CHARACTERIZING MICROBIAL POPULATION DYNAMICS DURING THE INITIAL STAGES OF COMPOSTING

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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May 2008

ABSTRACT

Composting is a solid state fermentation process that employs the use of complex relationships in microbial communities for the degradation of organic matter. By studying the process of composting, solid state fermentation, as a process may be better understood and characterized for the development of models in predicting scale up behavior. In addition, the study of compost microbial community provides essential information about the microbial community evolution and how this evolution drives changes in physical variables such as temperature, pH and oxygen concentration.

In this study, 3 reactors were designed and built to a working volume of 50L. Each reactor was equipped with 7 axial sampling ports up to a height of 0.7m above a perforated plate used to hold the substrate bed, with a 10cm interval between ports. The reactors were loaded with a mixed substrate of dog food and switchgrass with a C/N ratio of 15/1, with each reactor being started off at different moisture contents: 70%, 65% and 60% moisture content wet basis. This is accomplished by keeping a constant dry weight for the substrates and varying the volume of water added. The reactors were then allowed to run for at least 96 hours. Temperature and effluent gas concentrations were monitored online for the duration of the process and at pre-defined times, samples were collected and analyzed for pH using a pH meter, moisture content using oven drying and yeast, bacteria and fungi content using probe hybridization.

The results showed that moisture content is a key factor in determining the microbial community evolution, with the highest moisture content reactor generally producing the largest amount of detectable microbial DNA initially and at most points throughout the process. The temperature, effluent gas and pH profiles were also

consistent with the trends observed for the microbial community data. Furthermore, it was observed that fungi were detected at all sampled points during the process, suggesting that fungi play an important role in the composting process.

BIOGRAPHICAL SKETCH

Linelle Fontenelle was born in the small community of Grace on the island of St. Lucia. She is the third child of four siblings and the proud aunt of one niece and one nephew. She came to the United States by way of Florida tin 2000 to pursue a higher education. She completed her Associate's degree at the Central Florida Community College and then moved to New York City to complete her Bachelor of Engineering degree at the City College of New York. Linelle loves to travel and has taken on the challenge of visiting a new country each year. She also loves the outdoors: hiking, visiting waterfalls and beach activities like kayaking and snorkeling. Linelle also loves New York City because of its rich cultural and ethnic diversity.

To my parents: Jacinta & Felix Fontenelle

ACKNOWLEDGMENTS

I would like to first of all thank God for bringing me to this point in my life and for providing me with the guidance that I need (almost sounds like I'm accepting a Grammy ☺; maybe in my next lifetime). My parents and the rest of my family also need to be thanked for their continued support and their never-ceasing belief in me. I would also like to thank my advisor Larry Walker for taking me under his wing and nurturing me in my growth and development as a researcher. Stephane Corgie also needs to be thanked for guiding me through the molecular biology aspects of this project and for being supportive. In need of great recognition is Doug Cavney who taught me how to use the tools in the machine shop and provided me with any other support that I needed very willingly. I would also like to thank the other members of the Walker Lab for their support and encouragement and the New York State Foundation for Science, Technology and Innovation (NYSTAR) for providing funding for my work.

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Chapter 1

Introduction

Solid state fermentation (SSF), which is the growth of microorganisms on a moistened substrate in the absence of free flowing water, is commonly exemplified via the process of composting. SSF has been used for thousands of years, particularly in Asian countries, in the production of food products such as soy sauce [3]. SSF holds many advantages over traditional submerged fermentation, including cheaper production, higher product yields and lower energy requirements. SSF is more economically viable, by a factor of 100, over submerged fermentation for cellulose production [5]. Despite these advantages, SSF, has not; however, become a competitive technology in the western hemisphere because of the many engineering challenges associated with the process. As the microbes grow on the moistened solid substrate, their demand for oxygen and other nutrients changes, producing concentration gradients that contribute to overall mass transfer in the system. In addition, heat, which is a byproduct of the process is generated and must be properly managed for the best process efficiency. This leads to heat transfer aspects within the system which must be properly controlled. These engineering aspects greatly hinder the reproducibility, scale-up and standardization of SSF. Thus, there is a strong need to be able to predict SSF reactor behavior given a set of initial conditions.

