P was a limiting nutrient in the system. Therefore, in order to maximize the gas production, addition of P into the system through leachate recirculation is recommended. However, in this experimental set, the main focus was to evaluate the sole effect of enzyme addition, therefore nutrient was not added at this stage until the gas production was stabilized.

5.4.10 Substrate utilization in flow-through columns and comparison to the substrate conversion in batch experiments

The TOC and lignin of waste samples before and after flow-through column experiments were measured and the results are shown in Table 5-3.

Table 5-3: Substrate utilization in column experiments

Parameter	Before experiments	After experiments	Conversion (%)
TOC (mg/g)			
MnP enzyme added	431	115	73
Control	431	329	24
Lignin (% VS)			
MnP enzyme added	82	34	59
Control	82	77	6

The conversion of TOC in the enzyme-added column was 73% while in the control column TOC conversion was only 24%. Compared to the batch experimental results, 97% TOC conversion was observed in batch experiments with enzyme addition and only 26% in control batch reactor (refer Chapter 4). The mathematical model predicted that the TOC will be converted by 80% with enzyme addition (refer Chapter 6), but the actual conversion of 73% was somewhat less. A decreased contact between the waste and enzymes and other factors, such as channelling effects in flow through columns, could lead to the observed lower-than-expected efficiency.

Similarly, the percentage conversion of lignin in the enzyme enhanced flow-through column (59%) was lower than that observed in the batch experiments (68% in batch reactor). However, the lignin conversions in the control flow-through column and the batch reactors were nearly equal at about 6%. The control flow-through columns and the control batch reactors showed nearly similar TOC variation and lignin variation during

the experiments; therefore, it is reasonable to conclude that the main reason for a lower efficiency in the enzyme enhanced columns was the decreased contact between the waste and the enzyme.

5.4.11 Nutrient addition into the columns

After the stabilization phase in the first set of experiments, nutrient-augmented leachate was recirculated in both columns to enrich the waste with nutrients prior to starting the second set of experiments. It should be noted that there was about three months delay from the end of the stabilization phase of the first set to the start of the nutrient addition. The gas production and leachate parameters observed before and after nutrient augmented leachate recirculation experiments are summarized in Table 5-4.

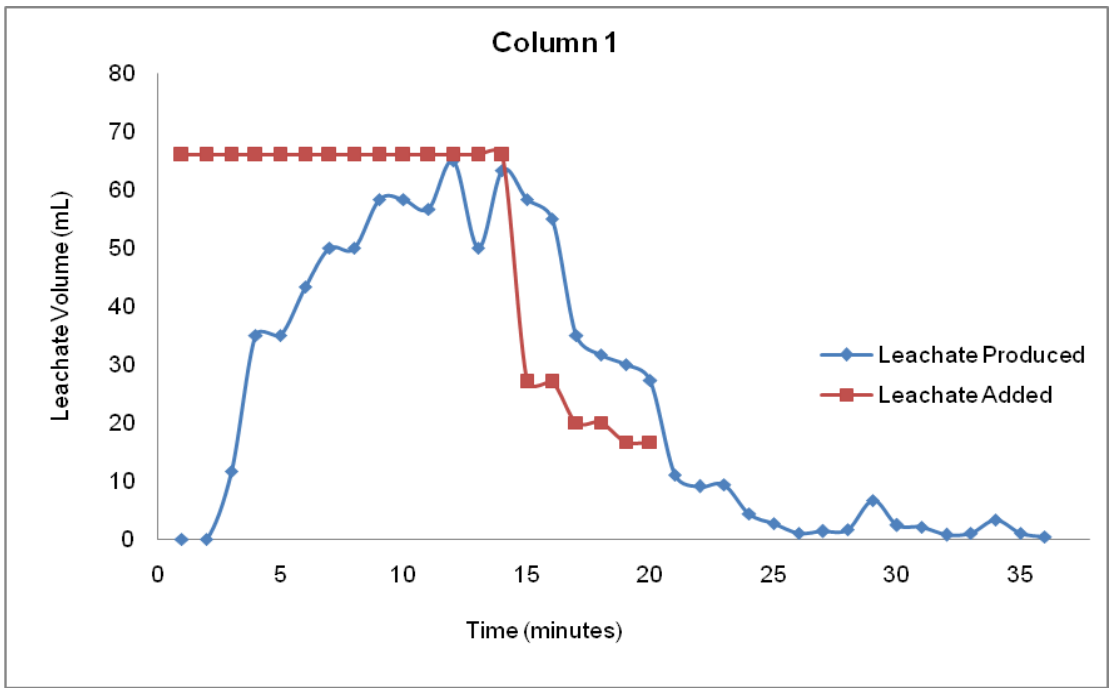
Table 5-4: Results from nutrient augmented experiments

Time (Day)	Control				Enzyme Added			
	Daily CH_4 (mL/day)	COD (mg/L)	TN (mg/L)	TP (mg/L)	Daily CH_4 (mL/day)	COD (mg/L)	TN (mg/L)	TP (mg/L)
0 (prior to nutrient addition)	0.0	1068	99.8	1.4	0.0	1700	189	2.9
1 (after nutrient addition)	24.7	1027	152.4	37.9	5.3	1432	238	63.4
2	46.2	984	107	26	9.8	1264	121	48.1
3	26.3	975	100.6	25.2	10.0	1246	139.8	43.7
4	11.4	1023	124	22.7	10.1	1173	143	31.4
5	3.1	1003	135	21.3	3.4	1118	149.5	27.8

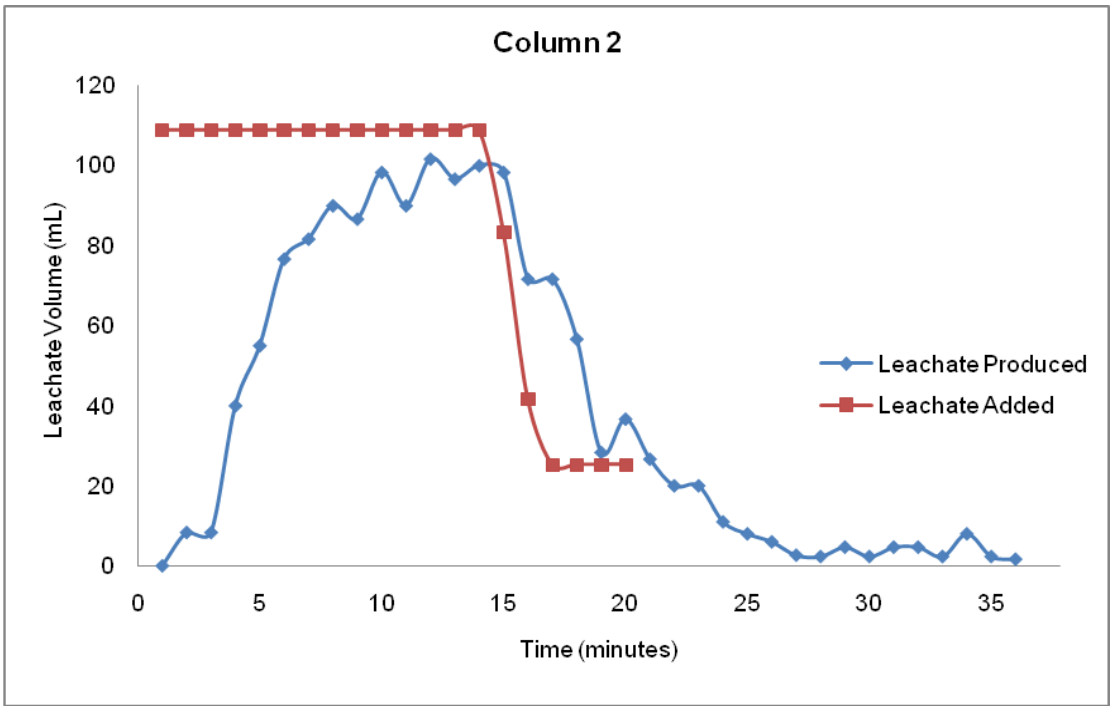
There was no gas production in the control and the enzyme-augmented columns before the nutrient addition. Both columns started producing methane after the nutrients were added into leachate prior to recirculation, with higher daily production in the control column compared to the enzyme added column. The possible reason is that there could be less organic substrate in enzyme added column as they degraded at a faster rate than in the control column. However, the columns were left un-operated (without regular leachate recirculation) for about 3 months prior to nutrient addition, therefore the spike in gas production could also be due to the moisture adjustment in the system. In about 5 days, the gas production declined again in both columns. The goal of adding the nutrients was successfully achieved, and TN and TP concentrations in the system were reached to the required levels.

5.4.12 Leachate hydrographs

To understand the leachate flow behaviour in the columns, leachate hydrographs were developed before starting the second set of batch experiments. The leachate hydrographs generated are shown in Figure 5-11.



(a)



(b)

Figure 5-11: Leachate hydrographs (a) column 1 (b) column 2

During the first 20 minutes of the experiment, approximately 1050 mL and 1740 mL of water were added to column 1 and column 2, respectively. Complete draining was observed in around 40 minutes in both columns and the collected volumes for column 1 and column 2 were 871 mL and 1424 mL, respectively. By analyzing these data, it was noticed that some amount of water was stored during each recirculation. On average, the water storage in column 1 was ~ 17%, while in column 2 the water storage was ~ 18%. A similar leachate flow behavior in both column 1 and column 2 was observed with an approximately 2-3 minutes breakthrough time (leachate breakthrough at the bottom of the column).

5.4.13 Optimizing the leachate recirculation process

5.4.13.1 Methane production

As described previously, a second set of flow-through columns was run to optimize the process parameters in order to lower the quantity of enzyme required in full-scale applications. First, both columns were operated as control columns (leachate recirculation without enzymes) for 30 days. Once the gas production has declined, the two columns were operated with two different conditions as shown in Table 5-5.

Table 5-5: Operational parameters in second set of flow-through column experiments

Parameter	column 1	column 2
Amount of MnP enzyme added (mg)	2	10
Number of leachate recirculations	7	1

Cumulative methane production trends are shown in Figure 5-12.

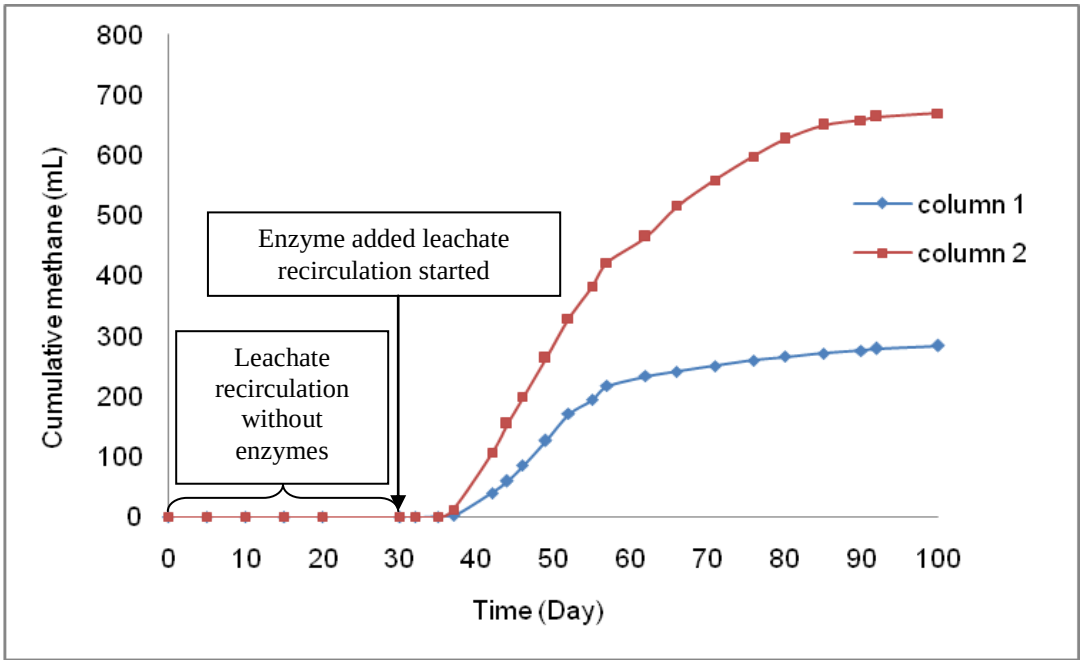


Figure 5-12: Cumulative methane production under different conditions

As evident by Figure 5-12, the gas production in both columns over the first 30 days was negligible. After about 8 days (lag phase) from enzyme addition, there was a significant increase in methane production in both columns. Column 1, with a lower quantity of enzyme and many recirculations, showed less cumulative methane; 284 mL during 100 days of operation. Column 2, with 5 times higher enzyme quantity than in column 1 and single recirculation event, produced nearly 2.4 times higher methane (669 mL) compared to column 1. This observation suggests that it may be possible to reduce the amount of enzyme by about 50%, but increase the number of recirculations. This would reduce the amount of enzyme that needs to be added at the beginning of the process.

5.4.13.2 Availability of carbon

Figure 5-13 shows the daily methane production and COD in leachate over time for column 1 and column 2. As explained previously, the gas production and COD were

found to be correlated. During the control experiment, between day 1 and day 30, the CODs in both columns were nearly equal, indicating the similarity of columns before enzyme augmentation. The COD in column 1 was measured after each recirculation event. The COD in leachate increased from 439 mg/L to 766 mg/L during day 30 to 35. A lag phase of methane production was observed during this period. Once the gas production was established in column 1, on day 35, a sudden drop in COD was observed. An increasing trend of COD was observed thereafter. Two major conclusions can be made from the COD and methane production data; COD was utilized in producing methane and each recirculation could re-introduce some un-bounded enzyme leading to an increase in COD after each recirculation event.

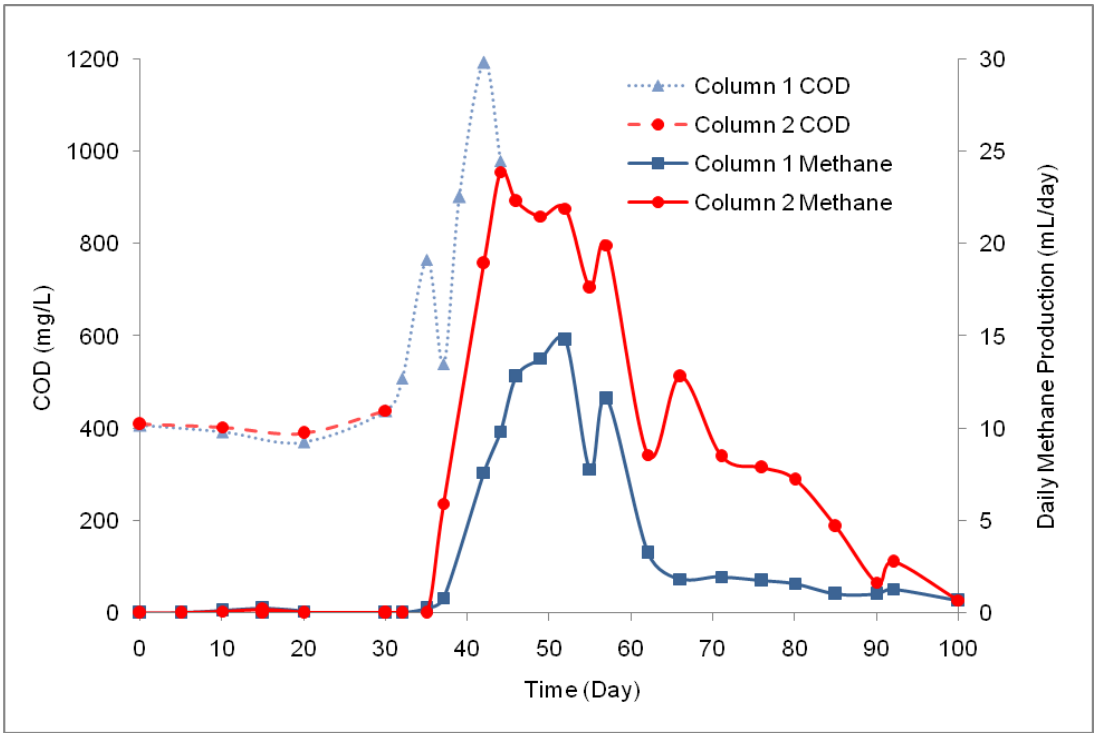


Figure 5-13: Daily methane production and COD in leachate

Leachate was recirculated 7 times in column 1 during the first 14 days after enzyme addition, *i.e.* day 30-42. The leachate was not recirculated thereafter; hence, the leachate parameters were not measured after day 42**.**

Since only one recirculation was carried out in column 2, the COD in column 2 was not measured after the enzyme was added. Therefore, a direct comparison of the two columns could not be made.

5.4.13.3 Availability of nutrients

The total nitrogen (TN) and the total phosphorous (TP) of leachate in column 1 after each recirculation event were measured and the results are shown in Figure 5-14.

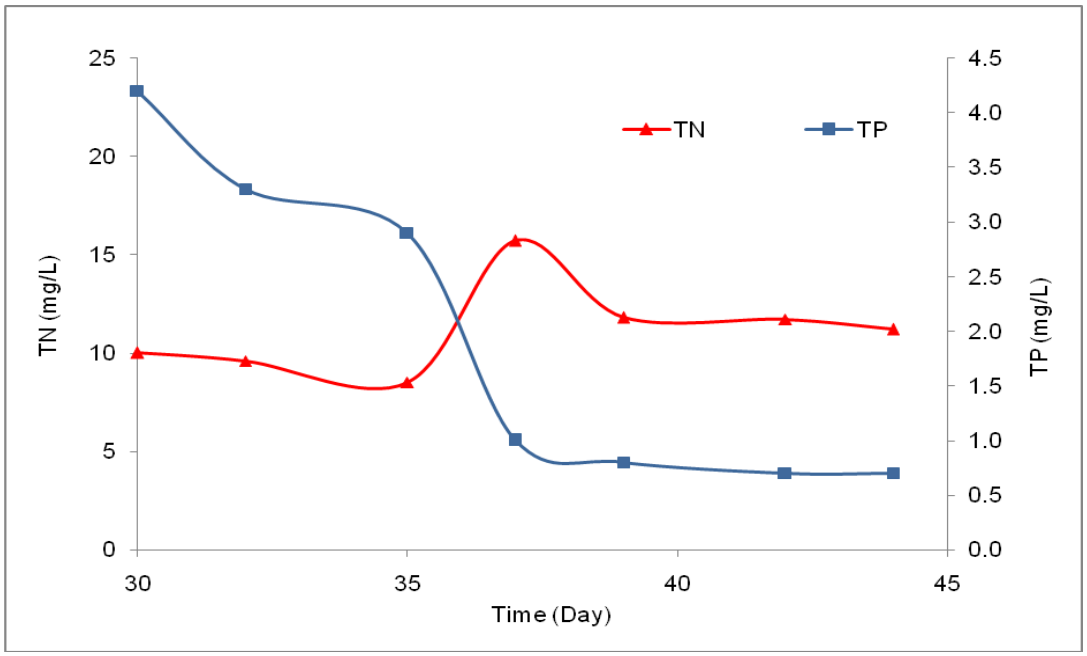


Figure 5-14: TP and TN in column 1

The TN variation over time was somewhat similar to the NH_3-N variation shown in Figure 5-9. There was a significant increase in TN from ~ 10 mg/L to 16 mg/L during the first 7 days (from day 30 to day 37) due to the decomposition of nitrogen-rich organic materials, such as protein. Thereafter, TN decreased to about 12 mg/L and remained nearly constant during the leachate recirculation period. This is mainly because anaerobic bacteria utilized N as a nutrient for their growth; however, at the same time, the

remaining N in the system could be re-introduced into the system through leachate recirculation leading to a constant TN in the system.

An approximately 76% decrease of TP from the initial value was observed during the first 7 days. The N and P are assumed to be macronutrients of anaerobic microorganism and were utilized during their metabolism (Mata-Alvarez, 2003). The TP remained nearly constant thereafter until day 42 due to the re-introduction via leachate recirculation.

The available and the required nutrient levels in terms of TN and TP in the system were compared based on COD:N:P ratio of 500:6:1 (Mata-Alvarez, 2003). Table 5-6 shows the experimentally measured COD, N, and P after each recirculation event and the theoretical levels of N and P to be present in the system for the experimentally measured COD.

Table 5-6: Comparison of experimental and theoretical nutrient (N & P) levels (column 1)

Time (Day)	Experimental			Theoretical - Based on COD:N:P ratio 500:6:1	
	COD (mg/L)	N (mg/L)	P (mg/L)	N (mg/L)	P (mg/L)
30	438	10	4.2	5.1	0.7
32	508	9.6	3.3	5.9	0.8
35	766	8.5	2.9	8.9	1.3
37	539	15.7	1.0	6.3	0.9
39	901	11.8	0.8	10.5	1.5
42	1192	11.7	0.7	13.9	2.0
44	980	11.2	0.7	11.4	1.6

The actual N levels in the system were higher than the required (theoretical) levels except on day 42. On day 42, there was a marked increase in COD compared to the other days; however, as the COD decreased, N in the system had reached closer to the required levels. Therefore, it is reasonable to conclude that the system contained sufficient N level during the experimental period.

As the available carbon (in terms of COD) increased, P was likely to become a limiting nutrient in the system. The experimentally measured P levels were lower than the required levels after day 37 even though nutrient-enriched waste was used for the experiments. Therefore, the addition of P nutrient into the system through leachate recirculation is recommended.

5.4.14 Comparison of results of first and second set of flow through column experiments

As discussed previously, similar trends were observed for methane yield and leachate quality such as pH, COD, TP, in the first and the second set of flow-through column experiments. A direct comparison of the two sets was not appropriate, especially because of the differences in initial waste composition and operating parameters. Partially degraded waste from the first set of experiments was used in the second set. The initial COD in leachate in the first set of experiments was nearly 10 times higher; ~4120 mg/L COD in first set of experiments compared to ~409 mg/L in the second set of experiments. Therefore, the second set of flow-through column experiments stabilized faster than the first set, but with less cumulative methane production. The cumulative methane production observed in the enzyme-augmented column in the first set was about 2000 mL at the end of the stabilization phase (after 110 days); column 2 in the second set stabilized in about 70 days with a cumulative methane yield of ~670 mg/L. In terms of methane-to-COD ratio, the second set of experiment showed about 3 times higher ratio than the first set of experiments; the reason could be the use of nutrient-enriched waste in the second set.

5.5 Conclusions and Recommendations

The process of leachate augmentation with enzymes was successfully scaled up to a set of flow-through columns. The increased waste degradation and gas production in enzyme-enhanced flow-through column, compared to the control column, confirmed that the leachate augmentation with enzymes before recirculation is a feasible option for enhancing the gas production in the gas declining phase of anaerobic waste degradation process. However, a lower efficiency in terms of gas production and substrate utilization was observed in flow-through column compared to the batch experiments, especially due to the decreased contact between the enzyme and the waste. Therefore, it is recommended to study the flow patterns in columns in detail to facilitate better contact.

The operational and scale up parameters identified through laboratory batch and flow-through columns are recommended for use in the field-scale application. The addition of nutrients, especially P, into the system prior to enzyme addition or with enzyme addition is recommended, depending on the existing nutrient levels in the landfill bioreactor.

Chapter Six: Mathematical Models

This thesis included the development of several simplified mathematical models to assist in the scale up of the collected data. The development and validation of the models are discussed in this chapter.

6.1 Types of Models

The main objective of developing mathematical models in this study was to facilitate the scale up process of anaerobic waste degradation aided by enzymes.

Three different models were developed for following purposes.

1. Use of an existing kinetic model to fit the results obtained in batch experiments.

1.1. Waste degradation kinetic model to identify the catalytic reaction mechanism and to obtain the reaction rate constants.

1.2. Enzyme decay model to obtain the enzyme decay constant due to enzyme deactivation.

1.3. Estimation of the activation energy associated in the enzyme enhanced and the control reactions.

2. Formulate and validate a scale-up model to determine the quantity of enzyme required in the flow-through columns and in a field-scale landfill bioreactor in order to obtain conversions that are similar to those obtained in batch reactors.

3. Formulate and validate a predictive model to predict the gas production in enzyme catalyzed reactions.

6.2 Kinetic Model

Kinetic models of solid waste biodegradation are used for the following reasons:

1. To study the rate of reactions, this is important for designing a bioreactor (Biswas *et al.*, 2006).
2. To understand the catalytic mechanisms of the reaction and the role of an enzyme in metabolism.

6.2.1 Theoretical framework and assumptions

6.2.1.1 Kinetics of enzyme catalyzed reactions

The Michaelis-Menten mono-substrate, double-intermediate enzyme catalyzed model (Taylor, 2002; Leskovac, 2003) was used to identify the reaction mechanism and to obtain the rate constants associated with the enzyme catalyzed reaction. The Michalis-Menten equation, which describes the rate of reaction catalyzed by a soluble enzyme, can be derived as below.

The overall rate of reaction depends on the catalytic activity of the enzyme in the waste hydrolysis reaction. It is assumed that enzyme catalyzed reaction follows the mechanism shown in Equation 6-1,

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_3} ES' \xrightarrow{k_4} E + P \qquad \textbf{Equation 6-1}$$

A single molecule of an enzyme (E) combines reversibly with a single molecule of substrate (S) to form an enzyme-substrate complex (ES), which is transformed into another different enzyme-substrate complex (ES') before release of free enzyme (E) and final product (P).

By using pseudo-steady-state hypothesis, the rate of formation and dissociation of the intermediates are assumed to be zero,

$$\frac{d[ES]}{dt} = 0 = \frac{d[ES']}{dt}$$ **Equation 6-2**

The rate expression, which describes the rate of change of concentrations of intermediates, ES and ES', can be written as,

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_3)[ES]$$ **Equation 6-3**

$$\frac{d[ES']}{dt} = k_3[ES] - k_4[ES']$$ **Equation 6-4**

Substituting Equations 6-3 and 6-4 in Equation 6-2,

$$[ES] = \frac{k_1[E][S]}{k_2 + k_3}$$ **Equation 6-5**

$$[ES'] = \frac{k_3[ES]}{k_4}$$ **Equation 6-6**

Similarly, the rate expression for change of concentration of products, P, can be written as,

$$\frac{d[P]}{dt} = k_4[ES']$$ **Equation 6-7**

Substituting Equations 6-5 and 6-6 into Equations 6-7, the reaction velocity, v', can be obtained.

$$v' = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{k_1 k_3[E][S]}{k_2 + k_3}$$ **Equation 6-8**

Assuming that the enzyme is conserved during the process (*i.e.* no short-term de-activation), the mass balance equation for the enzyme, E, can be written as,

$$[E]=[E_o]-[ES]-[ES'] \qquad \textbf{Equation 6-9}$$

Substituting Equations 6-5, 6-6, and 6-9 in Equation 6-8,

$$v' = \frac{k_1 k_3 k_4 [S][E_o]}{(k_2 k_4 + k_3 k_4) + (k_1 k_4 + k_1 k_3)[S]} \qquad \textbf{Equation 6-10}$$

Re-arranging Equation 6-10,

$$v' = \frac{\left(\frac{k_3 k_4}{k_3 + k_4}\right)[S][E_o]}{\left(\frac{k_4(k_2 + k_3)}{k_1(k_4 + k_3)}\right) + [S]} \qquad \textbf{Equation 6-11}$$

By defining the following relations,

$$V_{\max} = \left(\frac{k_3 k_4}{k_3 + k_4}\right)[E_o] = k_{cat}[E_o] \qquad \textbf{Equation 6-12}$$

$$K_M = \left(\frac{k_4(k_2 + k_3)}{k_1(k_4 + k_3)}\right) \qquad \textbf{Equation 6-13}$$

$$k_{cat} = \left(\frac{k_3 k_4}{k_3 + k_4}\right) = \frac{V_{\max}}{[E_o]} \qquad \textbf{Equation 6-14}$$

Equation 6-11 can be written in the form of the Michaelis-Menten equation,

$$v' = -\frac{d[S]}{dt} = \frac{V_{\max}[S]}{K_M + [S]} \qquad \textbf{Equation 6-15}$$

where,

$V_{\max}$ is the maximum rate of reaction (velocity) (g_{TOC}/g_{DS}.day),

K_M is Michaelis constant (g_{TOC}/g_{DS}),

k_{cat} is the catalytic rate constant (1/day).

Equation 6-15 can be integrated to linearize as follows,

$$\int_{[S_o]}^{[S_t]} \left[\frac{K_M + [S]}{V_{\max}[S]} \right] d[S] = \int_{to}^{t} -dt \qquad \textbf{Equation 6-16}$$

$$\left(\frac{[S_o]-[S_t]}{t_t - t_o} \right) = V_{\max} - K_M \frac{\ln\left(\frac{[S_o]}{[S_t]} \right)}{(t_t - t_o)} \qquad \textbf{Equation 6-17}$$

where, $[S_o]$ and $[S_t]$ are the initial concentration of substrate at t_o and the concentration of substrate at time, t_t, respectively.

According to Equation 6-17, a plot of $\left(\frac{[S_o]-[S_t]}{t_t - t_o} \right)$ versus $\frac{\ln\left(\frac{[S_o]}{[S_t]} \right)}{(t_t - t_o)}$ should give a straight line with a slope equal to K_M and intercept equal to $V_{\max}$.

6.2.1.2 Kinetics of un-catalyzed reaction

Conversion of substrate (S) to product (P), when it is not catalyzed by enzyme, can be represented as,

$$S \xrightarrow{k} P \qquad \textbf{Equation 6-18}$$

where, k is the un-catalyzed rate constant (1/day) independent of concentration, but dependent on the temperature.

Assuming that the waste degradation follows the first order kinetics, the velocity of the un-catalyzed reaction, $v^{"}$, can be written as,

$$v^{"} = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = k[S] \qquad \textbf{Equation 6-19}$$

Integrating Equation 6-19,

$$\int_{[S_o]}^{[S_t]} \frac{d[S]}{[S]} = \int_{to}^{t} -k\,dt \qquad \textbf{Equation 6-20}$$

$$\left(\ln \frac{[S_t]}{[S_o]}\right) = -k(t_t - t_o) \qquad \textbf{Equation 6-21}$$

where, $[S_o]$ and $[S_t]$ are the initial concentration of substrate at t_o and the concentration of substrate at time, t_t, respectively.

The slope of the straight line of $\left(\ln \frac{[S_t]}{[S_o]}\right)$ versus $(t_t - t_o)$ plot is equal to the rate constant, k.

6.2.2 Model inputs

The results obtained from the batch experiments for the enzyme catalyzed (MnP enzyme at its best combination of 0.3 mg enzyme dose and 0.0046 E:H_2O_2 ratio) and un-catalyzed reactions were fitted to Equation 6-17 and Equation 6-21, respectively, to estimate the maximum rate of reaction and the rate constants. Substrate utilization results shown in

Chapter 4 (Figure 4-18) in terms of TOC in waste (g/g) were used. The TOC results obtained from the experiments (raw data) were rearranged to use in the model as shown in Table 6-1.

Table 6-1: Model inputs

Time (Day)	Enzyme (MnP) Catalyzed Reaction			Un-Catalyzed Reaction	
	Raw data	Y	X	Raw data	Y
	$[S_t]$ (g_{TOC}/g_{DS})	$\left(\frac{[S_o]-[S_t]}{t_t-t_o}\right)$	$\frac{\ln\left(\frac{[S_o]}{[S_t]}\right)}{(t_t-t_o)}$	$[S_t]$ (g_{TOC}/g_{DS})	$\left(\ln\frac{[S_t]}{[S_o]}\right)$
0	0.431			0.431	
2	0.354	0.039	0.099	0.429	0.005
4	0.279	0.038	0.109	0.418	0.031
7	0.182	0.036	0.123	0.408	0.056
9	0.134	0.033	0.130	0.385	0.114
11	0.096	0.030	0.136	0.375	0.141
14	0.055	0.027	0.147	0.362	0.177
16	0.040	0.024	0.148	0.351	0.207
18	0.029	0.022	0.150	0.344	0.226
21	0.021	0.020	0.143	0.341	0.235
24	0.021	0.017	0.127	0.336	0.251
27	0.019	0.015	0.115	0.333	0.259
29	0.018	0.014	0.109	0.329	0.271
31	0.018	0.013	0.103	0.321	0.295
34	0.016	0.012	0.096	0.317	0.308
36	0.016	0.012	0.092	0.316	0.311
38	0.014	0.011	0.090	0.316	0.312

6.2.3 Model evaluation and discussion

Figure 6-1and Figure 6-2 show the model fitting plots for enzyme catalyzed and un-catalyzed reaction, respectively. It should be noted that for both the enzyme catalyzed and the un-catalyzed reactions only the results up to day 21 were used to evaluate the model parameters; the rest were used to validate the predictive model.

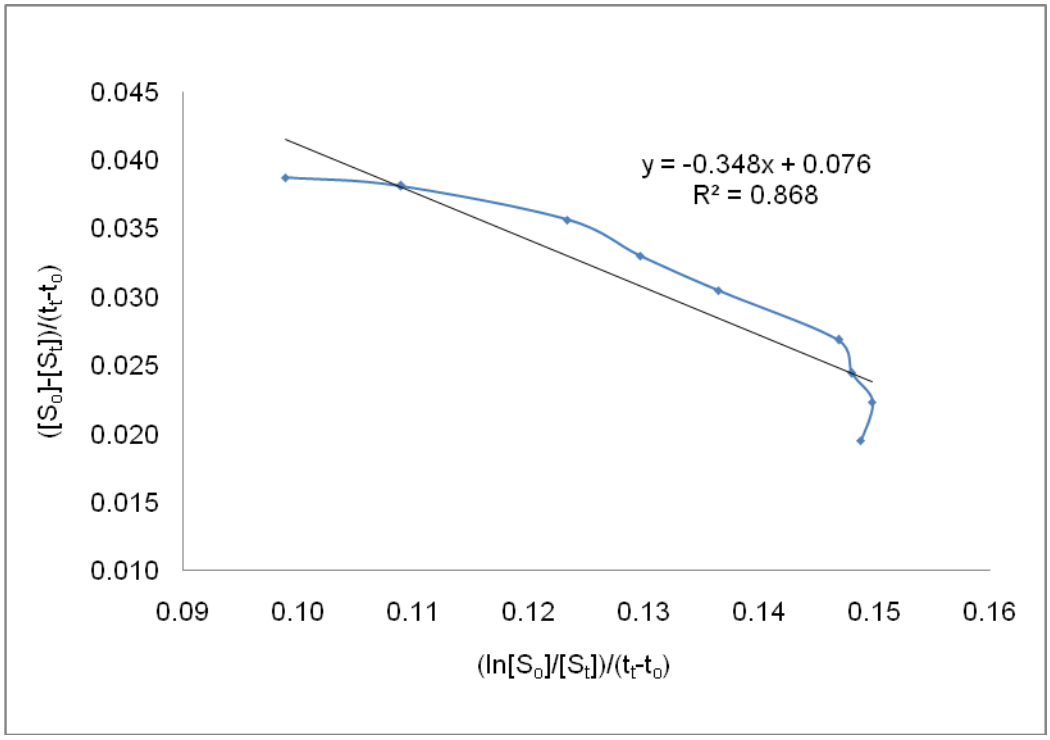


Figure 6-1: Kinetic model fitting- enzyme (MnP) catalyzed reaction

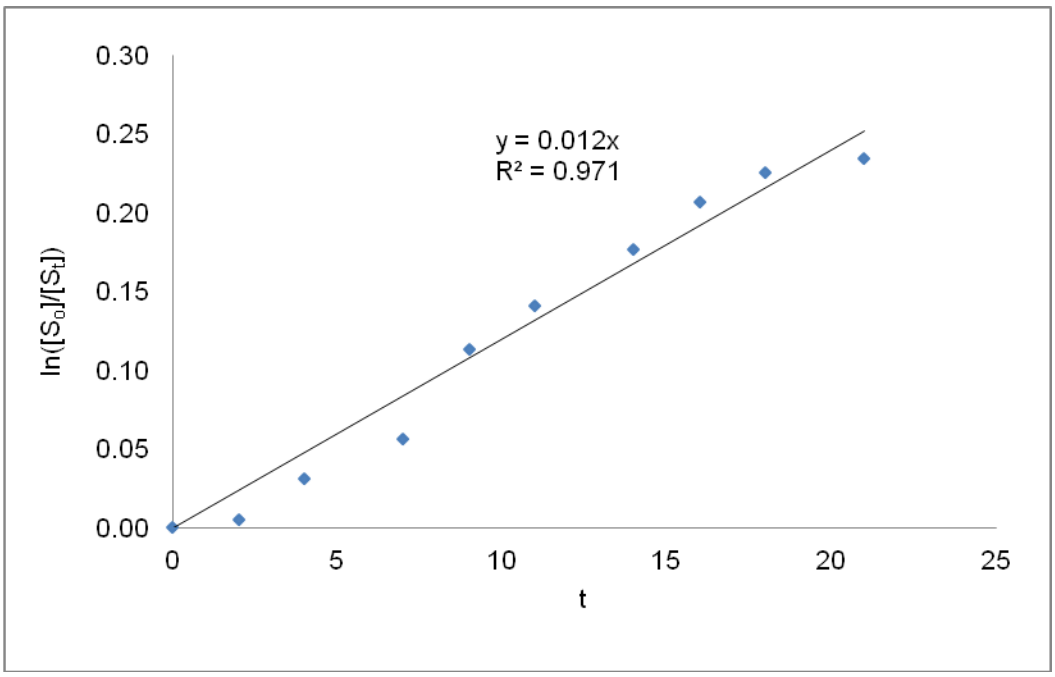


Figure 6-2: Kinetic model fitting- un-catalyzed reaction

As shown in Figure 6-1 and Figure 6-2, the experimental results showed a good fit with the kinetic models with the root mean square deviation (R^2) of 0.87 in enzyme catalyzed reaction and 0.97 in un-catalyzed reaction. Therefore, it can be concluded that the enzyme catalyzed reaction of anaerobic waste degradation followed the mono-substrate, double-intermediate mechanisms as described in Equation 6-1. However, under the pseudo-steady-state assumption, it is not possible to identify the enzyme substrate intermediates in a reaction pathway (Leskovac, 2003). The un-catalyzed waste degradation reaction followed the first order kinetics as shown in Equation 6-18.