In this study, composting was the studied SSF process. Composting is useful for the reduction of municipal solid wastes and for the destruction of potentially hazardous pathogenic organisms. In our research group, where one of the main goals is the sustainable production of cellulosic biofuel, the proper disposal of cellulosic wastes is an essential step. Composting of the agricultural wastes produces a

biologically stable humic substance that may be used as a soil additive, thereby efficiently reducing the amount of waste generated. In composting, microbial activity and growth lead to the degradation of organic material. This degradation occurs via the formation of complex microbial communities that work in a delicate balance to drive changes in the temperature and pH of the compost pile. Advances in molecular ecology methods such as probe hybridizations and genetic fingerprinting methods provide the tools necessary for monitoring the dynamics of microbial communities. The overwhelming majority of studies done on the microbial population of composts; however, have been qualitative in nature and this study represents the first of its kind to present quantitative data on multiple major groups present in a compost pile. Therefore the main objectives of this study are to:

- Design highly instrumented 50L-working volume compost reactors
- Reach thermophilic conditions in the compost pile within 96 hours
- Track temporal and spatial variations in temperature, pH, moisture content and yeast, fungi and bacterial microbial groups
- Determine the impact of initial moisture content on reactor performance
- Optimize a protocol for the extraction of DNA from compost samples
- Optimize the detection of bacteria, fungi and yeast from compost samples
- Quantify bacteria, fungi and yeast populations during the initial stages of composting by using probe hybridization

By obtaining quantitative information about the microbial community of a compost pile, such data may, in the near future, be incorporated into a model to understand and predict reactor performance. In the short term, this quantitative data may be used to calculate an empirical heat generation term in an energy equation, but in the long term, may be used on its own to turn molecular ecology into a predictive science that directs environmental biotechnological processes.

Chapter 2

Literature Review

2.1 Solid State Fermentation

2.1.1 General

2.1.1.1 Definition and Uses

The growth of microorganisms on a moistened solid substrate in the absence of free flowing water describes the technological process known as solid state fermentation (SSF). It is an old and familiar technology that dates back to the year 1000 BC when it was used to produce soy sauce [6] and since then, SSF has evolved as a reputable method for a broad range of applications particularly in Asian countries [5]. SSF is characterized by moisture contents in the range of 40% to 80% [7], with the water being held within the substrate particles where it supports microbial growth.

SSF has been used in numerous applications; such as, the production of enzymes, bio-control agents, organic acids, antibiotics, biopesticides, biofuels and a broad range of food products [3, 4]. SSF is also being used for the development of bioprocesses such as biopulping, bioremediation and biodegradation of hazardous compounds [8]. Furthermore, highly aerated SSF processes are employed around the world today in the composting of organic wastes yielding a stabilized soil amendment.

Optimal design and operation of mixed culture SSF is challenging given the high coupling of physical state variables; such as, temperature, moisture content, and the complex biochemical activities associated with the microbial ecology of the organic matrix. Thus, SSF technology faces many engineering challenges including heat and mass transfer limitations, reproducibility and standardization. These

challenges must be overcome before SSF may be viewed as a leading technology in the western world [5].

2.1.1.2 Advantages over Submerged Fermentations

In the Western world, submerged liquid fermentations have surpassed the use of SSF as a viable technology because of the lack of reproducibility of results [5]. Submerged fermentations are easier to control and thus result in a standardization of products fermented under very controlled conditions. Poor process control (temperature, pH, oxygen concentration) and longer process times characterize SSF reactors due to the heterogeneity of the substrate and the reaction environment [9]. Furthermore, this leads to major difficulties in reactor scale up.

Despite the challenges associated with SSF, this technology offers several advantages over submerged fermentation. For example, the estimated cost of production of cellulases via SSF is US \$0.20 kg^{-1}, whereas the cost rises to US\$20 kg^{-1} when using submerged fermentations [10]. In another study, tannase enzyme activity, using *A. niger* Aa-20 as the microorganism, increased from 2,500 IU L^{-1} in submerged fermentation to 12,000 IU L^{-1} in SSF [10]. More specifically, the advantages of SSF over submerged fermentations include:

(a) Lower capital investments and operating costs as a result of the simplicity of the required equipment with regard to design and spatial requirements [9].
(b) Higher product yields per unit reactor volume [9]. In the case of exo-pectinase, its production was found to be 50 times higher in SSF when compared to submerged fermentation, using *A.niger* CH4 as the microorganism [10].