By fitting the results to a linear function, using the least square method, the slope and the intercept of the lines were obtained. The kinetic parameters were then determined and these are summarized in Table 6-2.

Table 6-2: Kinetic parameters

Enzyme Catalyzed Reaction	
V_{max}	0.076 g_{TOC}/g_{DS}.day
K_M	0.348 g_{TOC}/g_{DS}
k_{cat}	506.7 1/day
Un-Catalyzed Reaction	
k	0.012 1/day

V_{max}, the maximum rate of reaction (velocity) achieved by the system at saturating substrate concentration, was 0.076 g_{TOC}/g_{DS}.day. Equation 6-15 can be further simplified; at high substrate concentrations, the reaction occurs at its maximum velocity, V_{max}. K_M, the Michaelis constant (a combination of elementary reaction rate constants), the substrate concentration at which the reaction rate is at half maximum velocity, was equal to 0.348 g_{TOC}/g_{DS}. The smaller the K_M is, the lower will be the substrate concentration at which the velocity (v) approaches V_{max}, and it indicates the high affinity of substrate to the enzyme. The catalytic turn over number, k_{cat}, gives a better indication of effectiveness of the substrate conversion that can be compared to the rate constant, *k,* in the un-catalyzed reaction. Comparing the rate constants of the enzyme catalyzed and un-catalyzed reactions, the enzyme catalyzed reaction at optimum conditions proceeded about 42,000 times faster than that of un-catalyzed reaction; *i.e.* 506.7 per day versus 0.012 per day.

Several researchers have suggested that the peroxidase enzyme catalyzed oxidation for certain substrates with hydrogen peroxide follows a “Ping Pong” mechanism (Leskovac, 2003; Cullen & Kersten, 2004) in which the first product is released before the second substrate is bound. In that approach, H_2O_2 was considered to be the first substrate and the waste molecules to be the second substrate. However, as a first approach, our attempt to

fit the experimental results to the Ping-Pong bi substrate model gave a poor fit, suggesting that our system of waste and enzymes was not governed by the Ping Pong mechanism.

A comparison of the kinetic constant with those reported in literature was not possible due to the unavailability of similar results from a similar study at this time. However, some enzyme reaction rates could be million times faster than those of comparable un-catalyzed reactions (Leskovac, 2003).

Finally, it may be argued that the present mechanism is of little relevance to most real systems as it applies only to enzyme catalyzed reactions where a single substrate is converted irreversibly to a single, non inhibitory product. In fact, a real system of heterogeneous waste and enzyme could behave in a complex manner, but our experimental results were correlated very well indicating that the framework used becomes a good approximation for explaining the process mechanism.

6.2.4 Enzyme decay model

As described by Dunn *et al*. (2003), biocatalysts (enzymes) in reactors usually undergo irreversible conformational changes known as denaturation. This often causes an exponential decrease of activity with time and can be described by a first order reaction rate process as below,

$$\frac{d[E]}{dt} = -k_E[E] \qquad \textbf{Equation 6-22}$$

where, $[E]$ is the enzyme concentration at any time and k_E is the enzyme decay constant.

By integrating and linearizing Equation 6-22,

$$\int_{[E_o]}^{[E_t]} \frac{d[E]}{[E]} = \int_{to}^{t} -k_E\, dt \qquad \textbf{Equation 6-23}$$

$$\left(\ln\frac{[E_t]}{[E_o]}\right) = -k_E(t_t - t_o) \qquad \textbf{Equation 6-24}$$

where, $[E_o]$ and $[E_t]$ are the initial concentration of enzyme at time 0, t_o , and the concentration of enzyme at any time, t_t , respectively.

k_E can be obtained from the slope of $\left(\ln\frac{[E_t]}{[E_o]}\right)$ versus $(t_t - t_o)$ plot.

Enzyme activity (U/L) test results obtained from batch experiments were used as inputs to the model. As described in Chapter 4, only the enzyme activity data obtained up to day 6 were useful. Those were fitted to Equation 6-24 and the plot is shown in Figure 6-3.

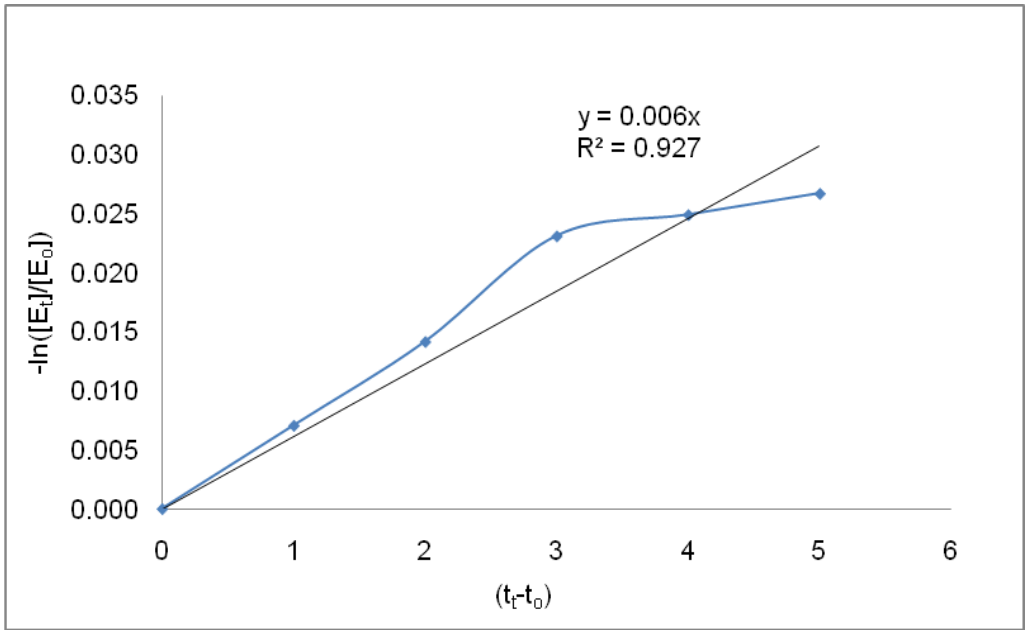


Figure 6-3: Enzyme decay model fitting

As shown in Figure 6-3, the model showed a good fit to the experimental data with R^2 of 0.927. The enzyme decay constant (k_E) was obtained from the slope as 0.006 per day. It was noticed that the enzyme decay was very slow compared to both the enzyme catalyzed and un-catalyzed reaction rates (refer Table 6-2) suggesting that the enzyme could stay active until the completion of the process of waste degradation. The linear relationship obtained (*i.e.* y = 0.006 x) could be used to predict the enzyme activity at any time ($[E_t]$) during the flow-through column experiments or in field experiments. It can also be used to estimate the time required for complete denaturation of the enzyme.

6.2.5 Estimating activation energy

The dependence of the reaction rate constant on temperature and the activation energy of the reaction were evaluated using the Arrhenius equation (Smith, 1981). As described in Chapter 4, batch experiments were conducted at two different temperatures, 35 ^{0}C and 45 ^{0}C. The rate constants of enzyme catalyzed and un-catalyzed reactions at two temperatures were obtained by fitting the experimental data to Equation 6-17 and Equation 6-21, respectively. The activation energy of both the reactions were then estimated using the linearized Arrhenius equation (Smith, 1981) shown in Equation 6-25.

$$\ln k = \ln A' - \frac{E_a}{RT} \qquad \textbf{Equation 6-25}$$

where, k = reaction rate constant, A' = pre exponential factor, E_a = activation energy (J/mol), R =universal gas constant (J/mol. K) , and T = absolute temperature (K).

At two different temperatures, T_1 and T_2 , Equation 6-25 can be written as,

$$\ln k_1 = \ln A' - \frac{E_a}{RT_1} \qquad \textbf{Equation 6-26}$$

$$\ln k_2 = \ln A' - \frac{E_a}{RT_2}$$ **Equation 6-27**

Solving Equation 6-26 and 6-27,

$$E_a = \frac{R \ln\left(\frac{k_1}{k_2}\right)}{\left(\frac{1}{T_2} - \frac{1}{T_1}\right)}$$ **Equation 6-28**

The estimated rate constants at specific temperature and the activation energy of both the enzyme catalyzed and un-catalyzed reactions are summarized in Table 6-3.

Table 6-3: Rate constants at different temperatures and activation energy

Parameter	Enzyme Catalyzed Reaction	Un-Catalyzed Reaction
T (K)	Rate Constant - k (1/day)	
308	506.7	0.012
318	581.8	0.018
	Activation Energy - E_a (kJ/mol)	
	11.3	34.1

The high rate constants at higher temperatures were attributed to the enhanced microbial activity at relatively high temperatures. Activation energy of the un-catalyzed reaction was about 3 times higher than that of the enzyme catalyzed reaction. This supports the fact that enzymes could lower the activation energy of the reaction. However, MnP enzyme in our system not only lowered the activation energy, but increased the available carbon, leading to a higher rate of reaction.

Overall, the validation of the kinetic model for substrate utilization and enzyme decay and estimation of activation energy in this study were only limited to the best combination of enzyme, enzyme dose, and E:H_2O_2 ratio as identified from the factorial batch experiments, and control reactions, because only the best combination was used in the scaling up process and for further experimentation. This was also due to the practical complexity of measuring substrate utilization over time in batch experiments; each reactor had to be discontinued once the sample was obtained for testing. As an alternative method for comparison, the rate constants and activation energy of all the reactions including the best combination could be obtained by relating the kinetics to the product formation instead of using substrate utilization. However, in that case, the yield coefficient of substrate to product should be estimated theoretically from stoichiometry or obtained experimentally for each combination.

6.3 Scale Up Model

To aid the scale up process, a mathematical model was formulated and then validated using the experimental results. The primary objective of developing a scale up model was to determine the quantity of enzyme required in flow-through columns or in field-scale operation to obtain the similar yield as obtained in batch reactor tests.

6.3.1 Model formulation

6.3.1.1 Assumptions

The reactions were assumed to be only kinetically limited (Pseudo homogeneous assumption). Mass transfer limitations including diffusion resistances were assumed to be negligible (Fogler, 2005).

The enzyme used was soluble in water. Therefore, it was assumed that the enzyme was readily available and only needed to be in contact with substrate for the reaction to be accelerated and the enzyme can reach the solid substrate without any resistance.

6.3.1.2 Conceptual model and model formulation

In developing the model, the following assumptions were made: the fresh catalyst enters the top of the flow-through column at a flow rate of *U*, moves through the reactor as a compact packed bed as shown in Figure 6-4 and the concentration in elemental volume (Δv) is constant.

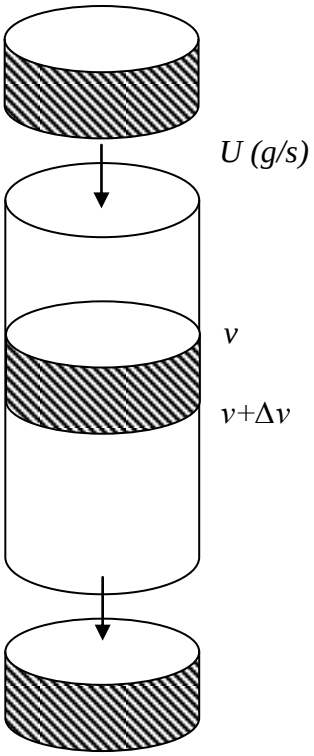


Figure 6-4: Flow-through column reactor scheme

The substrate packed in the flow-through column is the stationary phase with no incoming or outgoing elements. The enzyme in leachate is the mobile phase. Neglecting the substrate particles entering the system with the leachate, the mole balance of substrate (over a volume of Δv) can be written as,

$$\begin{pmatrix}\text{Rate of generation of}\\ \text{substrate in the system}\end{pmatrix}=\begin{pmatrix}\text{Rate of accumulation of}\\ \text{substrate within the system}\end{pmatrix} \qquad \textbf{Equation 6-29}$$

$$\int_{\Delta v} -r_s dv = \frac{dN_s}{dt} \qquad \textbf{Equation 6-30}$$

where, r_s is the rate of reaction and N_s is the moles of substrate accumulated.

Δv is chosen small enough so that there is no spatial variation of reaction rate within the sub volume, therefore,

$$\int_{\Delta v} -r_s dv = -r_s dv = -r_s v \qquad \textbf{Equation 6-31}$$

Substituting Equation 6-31 in Equation 6-30,

$$\frac{dN_s}{dt} = -r_s v \qquad \textbf{Equation 6-32}$$

Neglecting the waste settlement at this point, for constant volume system, the concentration of substrate, $[S]$, can be related as ,

$$\frac{d(N_s / v)}{dt} = -r_s \qquad \textbf{Equation 6-33}$$

$$\frac{dS}{dt} = -r_s \qquad \textbf{Equation 6-34}$$

The substrate conversion at any time, $[S_t]$, can be written in terms of substrate conversion, X_s ,

$$X_S = \frac{\text{moles reacted}}{\text{moles initial}} \qquad \textbf{Equation 6-35}$$

$$[S_t] = [S_o](1 - X_s)$$ **Equation 6-36**

where, $[S_o]$is the initial substrate concentration.

Taking the derivative of Equation 6-36 and equating to Equation 6-34,

$$\frac{dS_t}{dt} = -S_o \frac{dX_s}{dt} = -r_s$$ **Equation 6-37**

Enzyme deactivation could affect the rate of reaction; the total concentration of active site of an enzyme, which is accessible to the substrate, can change with time (Fogler, 2005). Therefore, the activity of enzyme at any time t, $a(t)$, can be written as,

$$a(t) = \frac{\text{rate of reaction on a catalyst that has been used for time } t}{\text{rate of reaction on a fresh catalyst}}$$ **quation 6-38**

$$a(t) = \frac{-r_s'(t)}{-r_s'(t=0)}$$ **Equation 6-39**

Rearranging Equation 6-39,

$$-r_s'(t) = a(t)\left[-r_s'(t=0)\right]$$ **Equation 6-40**

As shown in Section 6.2.4, the enzyme undergoes a first order decay. Therefore, the change of enzyme activity, $\frac{da}{dt}$, can be represented as,

$$\frac{da}{dt} = -k_E\, a$$ **Equation 6-41**

where, k_E is the enzyme decay constant.

Rearranging Equation 6-41,

$$dt = -\frac{1}{k_E}\frac{da}{a} \qquad \textbf{Equation 6-42}$$

Next, the contact time of enzyme with the substrate is related to the weight of the catalyst. Considering the point *v* in the reactor in Figure 6-4, where the substrate is in contact with enzyme weight (*W*), and assuming that the enzyme moves with leachate through the bed at a rate of *U*, the time, *t*, that the enzyme has been in contact with the substrate when the enzyme reaches a point *v*, can be written as,

$$t = \frac{W}{U} \qquad \textbf{Equation 6-43}$$

Differentiating Equation 6-43,

$$dt = \frac{dW}{U} \qquad \textbf{Equation 6-44}$$

Substituting Equation 6-42 on Equation 6-44,

$$\frac{dW}{U} = -\frac{1}{k_E}\frac{da}{a} \qquad \textbf{Equation 6-45}$$

Integrating Equation 6-45,

$$\int_{o}^{a(t)} \frac{da}{a} = \int_{o}^{W} -\frac{k_E}{U} dW \qquad \textbf{Equation 6-46}$$

Solving Equation 6-46,

$$\ln\left(a\right)_o^{a(t)} = -\frac{k_E}{U}\left(W\right)_o^W \qquad \textbf{Equation 6-47}$$

Enzyme activity at time *t*,

$$a(t) = e^{-\frac{k_E W}{U}} \qquad \textbf{Equation 6-48}$$

Combining Equations 6-37, 6-40, 6-44 and 6-48 and relating to the kinetic Equation 6-15, the following relationship can be obtained,

$$-U\frac{dX_s}{dW} = e^{-\frac{k_E W}{U}}\frac{v_{\max}\left(1-X_s\right)}{K_M + S_o\left(1-X_s\right)} \qquad \textbf{Equation 6-49}$$

Integrating Equation 6-49,

$$-\frac{U}{v_{\max}}\int_0^{X_s}\left[\frac{K_M + S_o\left(1-X_s\right)}{\left(1-X_s\right)}\right]dX_s = \int_0^W e^{-\frac{k_E W}{U}}dW \qquad \textbf{Equation 6-50}$$

Solving Equation 6-50,

$$-\frac{K_M}{v_{\max}}\ln\left(1-X_s\right) + \frac{S_o}{v_{\max}}X_s = \frac{1}{K_E}\left[1-e^{-\frac{k_E W}{U}}\right] \qquad \textbf{Equation 6-51}$$

Equation 6-51 can now be used to estimate the amount of enzyme, *W*, to reach a specific conversion of substrate (X_s).