(c) Lower risk of contamination from bacteria and yeast due to the low moisture content [11]. It has been found that the degradation of enzymes by contaminating proteases is 8 times higher in submerged fermentation than in SSF [10].

(d) Lower energy demands because of the lower requirements for aeration, water and mechanical agitation [9]

(e) Required carbon sources are less expensive and more abundant - agricultural residues are often used as raw materials [9].

(f) Production of enzymes that are more stable at higher temperature and extreme pH [12]

(g) The use of mixed cultures to take advantage of synergistic effects during growth [10]

2.1.2 Solid State Fermentation Operating Conditions

Environmental variables such as temperature, moisture and pH dictate the conditions under which a solid state reactor is operating and thus, play an essential role in determining microbial growth and the nature of the product formed. The effects of these operating conditions will be discussed in this section.

2.1.2.1 Moisture Content

Understanding and managing the transport of moisture within a solid state reactor is essential for the manipulation of an SSF process. The moisture content of the substrate is a key factor in determining the synthesis and secretion of desired products via involvement in the SSF processes of enzymatic action, biomass growth, and

nutrient and gas transport [13]. If the moisture content is too low, some microorganisms will not have enough water to sustain growth. In addition, substrate swelling, which is necessary for microbial activity on the substrate, will not occur. On the other hand, if the moisture content is too high, the void spaces within the solid matrix will be filled with water that will inhibit the transfer of oxygen to the substrate particles and the transfer of metabolic products such as carbon dioxide away from the substrate particle and could also foster contamination of the solid matrix. Thus, an optimum moisture content must be found to maximize the output of a given SSF application such as enzyme production [14] or microorganism growth. While many microorganisms are able to grow on solid substrates, bacteria and yeasts grow on solid substrates containing 40 – 70% moisture, whereas filamentous fungi can grow extensively in low moisture environments.

The effect of moisture content in a given SSF system is best described using the term, water activity [11, 13, 15, 16]. Water activity, a_w, is defined at a given temperature using the following relationship:

$$a_w = \frac{P_s}{P_o} \tag{2.1}$$

where P_s is the vapor pressure of water in equilibrium with the substrate and P_o is the vapor pressure of pure water [9, 11]. For a microorganism, this is the amount of water available to it in its immediate surroundings for microbial activity [13]. Typical water activity values for solid substrates fall in the range of 0.95 – 0.98 [3]. Studies have shown that small changes in the value of the water activity of a given system can noticeably affect the growth and activity of microorganisms [14, 16]. Water activity is; however, challenging to measure and is seldom reported in the literature; so moisture content values are typically used.

Variations in the water activity of an SSF process will occur during fermentation as a result of water evaporation through mass transfer and water production via metabolism. Also, high aeration rates can lead to significant drying of the organic matrix and a reduction in microbial activity; thus, to minimize drying, reactors are often aerated with saturated air [16-18] or water should be added periodically [19, 20] to the substrate bed.

2.1.2.2 Temperature

Temperature is arguably the most critical factor in any SSF process. It influences the type of metabolic product formed, the rate and extent of substrate degradation and product formation, the growth and metabolism of microorganisms, and the extent of heat and mass transfer processes [3, 9, 11, 21].

Temperature variations occur within an SSF reactor as a result of the buildup of heat produced from the metabolic activity of microorganisms. The heat accumulates because of the challenges associated with heat removal. One of the most common difficulties is that of uneven heating of the substrate matrix, resulting in the development of radial and axial temperature gradients[22]. Radial temperature gradients may be overcome by enclosing the fermenters in insulation and axial gradients are commonly combated through the forced aeration of saturated air through the substrate bed [2, 9, 11, 22]. Another strategy would be to regularly mix the substrate bed [20].