6.3.2 Evaluation of the model

Microsoft Office Excel Solver 2007 was used to solve Equation 6-51. As described in Chapter 5, two sets of flow-through column experiments were conducted. Therefore, the model was validated separately with the two sets of experimental results.

6.3.2.1 First set of column experiments

Equation 6-51 was solved to determine the amount of enzyme (*W*) needed to convert total organic carbon of waste by 80% (X_s), in the first set of flow-through column experiments. The model input and output parameters are shown in Table 6-4.

Table 6-4: Scale up model input and output parameters for the first set of flow-through column experiments

Parameter	Value	Source
V_{max} (g_{TOC}/g_{DS}.day)	0.076	From kinetic model & batch experimental results (Refer Table 6-2)
K_M (g_{TOC}/g_{DS})	0.348	From kinetic model & batch experimental results (Refer Table 6-2)
k_E (1/day)	0.006	From enzyme decay model & batch experimental results (Refer Section 6.2.4)
$[S_o]$ (g_{TOC}/g_{DS})	0.431	Batch experimental results (Refer Chapter 4, section 4.3.10.2)
X_s	0.8	Model input
U (mL/min)	10	From scale up approach (Refer Chapter 5, section 5.2)
***W* (mg)**	**12.0**	**Model output**

As model predicted, a flow-through column experiment was conducted by adding 12 mg of enzyme. The TOC in waste after the experiment was measured and it was used to

validate the model. The TOC in final waste was 115 mg/g (refer Table 5-3), that was equivalent to 73% of waste TOC conversion.

6.3.2.2 Second set of column experiments

The model shown in Equation 6-51 was used to estimate the amount of enzyme needed to achieve the specific substrate conversion in one cycle of leachate recirculation. In order to lower the quantity of enzyme while increasing the number of leachate recirculations, the developed model was run several times until the required waste conversion was achieved with a less quantity of enzyme assuming that the un-bounded enzyme to waste matrix remained in leachate after each cycle.

The model predictions are summarized in Table 6-5. The other model input parameters were similar to the parameters shown on Table 6-4.

Table 6-5: Scale up model predictions for second set of flow-through column experiments

Parameter	Value	Source
$[S_o]$ (g_{TOC}/g_{DS})	0.237	Column experimental results (Refer Chapter 4)
Column 1		
W (mg)	2.0	Model input in this case
X_s	0.8	Model output, ~ 0.2 in each cycle
# of cycles	**7**	**Model output**
Column 2		
# of cycles	1	Model input
X_s	0.8	Model input
***W* (mg)**	**10.0**	**Model output**

2 mg of enzyme was added to the column 1 and the leachate was recirculated 7 times as predicted from the model. 10 mg enzyme was added to the column 2 to achieve 80% waste TOC conversion in one cycle. After the experiments, the TOC of waste in each column was measured as an indication of the success of the model predictions. Initial TOC in waste was 0.237 g/g and the final TOC in column 1 and column 2 were 0.112 g/g and 0.093 g/g, respectively. TOC conversion in column 1 was 53% and in column 2, TOC conversion was 61%.

The model validation criteria described above are summarized in Table 6-6.

Table 6-6: Summary of model validation

Data set	Waste conversion %	
	Model	Experimental
First set of column	80	73
Second set of columns		
Column 1	80	53
Column 2	80	61

Although the enzyme quantity was calculated based on the 80% waste TOC conversion, the actual TOC conversions observed were relatively less than the model predictions. Insufficient contact between the waste matrix and the enzyme and the flow through variations such as channelling effects were thought to contribute to the lower than expected efficiency. Furthermore, the actual TOC conversion in the second set of flow-through column experiments was relatively lower than that of the first set of experiments. In the first set, the experimental period was longer (nearly 5 months); therefore, the leachate was recirculated many times to avoid the interferences that could arrive from the moisture deficiency when there was no leachate recirculation. This could allow a better contact of enzyme and waste matrix throughout the experimental period compared to the second set. In the second set, in both the columns, there were only a limited number of

recirculations, which could affect the efficiency of the waste degradation process as leachate recirculation throughout the operation is an important factor for a higher efficiency of the process (Suna Erses & Onay, 2003; Sponza & Agdag, 2004; Bilgili *et al.*, 2007).

However, the model can be considered as a good approximation because the conversion ratio observed in the real system was reasonably closer to the theoretical predictions. It is, therefore, recommended to use the developed scale up model to estimate the amount of enzymes required in the field scale application and carry out leachate recirculations as many times as possible to achieve the required waste conversion. It would allow enzyme to have a better contact with waste while providing the expected improvements with leachate recirculation alone such as providing enough moisture, re-introducing nutrients, and media for microbial movements in the system.

6.4 Predictive Model

A predictive model was developed to predict the methane production in enzyme enhanced systems. The developed model was then validated with the experimental results.

6.4.1 Conceptual model

As shown in Figure 6-5, a waste cell consists of solid, liquid, and gas phases. The solid phase includes organic solid waste, inorganic matter, and non degradable inert material. The organic solid waste could be divided into three fractions according to the degree of biodegradability; cellulose, hemicellulose, and lignin. However, those fractions are eventually converted to gas. The solid first dissolves into the leachate by enzymatic hydrolysis. In the liquid phase, various aqueous species such as acetate and water soluble monosaccharides, are in equilibrium. The species in the liquid phase are available to the biomass for conversion into gas. Biomass is a coexisting component in solid and aqueous phase. Biomass can undergo self decay or eventually can convert into gas. The gas phase mainly consists of methane and carbon dioxide.

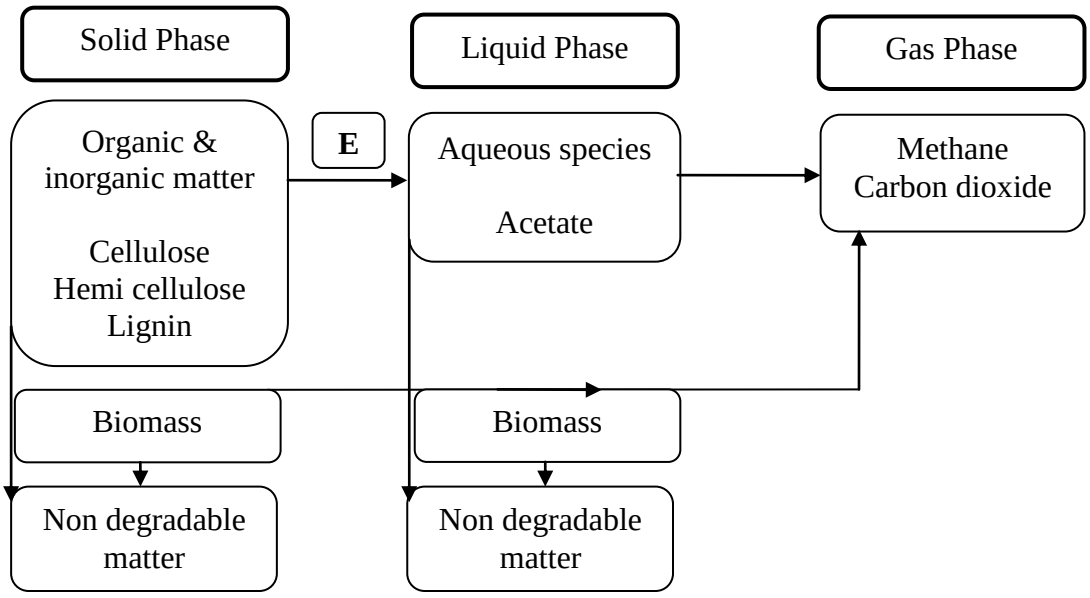


Figure 6-5: Simplified carbon degradation pathways

6.4.2 Model formulation and assumptions

The application of mass balance coupled with the Michaelis-Menten enzyme kinetics in a set of ordinary differential equations (ODE) are shown in Equation 6-53 to Equation 6-59. Solutions to the ODE provide the concentration of organic carbon sources at any time in each phase.

When formulating the model, any inhibitory effects were neglected as the experiments were run at near optimum conditions. The growth and decay of microbial mass were not accounted in this model since the model pathway assumed that microbial mass will be eventually mineralized to gas (methane and carbon dioxide). For simplicity, in the enzyme catalyzed reaction, the gas produced under control conditions was not accounted for. The enzyme decay was assumed to be negligible for the time period considered.

6.4.2.1 Solid hydrolysis

Hydrolysis of solid substrate plays an important role in waste degradation process. Lignin-rich waste hydrolysis is assumed to be dominated by the enzyme addition; hence the process is governed by enzyme kinetics. Therefore, the waste hydrolysis in enzyme catalyzed reaction can be represented as (similar to Equation 6-15);

$$\frac{d[S]}{dt} = -\frac{V_{\max}[S]}{K_M + [S]} \qquad \textbf{Equation 6-52}$$

where, $[S]$is the solid organic carbon concentration (g_{TOC}/g_{DS}), other variables were similar to the variables explained in Equation 6-15.

6.4.2.2 Aqueous carbon

Aqueous carbon is produced during solid carbon hydrolysis and is consumed during its conversion to gas as represented in Equation 6-53. For simplicity, all transformation processes involved in acetogenesis and acedogenesis were lumped together as a single step in the model.

Aqueous (water soluble) Carbon;

$$\frac{d[L]}{dt} = \left[\frac{d[L]}{dt}\right]_{formation} - \left[\frac{d[L]}{dt}\right]_{consumption} \qquad \textbf{Equation 6-53}$$

where, $[L]$is the aqueous organic carbon concentration (g_{DOC}/g_{DS}).

For enzyme catalyzed reaction, Equation 6-53 and Equation 6-52 can be related as,

$$\frac{d[L]}{dt} = \left[Y_{S-L}\frac{V_{\max}[S]}{K_M + [S]}\right] - K_L[L] \qquad \textbf{Equation 6-54}$$

Y_{S-L} is yield coefficient of converting solid carbon to aqueous carbon and K_L is the aqueous carbon degradation rate constant.

6.4.2.3 Formation of methane

Formation of methane from aqueous carbon is mathematically described in Equation 6-55.

$$\frac{d[G]}{dt} = Y_{L-G}\left[\frac{d[L]}{dt}\right]_{consumption} \qquad \textbf{Equation 6-55}$$

where, $[G]$ is the gaseous (methane) carbon concentration (g_{CH4-C}/g_{DS}) and Y_{L-G} is yield coefficient of converting aqueous carbon to methane carbon.

Equation 6-55 can be simplified as,

$$\frac{d[G]}{dt} = Y_{L-G}K_L[L] \qquad \textbf{Equation 6-56}$$

6.4.3 Model evaluation

The derived ODEs shown in Equations 6-52, 6-54, and 6-56 for enzyme catalyzed reaction were solved using the ODE45 solver in MATLAB. The methane production predictive model was then validated using both the batch experimental flow-through column experimental results.

6.4.3.1 Model calibration and validation using batch experimental results

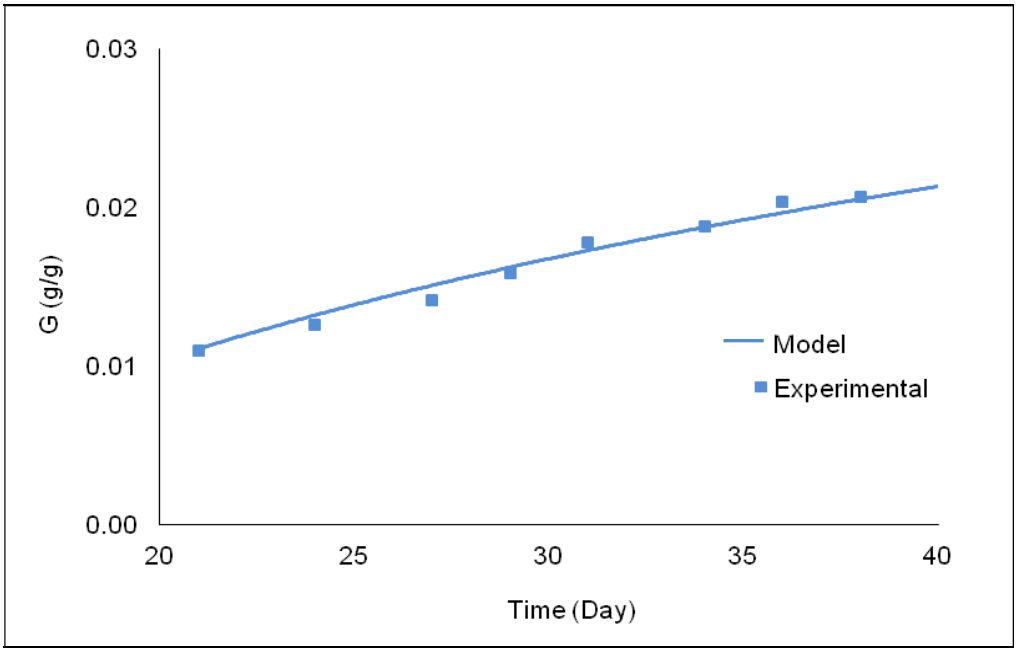
The cumulative methane yields from day 0 to day 21 were used to calibrate the model, hence to estimate the kinetic constants and the experimental yield coefficients. The

estimated model input parameters using batch experimental results up to day 21 are shown in Table 6-7.

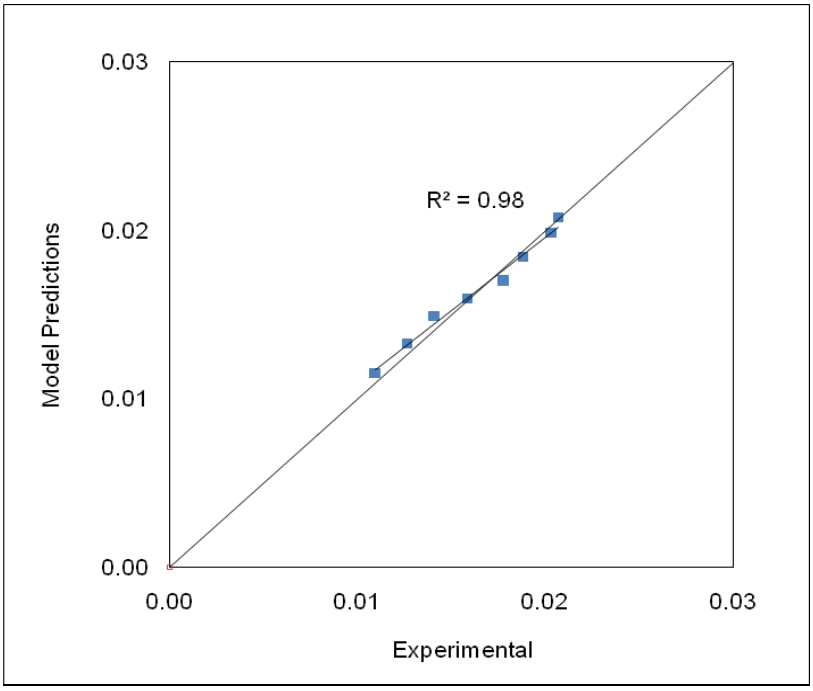
Table 6-7: Model parameters

Parameter	Value
V_{max} (g_{TOC}/g_{DS}.day)	0.076
K_M (g_{TOC}/g_{DS})	0.348
$[S_o]$ (g_{TOC}/g_{DS})	0.431
$[L_o]$ (g_{DOC}/g_{DS})	0.386
K_L (1/day)	0.01
Y_{S-L}	0.8
Y_{L-G}	0.15

The cumulative methane yields from day 22 to day 40 were used to validate the gas production predictive model. The experimentally measured and corresponding model predictions are shown in Figure 6-6.



(a)



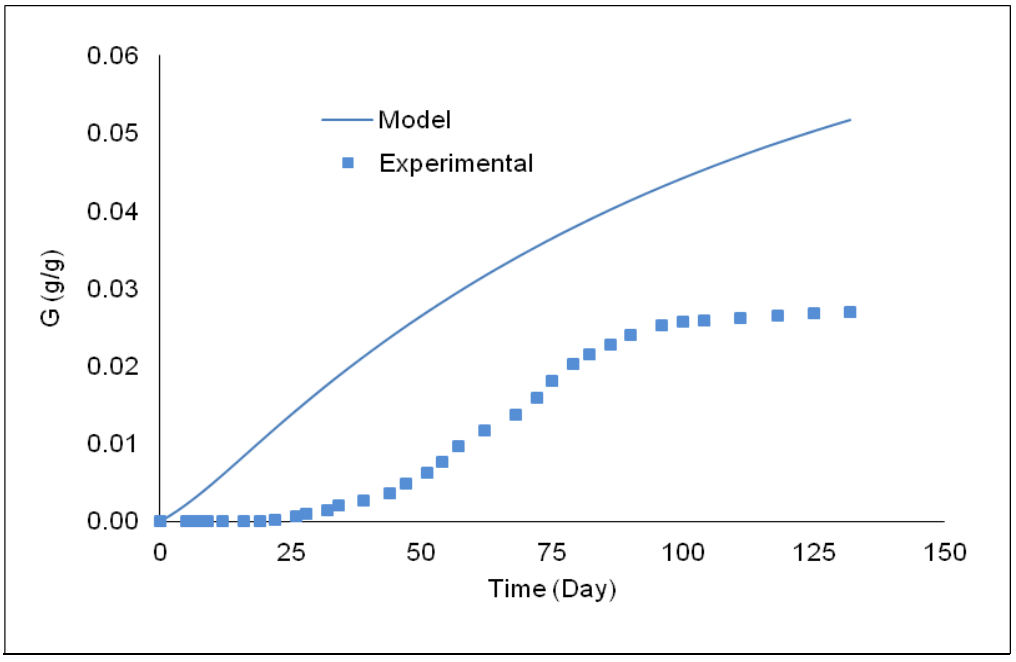
(b)

Figure 6-6: Validation of the methane production predictive model using batch experimental results (a) trend (b) model vs experimental

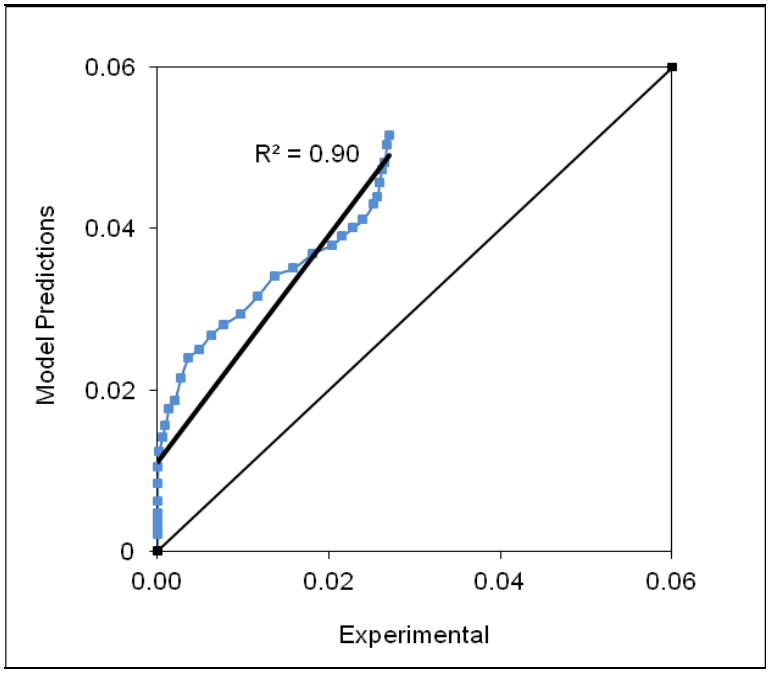
As can be seen from Figure 6-6, the developed model is able to predict the methane production very well. The model predictions versus experimental data plots show a good fit between the model and the experimental data with R^2 of 0.98. Furthermore, the graph shows a good association and good coincidence; thus, the model can be classified as a good model for predicting methane production in enzyme catalyzed anaerobic waste degradation process.

6.4.3.2 Model validation using flow-through column experimental results

As shown in Figure 6-7, the predictive model was also validated using the flow-through column experimental results.



(a)



(b)

Figure 6-7: Gas production predictive model validation using flow-through column experimental results (a) trends (b) model vs experimental

As shown in Figure 6-7 (a), the predicted trend for methane production is similar to the experimental results, but the experimental values showed a downward shift to the model predictions. This was mainly due to the use of batch experimental results to calibrate the model, hence the use of over-estimated yield coefficient in the model. As discussed in Section 5.4.2, the methane yields in the flow-through columns were lower than the batch reactors due to the insufficient contact between the enzymes and waste and the less controlled experimental conditions in flow-through columns compared to the batch reactors.

As can be seen from Figure 6-7 (b), a good association between the model and experimental values was observed with R^2 of 0.9. But, a positive bias in the model prevented a good agreement between the model and the experimental values. Therefore,

it is recommended to use the methane production predictive model with caution; the model may need to be re-calibrated with additional data at different conditions prior to its use.

Overall, in order to apply any model, two factors must be considered. The model must be complex enough to be realistic, but simple enough that fitted parameters can be easily interpreted and used for design purposes as well as being consistent with the observed experimental behaviour (Cecchi *et al.*, 1990). However, due to the complexity of the biological processes associated with anaerobic digestion, developing a detailed mathematical model that reflects the biological complexities is not an easy task; hence most models described in the literature are simplifications (Komilis, 2006; Slezak *et al.*, 2010).

6.5 Conclusions and Recommendations

The kinetic model for the enzyme catalyzed and un-catalyzed reactions defined the process mechanism quite well as evident from the model validation. The experimental results showed good agreement with the kinetic model predictions. The developed mathematical models, the scale up model and the predictive model, were validated using laboratory experimental results, and observed good agreements with the model predictions. The models can be used in scaling up the process to field scale operation and can be extended to different systems with modifications as required.

As described previously, the models discussed in this study were simplifications of the real system. The models can be also extended by incorporating many other factors such as the change of microbial masses, substrate or/and product inhibition, mass transfer resistances.

The predictive model developed in this study only considered the generation of gas as it was the main factor used to evaluate the effect of enzyme addition, but gas and leachate transportation in a waste cell are also important factors to be considered. A model

incorporating the basic concepts to simulate the spatial variation in addition to the temporal variation of gas and leachate transportation in landfill bioreactors is recommended to better understand the overall system (El-Fadel *et al.*, 1996; Gholamifard *et al.*, 2008). Such a model coupled with biological and hydraulic behaviours could be used to optimize the time and cost of operating bioreactor landfills by optimizing the volume of injected leachate, number and space of injection devices, and duration of leachate recirculation, in addition to the gas and leachate generation (Gholamifard *et al.*, 2008).

Chapter Seven: Development of Methodology for Field Application

The final objective of this study was to develop a comprehensive methodology for the application of enzyme augmented leachate recirculation in field-scale landfill bioreactors. The possibility of applying this novel method at the Calgary biocell was examined. This chapter includes the proposed methodology for field application, an overview of the Calgary biocell, and details of the experiments carried out at the Calgary biocell.

7.1 Proposed Methodology for Field Application

Based on the experience gained through the laboratory experiments and the developed mathematical models, a comprehensive methodology for the field application was developed. The proposed methodology is presented in Figure 7-1.

The first step is to decide whether the enzyme addition to the field-scale landfill bioreactor is a necessary step. The existing parameters of the bioreactor such as leachate quality and the gas production trends should provide important information on making a decision. The gas production could decrease due to three main factors; nutrient limiting, toxicity, and carbon limiting. If system has nutrient or toxicity issues, the additions of nutrients or avoiding toxicity are recommended prior to enzyme addition. Only if system is carbon limiting, the enzyme addition is recommended.

Establishing a baseline control leachate recirculation system in the field-scale landfill bioreactor without enzyme is an essential requirement; it will help to accurately evaluate the impact of enzyme addition. Depending on the exiting leachate recirculation process at the bioreactor, water addition after completely draining the existing leachate and study the leachate flow behaviours could be important steps prior to enzyme addition.

The scale up model developed in this study (refer Chapter 6 - Section 6.3) is proposed to use to estimate the quantity of enzyme need to be added to the field-scale landfill bioreactor. The activated enzyme should be added to the leachate before recirculation.

Leachate parameters and gas production and composition should be measured over several months followed by enzyme augmented leachate recirculation to monitor the effect of enzyme addition.

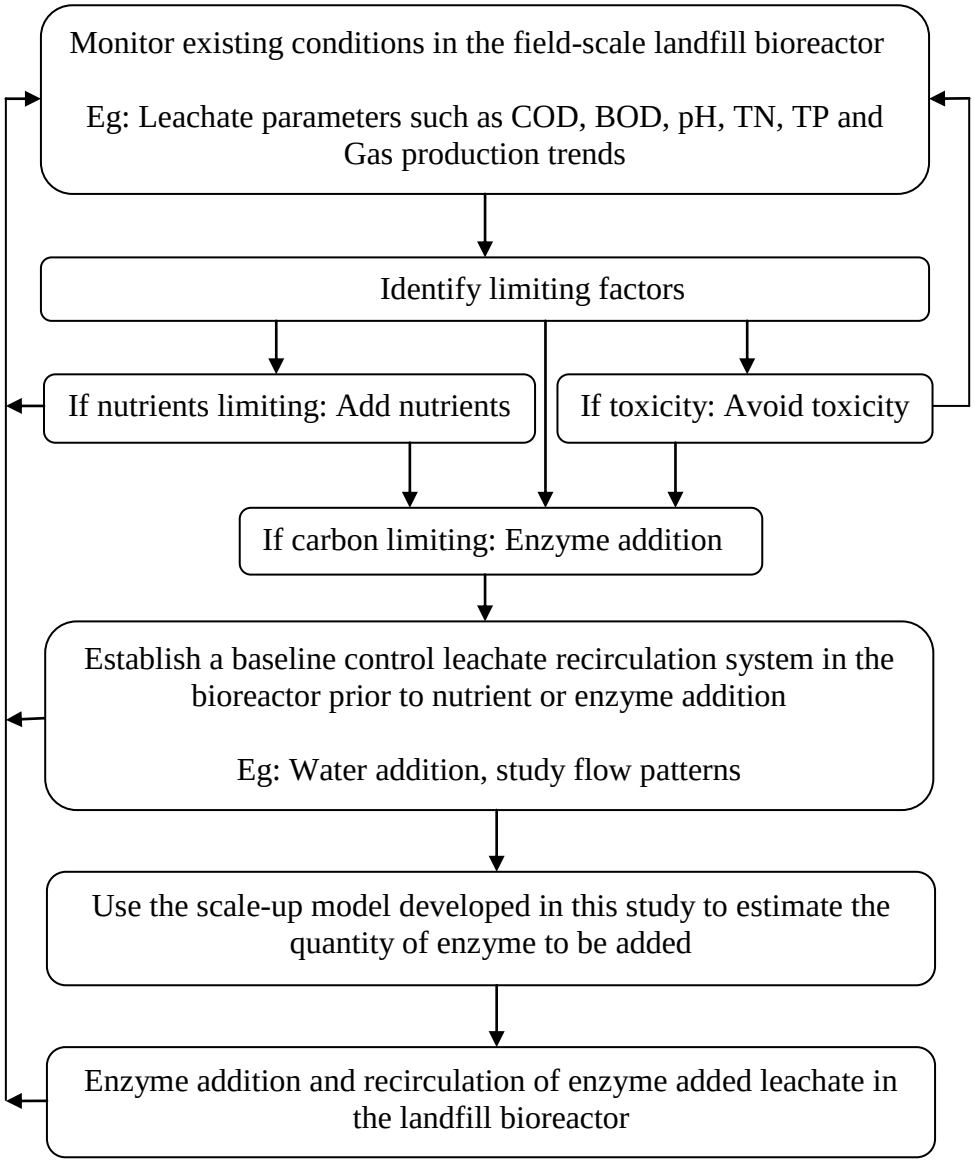


Figure 7-1: Proposed methodology for field application

As an example, the possibility of applying this methodology at the Calgary Biocell was examined.

7.2 Overview of Calgary Biocell

Calgary biocell, a full scale landfill bioreactor, was constructed in Calgary, Canada in 2004. The City of Calgary partnered with University of Calgary and local consulting firms to design, construct, and operate the biocell. It is not a permanent facility, the contents of the biocell will be removed once complete degradation of waste is achieved and a new biocell can be constructed, thereby reusing the space. Calgary biocell is also a research facility which has been using to investigate leachate recirculation, landfill gas (LFG) production, and waste settlement behavior in landfill bioreactors.

The biocell operation consists of three stages. In the first stage, the biocell is operated as an anaerobic bioreactor with leachate recirculation and gas collection. The collected landfill gas is used as a fuel source for electricity generation. After several years of anaerobic operation or once the gas production has stopped, the second stage will be initiated. In the second stage, air will be injected to initiate aerobic activity and to convert the cell into an ‘in-ground composter’. The third stage is the biocell mining to recover resources and space.

Calgary biocell is a unique facility and first of its kind where the three processes, anaerobic bioreactor, aerobic bioreactor, and mining are applied in one landfill cell (Hettiaratch *et al.*, 2007; Hunte, 2010). Currently, the biocell is operated in its first stage and this research focused only the anaerobic phase of the biocell operation.

A schematic diagram of the biocell is shown in Figure 7-2.

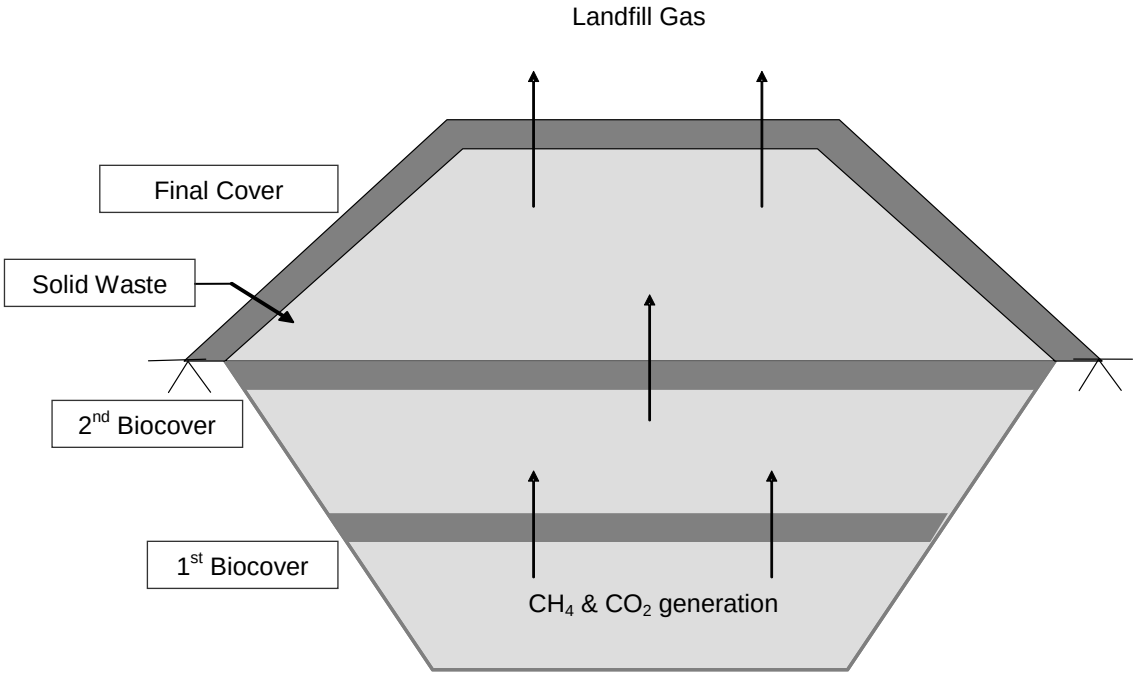


Figure 7-2: Schematic diagram of Calgary biocell

The biocell is located in City of Calgary Shepard landfill site. Shape of the biocell is like a pyramid. The biocell covers an area of 100 m x 100 m with a waste footprint of 85 m x 85 m. Height of the biocell is 15 m and it extends 10 m below the ground surface. Base of the biocell is 50m x 50m. Total amount of waste deposited in the cell was approximately 48,000 tonnes of residential MSW.

Components of the biocell include a bottom liner system, intermediate covers, and final cover system. There are two 500 mm thick intermediate cover layers with a vertical space of 5 m. These layers are methane oxidizing layers which contain a mixture of compost (25% by volume) and top soil (75% by volume). The composite liner system consisted of a geo-textile cushion layer, high density polyethylene geo-membrane liner, and compacted clay liner. Once the waste was placed, the biocell was covered with a final cover to prevent infiltration and minimize odours and LFG emissions. The final cover consists of a soil layer, low density polyethylene membrane, and a biocap layer (25%

compost and 75% topsoil by volume). The soil layers support vegetation on the cover and are intended to minimize erosion.

The biocell is equipped with a landfill gas collection system, a leachate collection, removal, and recirculation system, and automated monitoring system. Landfill gas extraction is undertaken by horizontal and vertical collector pipes and directed to the header pipe located on the site. Collected gas is conveyed under vacuum to the Shepard LFG utilization facility. Leachate generated within the cell is directed to the leachate sump. When the level in the sump reaches a specified value, leachate is recirculated to the cell. The pumps were set to start and stop automatically when the leachate level reaches a set point. The leachate injection system is operated in conjunction with the leachate collection and removal system. It consists of horizontal trenches installed with perforated pipes and vertical wells. An automated monitoring system made up of sensors, was installed in the cell and connected to a data acquisition system to measure parameters including temperature and moisture.

7.3 Experiments at the Calgary Biocell

7.3.1 Monitoring leachate parameters

Existing leachate parameters of the biocell were analyzed over a period of ~4 years to obtain baseline data to ensure that there were no other limiting factors such as nutrients (N and P) and toxicity (NH_3) on waste degradation prior to enzyme addition.

Leachate samples were collected once in 3-5 months and tested for pH, COD, BOD, TP, NH_3-N, and TN at our laboratory using the procedures described in Chapter 3. The test results are summarized in Table 7-1. Accuracy of the results was checked with the results obtained by biocell consulting group, which was tested in Calgary ALS laboratory.

Table 7-1: Biocell leachate test results

Parameter	2009 Apr	2009 June	2009 Sep	2009 Dec	2010 Mar	2010 May	2010 June	2010 Aug	2010 Nov	2011 Jan	2011 Apr	2011 Aug
pH	7.7	7.7	7.6	7.8	7.7	7.8	7.7	7.6	7.7	7.8	7.7	7.9
COD (mg/L)	3521	3784	3987	3239	4682	2730	3766	196	289	1094	829	4142
BOD (mg/L)	295	227	187	174	336	120	263	156	142	127	86	368
BOD:COD	0.08	0.06	0.05	0.05	0.07	0.04	0.07	0.80	0.49	0.12	0.10	0.09
TP (mg/L)	23	19	15	19	21	12	14	7	9	11	9	51
NH3-N (mg/L)	2390	2221	1178	1423	2280	1910	1327	988	221	627	472	2270
TN (mg/L)	2623	2452	1389	1772	2652	2199	1870	1123	326	689	517	2680

Following conclusions can be drawn from the leachate analysis results shown in Table 7-1.

- ✓ pH of leachate showed to be closer to neutral over the monitoring period; it is an indication of establishing the methanogenic conditions in the biocell.

- ✓ A specific trend for COD and BOD could not be observed over the given period of time. However, analyzing the variation of leachate parameters over time would not be reasonable at this point without following the leachate recirculation and truck-out plan as the leachate quality could be affected by many parameters such as frequency of leachate recirculation, recirculating the same leachate over a time, and the amount of leachate trucked-out or the amount of water being added during a given period of time.

- ✓ It is important to note that BOD:COD ratio at each test was very low. This suggests that the decrease in biodegradable organic fraction compared to the non-biodegradable fraction of waste.

- ✓ A specific trend for TP, NH3-N, and TN could not be observed during the study period. Again, the leachate recirculation and truck-out activities could affect this behaviour. However, as described in Chapter 5, based on COD:N:P ratio of 500:6:1(Mata-Alvarez, 2003), the biocell seems to contain enough nutrients within the system. Therefore, an addition of nutrients to the system prior to enzyme addition is not recommended.