Despite the difficulties associated with its regulation, temperature is the easiest SSF variable to monitor online and is arguably the most controllable. Several studies have been carried out where temperature was manipulated to optimize product growth or activity [23-25]. In these studies, the isothermal approach was used –

several fermentations were carried out at a range of temperatures, with each reactor being held at a constant temperature throughout. In addition, other studies have been done to model the temporal and spatial temperature patterns for a given reactor system [18, 23, 24, 26, 27], in order to make the system more predictable for scale up.

One such model [23] looked at the temporal distribution of temperature to determine whether maximum temperatures could denature growing enzymes. The model was developed for a well-mixed bioreactor and is described by the following equation:

$$\frac{dT}{dt} = \frac{F_a C_{pa}(T_a - T) + F_a\left(\lambda(H_a - H) + C_{pv}(H_a T_a - HT)\right) - hA(T - T_w) + BY_Q\frac{dX}{dt}}{C_{pb}B(1+W)} \qquad (2.2)$$

Where:

T = Bed temperature (°C)

T = time (hr)

F_a = Dry air flow rate (kg-dry-air hr^{-1})

C_{pa} = Dry air heat capacity (J kg-dry-air^{-1}°C^{-1})

T_a = Temperature of the air at the air inlet (°C)

λ = Enthalpy of evaporation of water at 0 °C (J kg-vapor^{-1})

H_a = Inlet air humidity (kg-vapor kg-dry-air^{-1})

H = Outlet air humidity (kg-vapor kg-dry-air^{-1})

C_{pv} = Water vapor heat capacity (J kg-vapor^{-1}°C^{-1})

h = Bioreactor wall heat transfer coefficient (J $hr^{-1}m^{-2}$°C^{-1})

A = Bed/cooling water jacket contact area (m^2)

T_w = Temperature of the cooling water (°C)

B = Total mass of solids in the bed (kg)

Y_Q = Heat yield from growth (J kg-biomass^{-1})

X = Biomass content of the solid substrate (kg-biomass kg-IDS^{-1})

C_{pb} = Moist substrate bed heat capacity (J kg-solid^{-1}°C^{-1})

W = Water content of the bed (kg-water kg-IDS^{-1})

Although this model was able to mimic for the most part the general temperature trends observed during SSF processes, it was not compared to a specific experiment to numerically evaluate its accuracy. The model also did not incorporate changes in the moisture content of the bed due to evaporation. The major assumptions used in this model were that the air leaving the reactor was in equilibrium with the solid bed that the microorganism growth did not affect the properties of the solid bed. This second assumption is surprising because the properties of the bed will change once microbes begin to grow on the substrate. Despite the shortcomings of this model, it is a good tool for predicting whether enzymes will denature during an SSF process under a given set of conditions.

Another model [18] developed specifically for temperature prediction in compost, employed an empirical heat generation term to better capture microbial heat production. This model, developed from an analytical solution is of the form:

$$\bar{T} = \frac{1}{c}\ln\left[\left(\frac{a'-a)}{a'}\right)e^{\theta a' c t} + \frac{a}{a'}\right] \qquad (2.3)$$

Where:

$\bar{T} = T - T_o$, where T = temperature and T_0 = initial temperature

c = Regression constant

a = Regression constant

$a' = a + RO_{2,0}$, where $RO_{2,0}$ is the rate of oxygen depletion at time and position 0

θ = Empirically determined coefficient

t = time

The assumptions used in this model included constant moisture content throughout the process, as well as a constant mass flux. Although this model produces temperature profiles that follow reasonably well with empirical data, some information was lost in the model because of the many simplifying assumptions that were made to obtain an analytical solution.

The temperature models presented above and the others previously mentioned are; however, substrate specific and so there does not exist one overarching model that will predict the temperature profile for any SSF system because of the difficulty associated with determining the amount of heat generated as a result of microbial activity [18].

2.1.2.3 Aeration

The necessity of oxygen (O_2) for high-rate microbe growth and activity dictates that the substrate bed must be continuously and uniformly oxygenated for optimal results. One way of achieving this is to periodically mix the substrate bed; however, it has been found that a continuous air supply is a more efficient way of providing oxygen to the solid matrix [15, 22]. Forced aeration of saturated air not only provided O_2 to the reactor bed, but also helped to sustain humidity requirements and to evenly dissipate some of the metabolic heat and gaseous metabolites generated.