In addition to the leachate analysis, the gas production rate was observed. The present biogas production rate in the biocell is ranged between 18-35 m^3/hr. On average, the biogas consists of approximately 60% CH_4 and 40% CO_2.

7.3.2 Preparatory phase before enzyme addition

In order to accurately evaluate the impact of enzyme addition at the biocell, establishing a baseline control leachate recirculation system in the biocell without enzyme was an essential requirement. Water addition to the biocell after completely draining the existing leachate was the main stage prior to enzyme addition.

7.3.2.1 Energy balance model for the biocell

Incoming water to be added to the biocell was expected to be very cold, around 2-5 ^{0}C. There were some concerns before adding cold water to the biocell as it could lower the biocell temperature, hence the microbial activity inside the biocell. Therefore, a model which accounts an energy balance of the biocell was developed to determine the temperature drop that could occur as a result of introducing cold water in to the biocell.

7.3.2.1.1 Energy balance model formulation

As shown in
Figure 7-3, the biocell could be treated as a control volume.

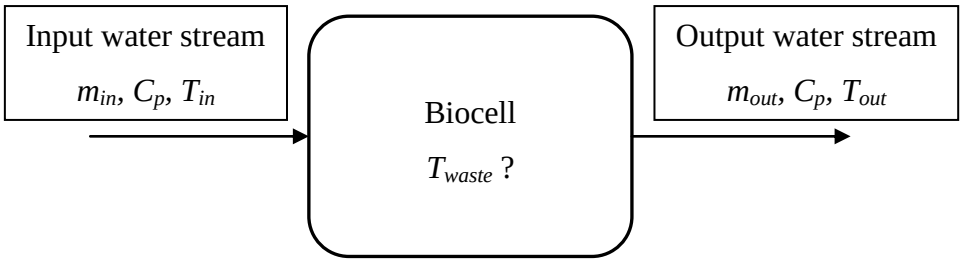


Figure 7-3: Energy balance model - Calgary biocell

Applying energy balance to the control volume, assuming no heat generation or heat losses during the period of leachate recirculation (~4 h),

$$E_{in} - E_{out} = \frac{dE_{Biocell}}{dt}$$ **Equation 7-1**

where, E_{in} and E_{out} are total energy entering and leaving the biocell, and $\frac{dE_{Biocell}}{dt}$ is the change in total energy of the biocell.

Equation 7-1 can be expanded as,

$$m_{in}C_pT_{in} - m_{out}C_pT_{out} = \frac{d(m_{Biocell}C_{p,Biocell}T_{Biocell})}{dt} \qquad \textbf{Equation 7-2}$$

where,

m_{in} and m_{out} = mass flow rate of incoming and outgoing water (kg/h)

C_p = specific heat capacity of water (kJ/kg.^{0}C)

T_{in} and T_{out} = temperature of incoming and outgoing water (^{0}C)

$m_{Biocell}$ = mass of waste in the biocell (kg)

$C_{p,Biocell}$ = specific heat capacity of solid waste (kJ/kg.^{0}C)

$T_{Biocell}$ = temperature of the biocell (^{0}C)

When calculating $T_{Biocell}$, the mass flow of incoming and outgoing water was assumed to be uniform and equal ($m_{in} = m_{out}$), assuming that the waste is saturated with leachate. Time duration of leachate recirculation was taken as 4 h. Assuming T_{in} and T_{out} does not change over time, we can treat this process as a uniform flow process (Cengel & Boles, 1998). Therefore, Equation 7-2 can be simplified as,

$$m_{in}C_pT_{in}\int_0^4 dt - m_{out}C_pT_{out}\int_0^4 dt = m_{Biocell}C_{p,Biocell}\int_{TBiocell}^{Twaste} dT \qquad \textbf{Equation 7-3}$$

where, T_{waste} is the temperature of waste during (at) the leachate recirculation (^{0}C) which needs to be determined through the energy balance.

Equation 7-3 can be solved as,

$$T_{waste} = \frac{\left[m_{in}C_pT_{in} - m_{out}C_pT_{out}\right]\times 4}{\left[m_{Biocell}C_{p,Biocell}\right]} + T_{Biocell} \qquad \textbf{Equation 7-4}$$

7.3.2.1.2 Energy balance model evaluation

The model developed was then solved to find the temperature drop of waste inside the biocell during cold water recirculation. The model input and output parameters are summarized in Table 7-2. It was assumed that the average biocell temperature to be 25 ^{0}C and the leachate leaving are at the same temperature as the biocell and the wetted volume to be only 20% of the waste matrix.

Table 7-2: Energy balance model parameters

Parameter	Value
Incoming leachate flow	
m_{in} (kg/h)	12000
C_p (kJ/kg.^{0}C)	4.2
T_{in} (^{0}C)	2
Outgoing leachate flow	
m_{out} (kg/h)	12000
C_p (kJ/kg.^{0}C)	4.2
T_{out} (^{0}C)	25
Biocell energy change	
$m_{Biocell}$ (kg)	10000000
$C_{p,Biocell}$ (kJ/kg.^{0}C)	0.9
$T_{Biocell}$ (^{0}C)	25

Model outputs	
T_{waste} (^{0}C)	24.5
Temperature drop (^{0}C)	**0.5**

As shown in Table 7-2, under worst case assumptions, the temperature could only drop by ~ 0.5 ^{0}C. There will not be any adverse effects to the microbial activity by adding 12 m^3 of 2 ^{0}C water over a 4 h time period; therefore, the cold water addition to the biocell was recommended.

7.3.2.2 Water addition to the biocell

The biocell was completely drained to remove the leachate which had been recirculating over a long period of time (~6 months). 12 m^3 of water was added in an hour period following a complete drainage, to saturate the biocell to avoid the effect of accumulated chemicals present in recirculated leachate over and over. Leachate generation rates after recirculation were recorded and a leachate hydrograph was developed. Two month later, 12 m^3 of water was added again to the biocell to avoid the possibility of the biocell to be moisture limited.

The leachate hydrograph developed during the first water addition is shown in Figure 7-4.

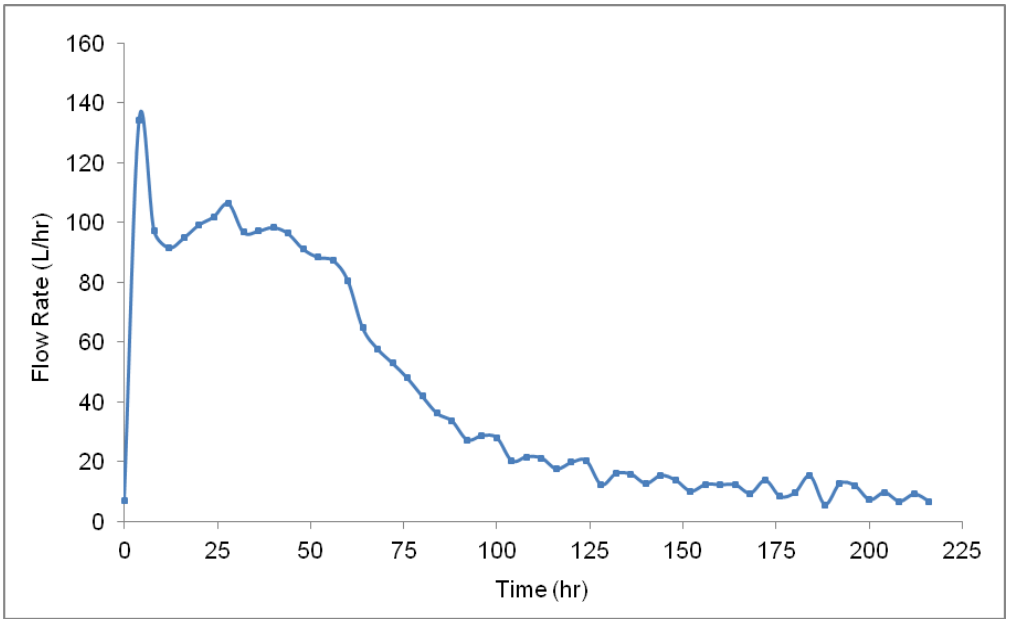


Figure 7-4: Leachate hydrograph for biocell

Water addition took place during the period of 0-1 h; drained leachate flow rate was measured in every 4 h until there was no significant drainage. Approximately 12000 L of water was added to the biocell and only about 8439 L of leachate was collected during the monitored drainage period. The water storage in the biocell was ~ 30%. A breakthrough time before the first drop of leachate reached at the bottom of the biocell was ranged between 1-4 h time period.

The quality of leachate produced was measured after water addition as they were useful for determining the existing organic content and the nutrient content in waste. Leachate analysis results before and after the water addition are shown in Table 7-3.

Table 7-3: Leachate parameters before and after water addition

Parameter	Before water addition	After water addition
pH	7.8	7.9
COD (mg/L)	3985	3656
BOD (mg/L)	370	336
BOD/COD	0.09	0.09
TP (mg/L)	56	49
NH3-N (mg/L)	2195	1985
TN (mg/L)	2720	2568

A significant change in leachate parameters could not be observed following water addition. They were closer to the parameters of leachate that had been accumulated over a time.

7.3.3 Proposed method for enzyme addition at Calgary Biocell

7.3.3.1 Determination of the quantity of enzyme to be added to the biocell

The scale up model developed and validated through laboratory experimental results (refer Chapter 6 - Section 6.3) was used to determine the amount of enzyme need to be added to the biocell to reach the specific waste conversion to gas. Based on the kinetic parameters obtained through lab experiments (refer Table 6-4), the enzyme (MnP) quantity to be added to the biocell was estimated as 1024 g which is equivalent to 20476 U of MnP enzyme.

7.3.3.2 Enzyme addition and recirculation of enzyme added leachate in biocell

It is proposed that activated enzyme should be added to the above ground water tank located in the site to undertake manual mixing. The enzyme added water should be allowed to drain into the leachate sump under gravity. It should be then pumped into the biocell at a low flow rate (as low as ~12 m^3/day). The same leachate should be recirculated through the biocell regularly without adding any extra water. Leachate parameters and gas production and composition should be measured over several months followed by enzyme addition.

7.4 Conclusions and Recommendations

The proposed methodology could be applicable to any existing landfill bioreactor at its later stage of waste degradation. As also noted previously, the methodology could be either improved or modified as required based on the existing processes of a particular landfill bioreactor.

The developed novel method needs to be validated using the field scale results. It is recommended to apply this method in the Calgary biocell when the biocell is ready or in any other full scale landfill bioreactor.

Chapter Eight: Conclusions, Recommendations, and Research Contributions

8.1 Conclusions

A summary of conclusions obtained from this study is presented below:

1) At first, the results observed from the batch experiments in this study showed that the leachate augmentation with enzymes before recirculation is a feasible option for enhancing the gas production in the gas declining phase of anaerobic waste degradation process. The optimum process parameters for the highest methane production were identified through a series of laboratory batch experiments.

2) The process of leachate augmentation with enzymes was successfully scaled up to an intermediate step, a set of flow-through columns, before recommended it in the field-scale tests.

3) The scale up approach used in this study was partially based on the theoretical concepts, kinetic and flow dynamic similarity, and partially based on the empirical procedure. A combination of these two methods seems to be successful in translating the process of enzyme augmented leachate recirculation form batch to pilot scale.

4) This research marks the single most comprehensive investigation of recirculating enzyme augmented leachate in landfill bioreactors undertaken to date. The operating criteria identified in this study would be essential in successful application of this methodology in full scale landfills. The outcome of this research will have a major impact on enhancing the gas production in anaerobic landfill bioreactors, by yielding the answer to the key question: ‘how to manipulate leachate with enzymes to maximize gas production in landfill bioreactors?’.

5) Overall, the process of augmenting leachate with peroxidase enzymes before recirculation offers considerable benefit over the conventional leachate recirculation technique currently in use, including the enhanced gas production and land reuse opportunities through rapid waste stabilization. Enhancing the gas production in landfills offers environmental and economic benefits.

6) The cost of enzymes would definitely become a major barrier for commercializing this novel approach in a larger scale. Commercially available peroxidase enzymes are quite expensive, for example, 100 mg of MnP, the best performed enzyme in this study, cost about 900 CAD. A potential alternative for this approach is therefore recommended below in Section 8.2.

8.2 Recommendations

The novel approach presented in this thesis still requires significant improvements. Continuous development of research is needed on both experimental and numerical aspects of enzyme added leachate recirculation process in enhancing waste degradation and gas production in landfill bioreactors. The following areas of potential future development are identified based on the experience gained throughout the study:

1) One of the possibilities to avoid the high cost associated with enzymes is the direct use of white rot fungi (WRF) instead of using enzymes. The WRF produces a family of enzymes called ligninases, which shows extensive biodegrative properties (Cookson, 1995). However, it has been observed that WRF do not grow well in anaerobic environments (Cookson, 1995). Two potential options for introducing WRF into the field-scale landfill bioreactors are recommended.

    a) Another aerobic bioreactor may need to operate simultaneously as shown in Figure 8-1. The WRF could grow separately in an aerobic bioreactor under their favorable growth conditions. The resultant enzymes may need to

characterize and quantify prior to feed into the anaerobic waste bioreactor through leachate recirculation.

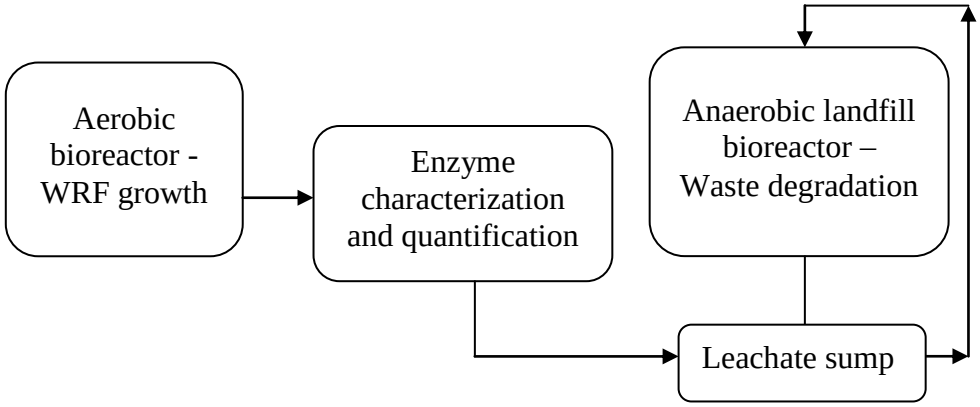


Figure 8-1: Schematic diagram for WRF process

b) When it comes to the application of WRF into the biocell specifically, WRF can introduce at its second stage; that is, once the biocell is switched into the aerobic stage from the anaerobic stage. It will not enhance the methane production, but it will assist in stabilizing waste at a higher degree.

There are also several other benefits of using WRF instead of enzymes. Among them, the most important is their capability of re-production. On the other hand, it would provide a solution to the long term enzyme inactivation behaviour.

2) As described in Chapter 4, non-conclusive results were observed in some of the microbial characterization tests and enzyme activity tests conducted to identify the enzymatic waste degradation mechanism in finer details. The standard analytical test methods used in this study may need revisions, which are applicable specifically to the leachate. Further experiments in this area are therefore recommended with the revised test methods.

3) The models developed in this study, scale up model and predictive model, were simplifications of the real system. It is recommended to extend these models by

incorporating other factors that have not been considered in this study including the change of microbial masses, mass transfer resistances, enzyme-waste contact behaviour, and flow and hydraulic patterns in the flow-through system.

8.3 Research Contributions

8.3.1 Refereed Journals

- ✓ Jayasinghe, P.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Steele, M.A., Enhancing gas production in landfill bioreactors: A flow-through column study on leachate augmentation with enzyme, Journal of Hazardous, Toxic and Radioactive Waste, 2012 (in press)
- ✓ Jayasinghe, P.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Kumar, S., 'Effects of enzyme additions on methane production and lignin degradation of landfilled sample of municipal solid waste', Bioresource Technology, 102(7), 4633-4637, 2011

8.3.2 Refereed Conferences

8.3.2.1 Peer Reviewed Full Paper + Oral Presentation

- ✓ Jayasinghe, P.A., Steele, M.A., Hettiaratchi, and J.P.A., Mehrotra, A.K., Enhancing energy recovery from waste: A study on nutrient and enzyme addition to enhance methane gas production, Paper presented at the '12th International Environmental Specialty Conference', Edmonton, June 2012
- ✓ Jayasinghe, P.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Preliminary Results from a Flow-Through Column Study on Leachate Augmentation with Enzyme, Paper presented at the '27th International Conference on Solid Waste Technology and Management', Philadelphia, March 2012
- ✓ Jayasinghe, P.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Optimization of Gas Production in Bioreactor Landfills by Leachate Augmentation, Paper presented at the 'Air & Waste Management Association's 103rd Annual Conference & Exhibition', Calgary, June 2010
    - ***Winner of the "PhD Platform Paper Award"***
    - ***Nominated paper for the "Young Professionals Best Paper Award"***

- ✓ Jayasinghe, P.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Operation of a Sanitary Landfill Cell for Optimum Anaerobic Degradation of Municipal Solid Waste, Paper presented at the '11th International Environmental Specialty Conference', Winnipeg, June 2010

8.3.2.2 Peer Reviewed Abstract + Oral Presentation

- ✓ Jayasinghe, P.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Enhancement of Waste Degradation in Bioreactor Landfills through Enzymatic Catalysis, Presented at the 'The UofC Graduate Conference', University of Calgary, May 2010
    - ***Winner of the "NSERC Award for the Best Oral Presentation"***

8.3.2.3 Peer Reviewed Abstract + Poster Presentation

- ✓ Jayasinghe, P.A., Steele, M.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Enhancing energy recovery from waste, Poster presented at the Canadian Prairie & Northern Section (CPANS), Air & Waste Management Association (AWMA), Calgary, April 2012
    - ***Winner of "the 1st prize" in the poster contest***
- ✓ Jayasinghe, P.A., Steele, M.A., Hettiaratchi, J.P.A., Mehrotra, A.K., Enhancing energy recovery from waste: A study on nutrient and enzyme addition to enhance methane gas production, Poster presented at the 'Global Clean Energy Congress and Exhibition', Calgary, November 2011

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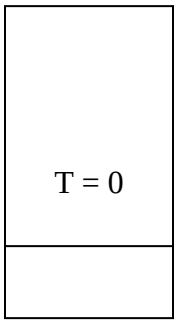
APPENDIX 1

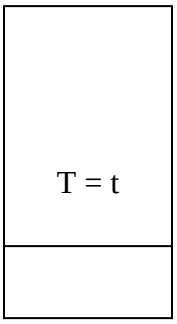
1) Methane Production Calculations

The methane production can be calculated using gas compositions as follows assuming that ideal gas behaviour in the gas phase.



$$n_{N2} = N_{Total}$$

$$p_i v_i = n_{N2} RT \qquad (1)$$



$$n_{N2} + n_{CH4} + n_{CO2} = N_{Total}$$

$$x_{N2} + x_{CH4} + x_{CO2} = 1$$

$$x_{N2} = (1 - x_{CH4} + x_{CO2})$$

$$p_{Total} v_i = N_{Total} RT \qquad (2)$$

Dividing equation (1) by (2);

$$\frac{p_i}{p_{Total}} = \frac{n_{N2}}{N_{Total}} = x_{N2} = (1 - x_{CH4} + x_{CO2}) \qquad (3)$$

Using Dalton's law for CH_4;

$$p_{Total} = \frac{p_{CH4}}{x_{CH4}} \qquad (4)$$

Substitute equation (4) in (3);

$$\frac{p_i}{p_{CH4}} = \frac{(1 - x_{CH4} + x_{CO2})}{x_{CH4}}$$

Rearranging,

$$p_{CH4} = \frac{p_i x_{CH4}}{(1 - x_{CH4} + x_{CO2})} \qquad (5)$$

Using Boyle's Law,

$$pv = \text{Constant}$$

$$p_i v_{CH4} = p_{CH4} v_i \tag{6}$$

Substitute equation (6) in (5);

$$v_{CH4} = \frac{v x^{o}_{N2} x_{CH4}}{(1 - x_{CH4} + x_{CO2})} \tag{7}$$

where,

v_{CH4} = Volume of methane in ml

x^{o}_{N2} = Initial composition of N_2 gas

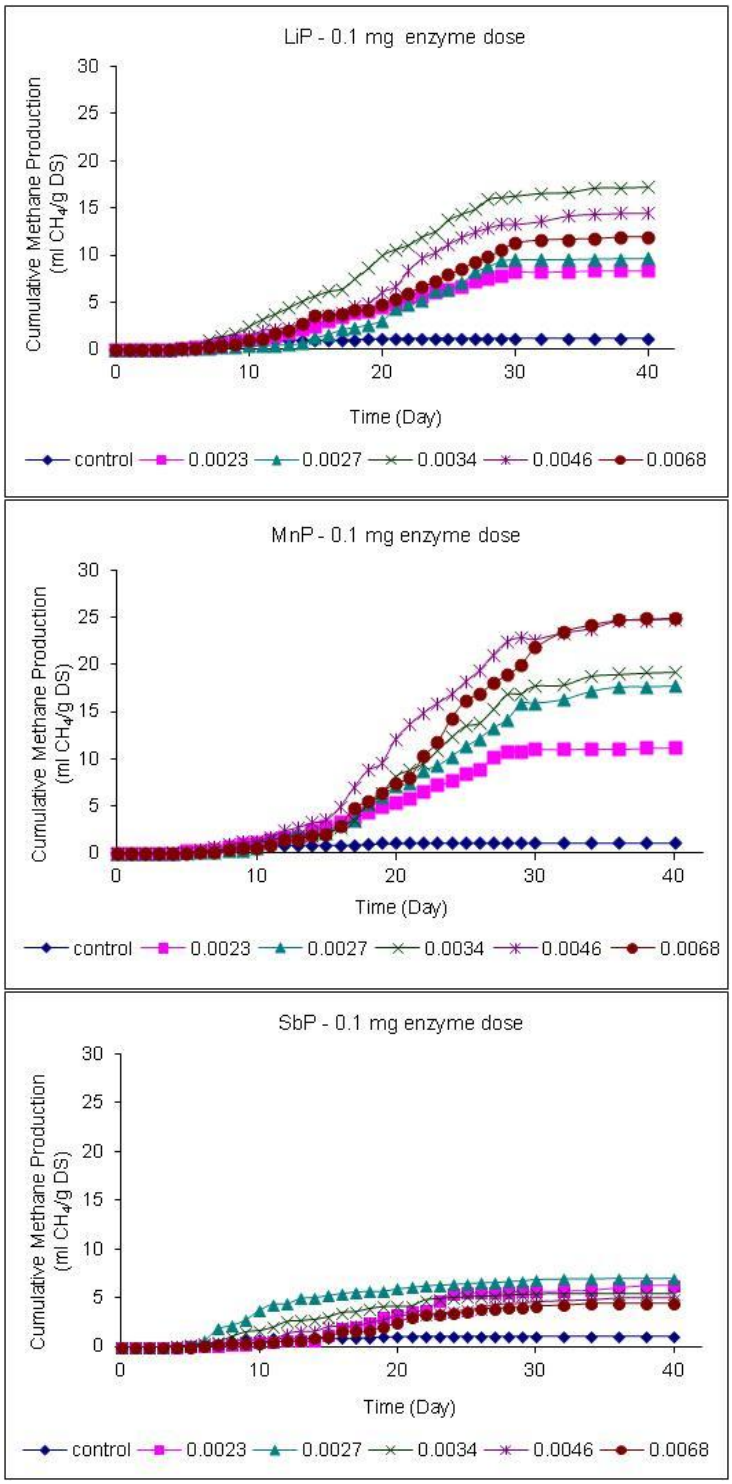
v = Volume of gas phase (125-15 = 110 ml)

x_{CH4}, x_{CO2} = mole fractions of methane and carbon dioxide at time t

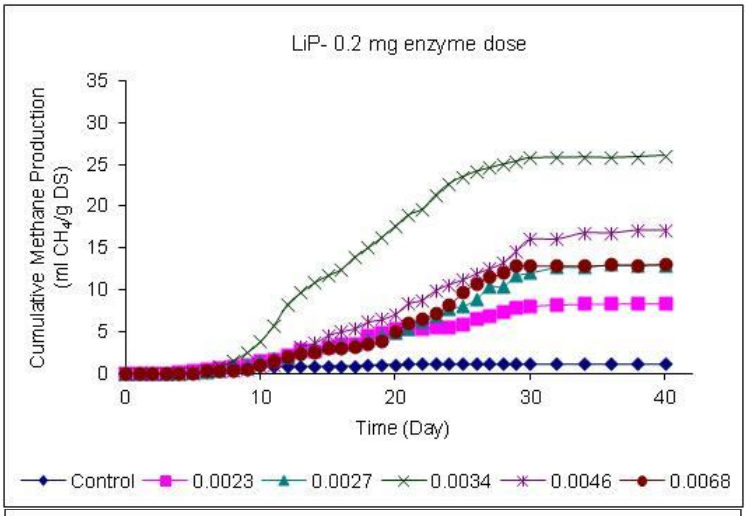
Net production of N_2 can be considered negligible in relation to CH_4 production.

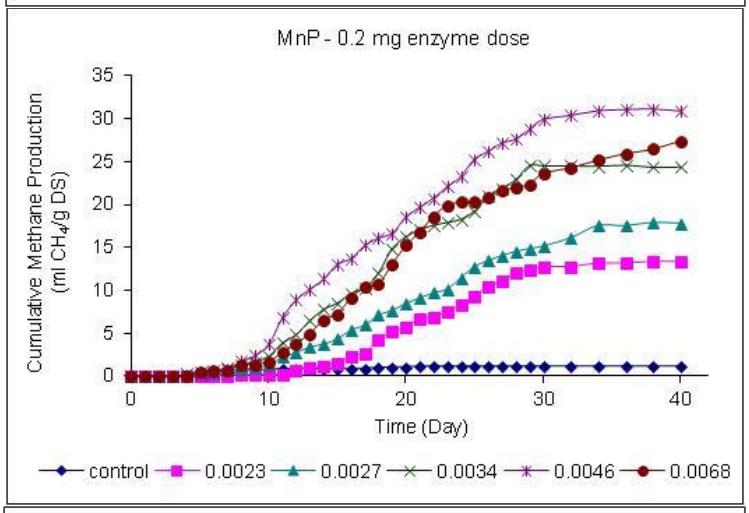
2) Cumulative methane production for different E:H_2O_2 ratio at different enzyme doses

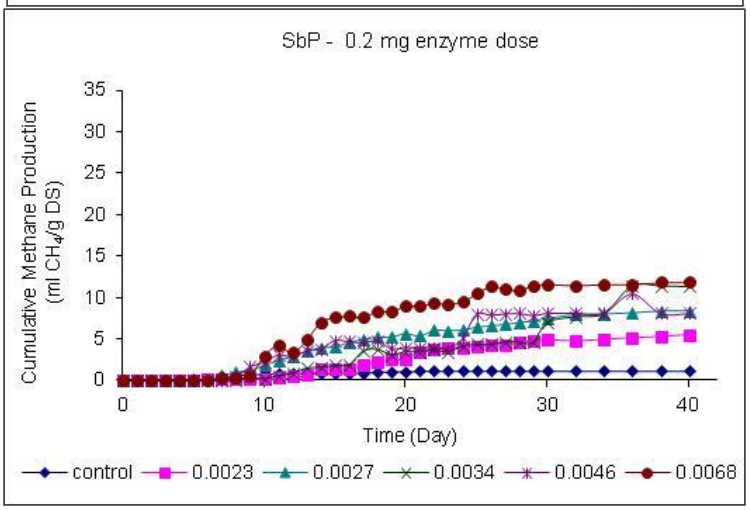
Cumulative methane production over time for different E:H_2O_2 ratio at 0.1 mg enzyme dose



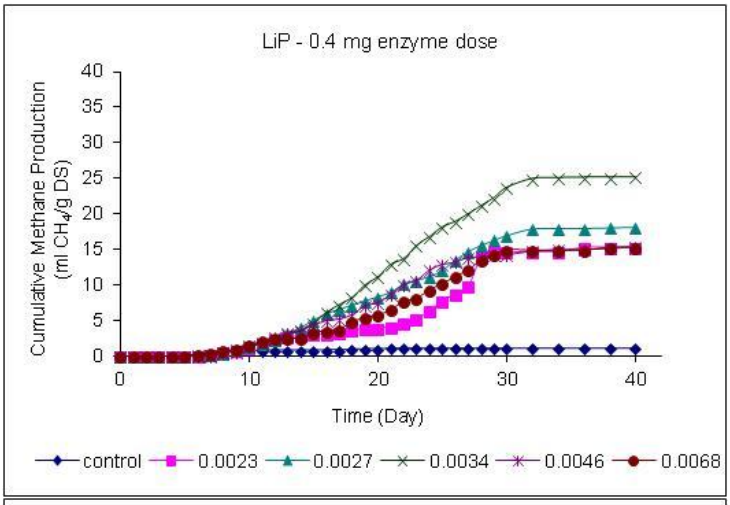
Cumulative methane production over time for different E:H_2O_2 ratio at 0.2 mg enzyme dose

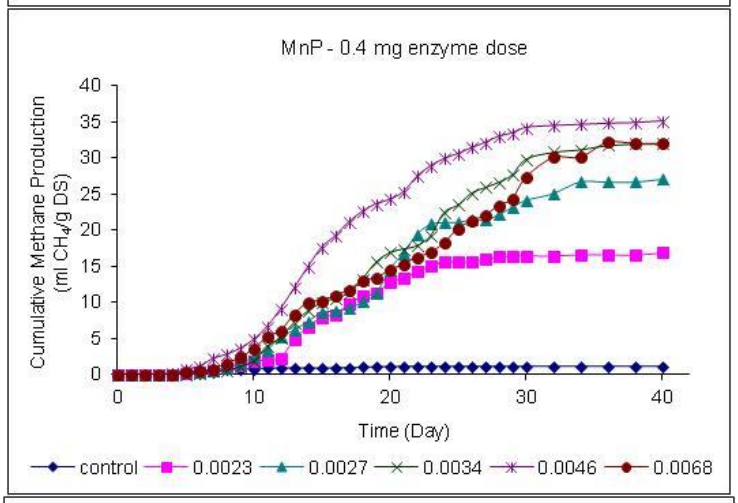


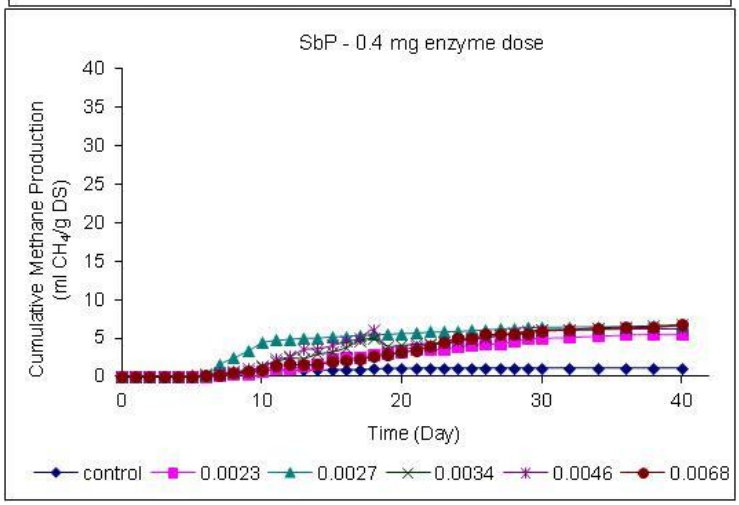




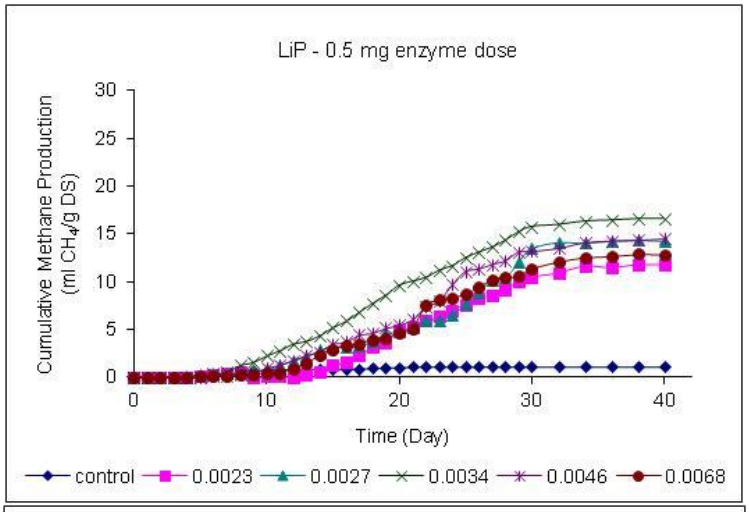
Cumulative methane production over time for different E:H_2O_2 ratio at 0.4 mg enzyme dose

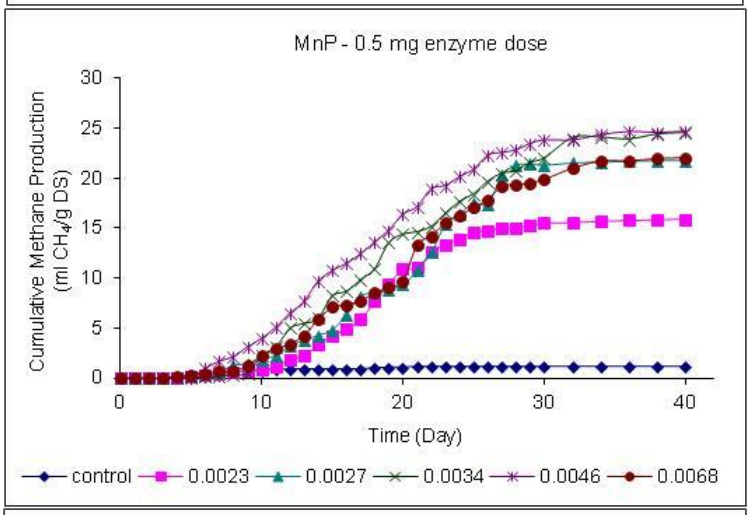


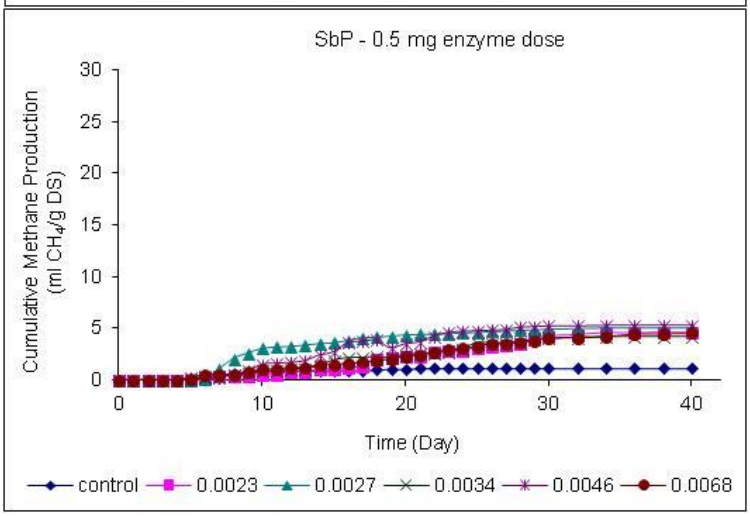




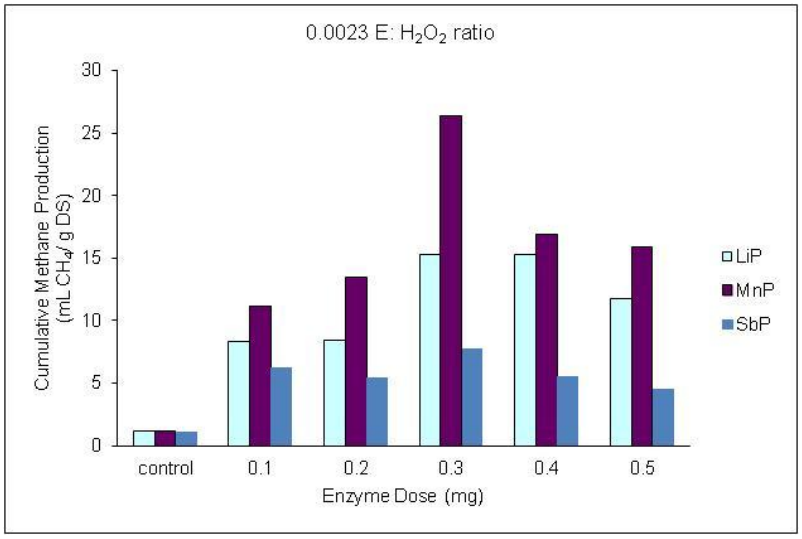
Cumulative methane production over time for different E:H_2O_2 ratio at 0.5 mg enzyme dose