As with the moisture content, an optimal aeration rate must be maintained to optimize microbial growth and activity. When the aeration rate is too low, mass transfer processes become limited and consequently either: slows down the growth of microorganisms if the flow rate is really low or generates more heat if the low flow rate is still able to sustain metabolic activity [22]. On the other hand, if the rate of

aeration is too high, the substrate bed will become dry as a result of rapid evaporation of water from the bed. This desiccation can lead to lower metabolic activity and dramatic changes in the microbial population and composition of the substrate bed. Aeration values vary in the literature from low values of 8L/min (200-L bioreactor) [28] to high values of 100L/min (770-L bioreactor) [18]. One way of maximizing the effects of extreme aeration rates is by using discontinuous flow rates [29]. In this method, the aeration rate is kept to a minimum at the beginning of the fermentation when the temperature is low and a high flow rate is applied later on in the process when the temperature has risen to a given set point.

2.1.2.4 pH

Changes in pH during SSF provide another indication of the activity of microorganisms within the system. These changes are associated with the hydrolysis of proteins in the substrate and the production of ammonia and organic acids at different stages in the process [13, 30]. Although pH is difficult to measure online because of the lack of free water, it is an important factor in determining the optimum conditions for product growth, particularly for enzymes. Buffers and other pH correcting agents, such as urea and ammonia, may be added to the solid substrate in an attempt to control the pH, but this is still not very effective at eradicating pH gradients from the system [11].

Typically, when cellulosic substrates are used, the initial pH is in the range of 4.5 – 6.5 [9]. Some studies have found that as fermentation proceeds, pH values quickly become basic in the initial phase as a result of the production of ammonia (NH_3) [30]; while other work has found that the pH becomes more acidic for a brief period of time before it rapidly becomes basic [31]. At that point, the pH either

stabilizes at a basic pH value around 9.1 [30] or the pH may become more basic as a result of a shift from bicarbonate alkalinity to hydroxide alkalinity due to carbon dioxide stripping [31]. However, the completed studies agree that different initial treatments such as different aeration rates have little effect on the pH trends [30, 31].

2.1.2.5 Substrate

The desired result of a given SSF process is the determining factor in the choice of substrate used. This selection is critical because the substrate will be the provider of nutrients and physical support for the growth of microorganisms. Listed in Table 2.1 are some substrates that have been used with various microorganisms and the products formed, while in Table 2.2, the chemical composition of some frequently used substrates are listed.

From Table 2.1, it is seen that different substrates can make the same product and that the same substrate can make different products. This is due to the effect of the nature of the inoculum added to the fermentation process. Inoculation provides the seed organism or organisms that will initiate substrate decomposition. *Aspergillus niger* is a common organism used to act on a variety of substrates in SSF. Table 2.3 lists some microorganisms and the SSF processes in which they are involved.

The chemical composition of substrates, as shown in Table 2.2 is important because it shows what inorganic nutrients are available to support microbial growth. In composting systems, nitrogen is the most closely examined nutrient because it is needed in high concentrations for cellular synthesis [32]. Also of importance is the carbon content of the substrate because the largest fraction of organic molecules in the cell is made up of carbon. For this reason, the carbon/nitrogen (C/N) ratio is often used as an important parameter in SSF systems to estimate the level of degradability of a substrate. In aerobic processes, microbes require C/N ratios of 15 to 30 [32].

Table 2.1 Substrates associated with various SSF products or processes [3]

Substrate	Microorganism	Product or Process
Alkali-treated sugar cane	*Trichoderma reesei* & *A. niger*	Cellulases
Composted chicken manure	*Trichoderma vierns*	Bioherbicide
Corn cob	*Phanerachaete chrysosporium*	Lignolytic enzymes
Dry coffee husks	*Aspergillus niger*	Citric acid
Eucalypt wood	*Phlebia radiate or Funalia trogii*	Biopulping pretreatment
Ground soybeans	*Bacillus subtilis*	Pyrazines
Impregnated hemp	*Coniothyrium minitans*	Bioinsecticide
Impregnated spent malt grains	*Xanthomonas campestris*	Xanthan gum
Impregnated sugar cane bagasse	*Aspergillus tamarii*	Caffeine removal
Municipal solid waste	*natural consortia*	Methane
Pineapple waste	*Aspergillus niger*	Citric acid
Rice bran	*Aspergillus niger*	Glucoamylase
Rice flour	*Colletotrichum truncatum*	Bioinsecticide
Sawdust	*Lentinula edodes*	Shiitake
Soy bran and wheat bran	*Aspergillus niger*	Pectinases
Steamed rice	*Aspergillus oryzae*	Kojic acid
Sugar cane bagasse pith	*Phanerochaete chrysosporium*	Bioremediation