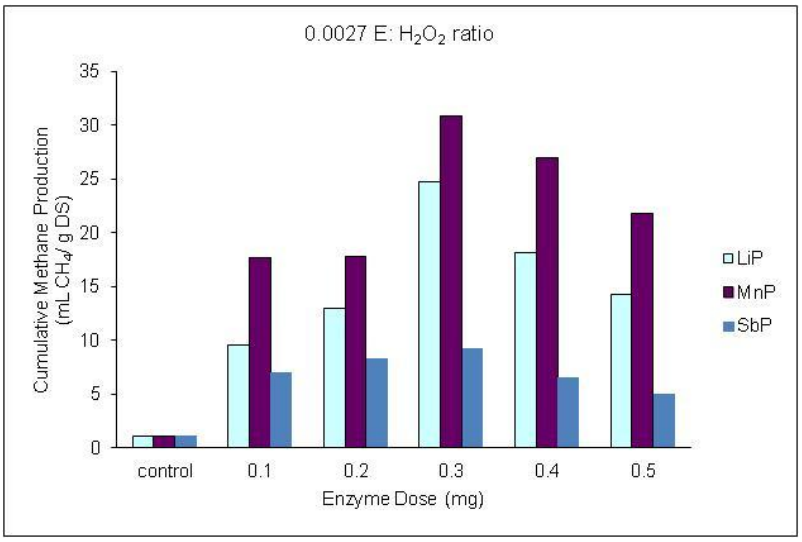


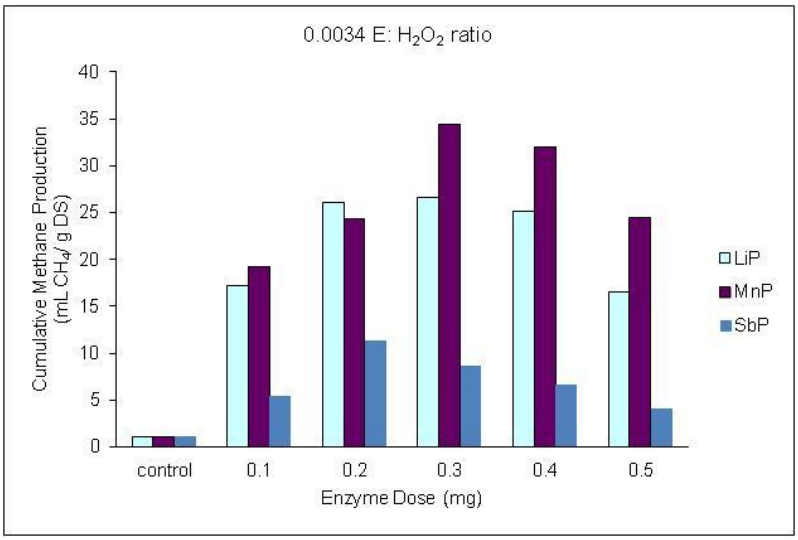


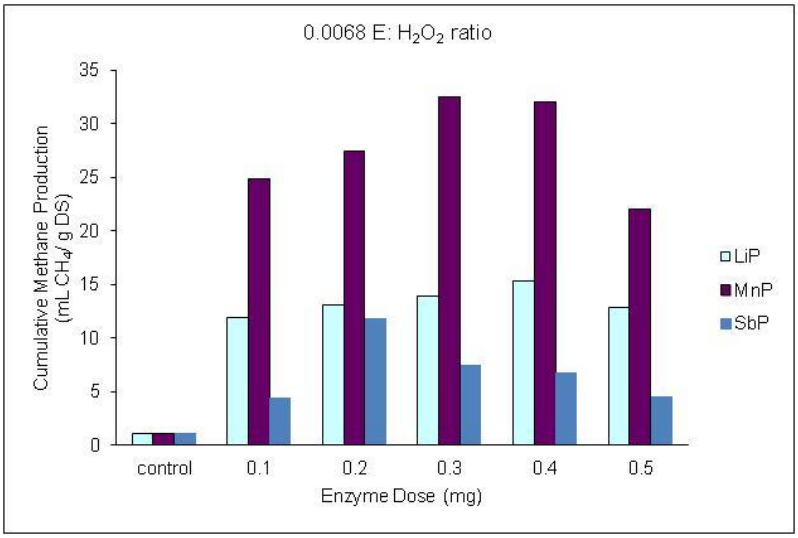


3) Effect of enzyme type on gas production at different levels of enzyme doses at different E:H_2O_2 ratio

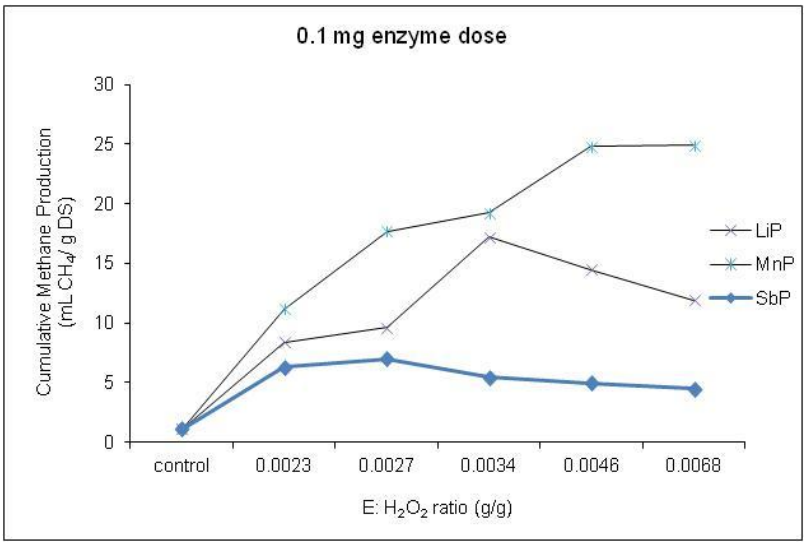


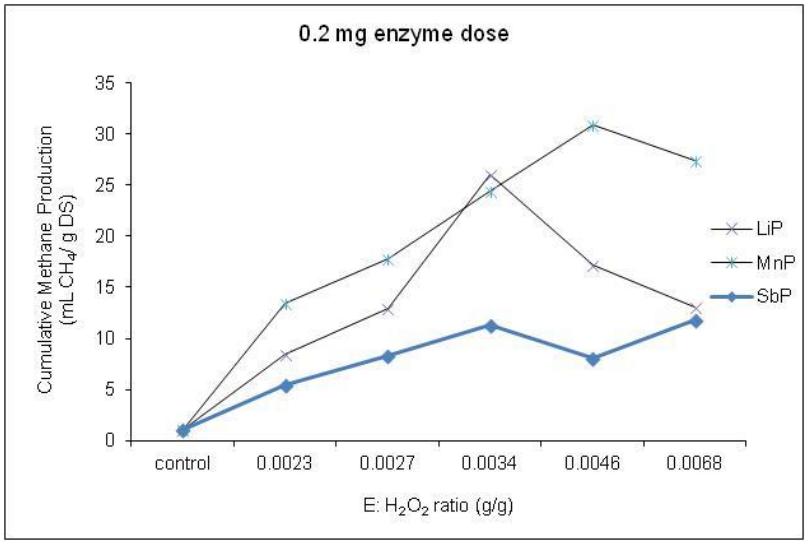


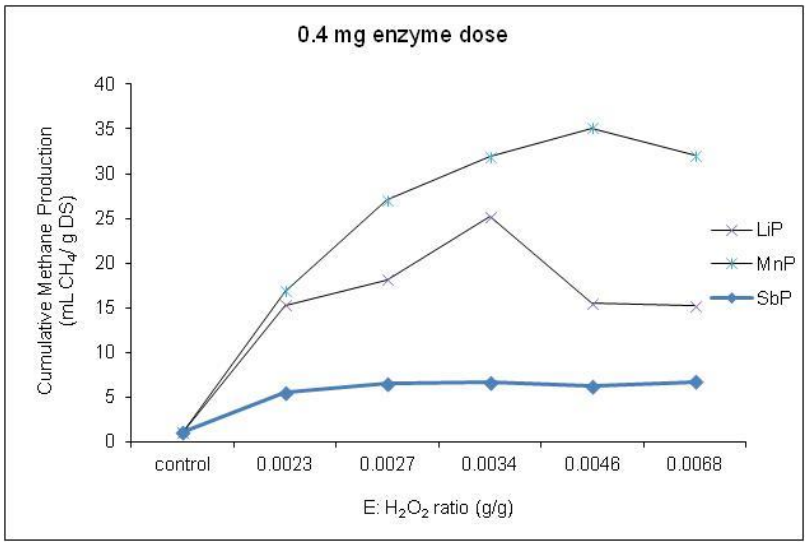

0.0034 E: H2O2 ratio
Cumulative Methane Production (mL CH4/ g DS)
40
35
30
25
20
15
10
5
0
control
0.1
0.2
0.3
0.4
0.5
Enzyme Dose (mg)
LiP
MnP
SbP

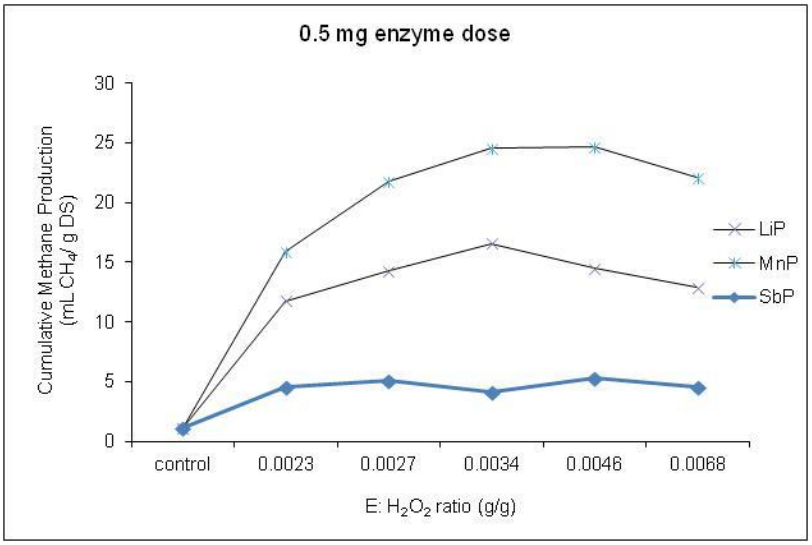

0.0068 E: H2O2 ratio
Cumulative Methane Production (mL CH4/ g DS)
35
30
25
20
15
10
5
0
control
0.1
0.2
0.3
0.4
0.5
Enzyme Dose (mg)
LiP
MnP
SbP

4) Effect of E:H_2O_2 ratio for different enzymes at different enzyme doses






0.4 mg enzyme dose
Cumulative Methane Production (mL CH_4/ g DS)
40
35
30
25
20
15
10
5
0
control
0.0023
0.0027
0.0034
0.0046
0.0068
E: H_2O_2 ratio (g/g)
LiP
MnP
SbP


0.5 mg enzyme dose
Cumulative Methane Production (mL CH_4/ g DS)
30
25
20
15
10
5
0
control
0.0023
0.0027
0.0034
0.0046
0.0068
E: H_2O_2 ratio (g/g)
LiP
MnP
SbP

5) ANOVA table for factorial experiments

R (response-cumulative methane production at day 40) versus A (enzyme type), B (enzyme dose), C (Enzyme:H_2O_2 ratio).

Factor	Levels	Values
A	3	LiP, MnP, SbP
B	5	0.1, 0.2, 0.3, 0.4, 0.5
C	5	0.0023, 0.0027, 0.0034, 0.0046, 0.0068

Source	Degree of freedom	Sum of squares	Mean square	$F_{calculated}$
A	(i-1)	SSA	MSA=SSA/(i-1)	F=MSA/MSE
B	(j-1)	SSB	MSB=SSB/(j-1)	F=MSB/MSE
C	(k-1)	SSC	MSC=SSC/(k-1)	F=MSC/MSE
A*B	(i-1)(j-1)	SSAB	MSAB=SSAB/(i-1)(j-1)	F=MSAB/MSE
A*C	(i-1) (k-1)	SSAC	MSAC=SSAC/(i-1)(k-1)	F=MSAC/MSE
B*C	(j-1) (k-1)	SSBC	MSBC=SSBC/(j-1)(k-1)	F=MSBC/MSE
A*B*C	(i-1) (j-1) (k-1)	SSABC	MSABC=SSABC/(i-1)(j-1)(k-1)	F=MSABC/MSE
Error	ijk(n'-1)	SSE	MSE=SSE/ijk(n'-1)	
Total	(n-1)	SST		

i= number of levels of factor A

j= number of levels of factor B

k= number of levels of factor C

n' = number of replications for each combination of factors A, B, and C

n= number of total observations

$$SSA = jkn' \sum_{i=1}^{3} (\overline{X_i} - \overline{\overline{X}})^2$$

$$SSAB = n' \sum_{i=1}^{3} \sum_{j=1}^{5} (\overline{X_{ij}} - \overline{X_i} - \overline{X_j} + \overline{\overline{X}})^2$$

$$SSE = \sum_{i=1}^{3} \sum_{j=1}^{5} \sum_{k=1}^{5} \sum_{n'=1}^{3} (\overline{X_{ijkn'}} - \overline{X_{ijk}})^2$$

$$\overline{\overline{X}} = \frac{\sum_{i=1}^{3} \sum_{j=1}^{5} \sum_{k=1}^{5} \sum_{n'=1}^{3} X_{ijkn'}}{3*5*5*3} \qquad \overline{X_i} = \frac{\sum_{j=1}^{5} \sum_{k=1}^{5} \sum_{n'=1}^{3} X_{ijkn'}}{5*5*3} \qquad \overline{X_{ij}} = \frac{\sum_{k=1}^{5} \sum_{n'=1}^{3} X_{ijkn'}}{5*3}$$

6) F Table:

CRITICAL VALUES OF F

For a particular combination of numerator and denominator degrees of freedom, entry represents the critical values of F corresponding to a specified upper tail area (α)

Denominator d.f.$_2$	Numerator, d.f.$_1$																			
	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	161.4	199.5	215.7	224.6	230.2	234.0	236.8	238.9	240.5	241.9	243.9	245.9	248.0	249.1	250.1	251.1	252.2	253.3	254.3
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38	19.40	19.41	19.43	19.45	19.45	19.46	19.47	19.48	19.49	19.50
3	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79	8.74	8.70	8.66	8.64	8.62	8.59	8.57	8.55	8.53
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96	5.91	5.86	5.80	5.77	5.75	5.72	5.69	5.66	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74	4.68	4.62	4.56	4.53	4.50	4.46	4.43	4.40	4.36
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06	4.00	3.94	3.87	3.84	3.81	3.77	3.74	3.70	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64	3.57	3.51	3.44	3.41	3.38	3.34	3.30	3.27	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35	3.28	3.22	3.15	3.12	3.08	3.04	3.01	2.97	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14	3.07	3.01	2.94	2.90	2.86	2.83	2.79	2.75	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98	2.91	2.85	2.77	2.74	2.70	2.66	2.62	2.58	2.54
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90	2.85	2.79	2.72	2.65	2.61	2.57	2.53	2.49	2.45	2.40
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80	2.75	2.69	2.62	2.54	2.51	2.47	2.43	2.38	2.34	2.30
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71	2.67	2.60	2.53	2.46	2.42	2.38	2.34	2.30	2.25	2.21
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65	2.60	2.53	2.46	2.39	2.35	2.31	2.27	2.22	2.18	2.13
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54	2.48	2.40	2.33	2.29	2.25	2.20	2.16	2.11	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49	2.42	2.35	2.28	2.24	2.19	2.15	2.11	2.06	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49	2.45	2.38	2.31	2.23	2.19	2.15	2.10	2.06	2.01	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46	2.41	2.34	2.27	2.19	2.15	2.11	2.06	2.02	1.97	1.92
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42	2.38	2.31	2.23	2.16	2.11	2.07	2.03	1.98	1.93	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35	2.28	2.20	2.12	2.08	2.04	1.99	1.95	1.90	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37	2.32	2.25	2.18	2.10	2.05	2.01	1.96	1.92	1.87	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34	2.30	2.23	2.15	2.07	2.03	1.98	1.94	1.89	1.84	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32	2.27	2.20	2.13	2.05	2.01	1.96	1.91	1.86	1.81	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30	2.25	2.18	2.11	2.03	1.98	1.94	1.89	1.84	1.79	1.73
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28	2.24	2.16	2.09	2.01	1.96	1.92	1.87	1.82	1.77	1.71
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27	2.22	2.15	2.07	1.99	1.95	1.90	1.85	1.80	1.75	1.69
27	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.25	2.20	2.13	2.06	1.97	1.93	1.88	1.84	1.79	1.73	1.67
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24	2.19	2.12	2.04	1.96	1.91	1.87	1.82	1.77	1.71	1.65
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22	2.18	2.10	2.03	1.94	1.90	1.85	1.81	1.75	1.70	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	2.09	2.01	1.93	1.89	1.84	1.79	1.74	1.68	1.62
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08	2.00	1.92	1.84	1.79	1.74	1.69	1.64	1.58	1.51
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99	1.92	1.84	1.75	1.70	1.65	1.59	1.53	1.47	1.39
120	3.92	3.07	2.68	2.45	2.29	2.17	2.09	2.02	1.96	1.91	1.83	1.75	1.66	1.61	1.55	1.50	1.43	1.35	1.25
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88	1.83	1.75	1.67	1.57	1.52	1.46	1.39	1.32	1.22	1.00

Source: Reprinted from Pearson, E. S. and H. O. Hartley, eds., Biometrika Tables for Statisticians, *3d ed.; 1966; by permission of the* Biometrika *Trustees,* London