Table 2.1 (Continued)

Sugar beet pulp or citrus waste	*Neurospoa sitophila*	Protein enrichment
Various fruit peel	*Saccharomyces cerevisiae*	Fermented food
Wheat bran	*Aspergillus oryzae*	Iturin

Table 2.2 Nutritional composition of common agricultural wastes used as substrates in SSF [4]

Substrate	C:N	Ca (%)	P (%)	Mg (%)	K (%)	S (%)	Na (%)	Cl (%)	Protein (%)	Fat (%)	Fiber (%)
Corn flour	34.0	0.02	0.31	0.09	0.26	0.12	0.03	0.05	8.8	3.6	2.3
Millet	26.1	0	0.3								
Rice bran	24.0	0.08	1.4	0.95	1.74	0.18	0.03	0.07	12.9	1.5	12.9
Rice husk	92.6										
Sorghum	27.4	0.03	0.3						8.9	2.8	2.2
Soy Hulls	27.3	0.4	0.15	0.14	0.72	0.09	0.04	0			
Soy meal	6.6	0.2	0.6	0.25	1.8	0.33	0.03	0.07	48	0.9	3.4
Wheat bran	18.9	0.07	1.01	0.61	1.18		0.002		15.7	4	10
Wheat middlings	18.4	0.15	0.9	0.5	1.2	0.15	0.17	0.03	16	4.3	7.8
Wheat straw	88.7										

Table 2.3 Main groups of microorganisms involved in SSF processes [2]

Microflora	**SSF Process**
Bacteria	
Bacillus sp.	Composting, Natto, amylase
Pseudomonas sp.	Composting
Lactobacillus sp.	Ensiling, Food
Yeast	
Endomicopsis burtonil	Tape, cassava, rice
Saccharomyces cerevisiae	Food, Ethanol
Fungi	
Aspergillus sp.	Composting, Industrial, Food
Monilia	Composting
Beauveria sp.	Biological control, Bioinsecticide
Aspergillus oryzae	Koji, Food, citric acid
Aspergillus niger	Feed, Proteins, Amylase, citric acid
Penicilium notatum, roqueffortii	Penicillin, Cheese

Research has also shown that the amount of time required for composting, increases with increasing C/N ratios; however, if the nitrogen present is part of a nondegradable organic molecule, it would not be available for degradation regardless of the C/N ratio [32].

The advent of producing alternative fuels from lignocellulosic biomass has also inspired work with a variety of substrates that are energy crops, with special attention being paid to grasses. SSF technology provides promise of cheaper production and better hydrolytic efficiencies [33] of substrates for the production of biofuels.

2.2 Engineering Aspects of SSF

To make SSF a competitive technology in the Western world, it must be more predictable and controllable so that reactor scale up would be possible without destroying the reliability of the process or hindering the formation of the desired product. This section discusses the major engineering aspects which play a role in the design of large scale SSF processes.

2.2.1 Mass Transfer Aspects

The transfer of mass in SSF reactors plays a critical role in determining the growth of microorganisms. The materials being transferred include nutrients, gases and enzymes which are transported at two scales – the mirco-scale and macro-scale [22]. The macro-scale phenomena include the bulk flow of air into and out of the reactors, natural conduction, convection and diffusion in systems not employing forced aeration, cooling of the reactors by conduction and convection, and the effects of agitation on the substrate bed. At the micro-scale, transfer is dependent on the

growth mechanism of the microorganisms and is further subdivided at the particular level.

In aerated SSF reactors, the bulk flow of air serves to cool the solid matrix via evaporative cooling and to provide oxygen to growing microorganisms. The process of transferring oxygen from the bulk air flow to the cells of the microorganism takes place both at the inter- and intra-particular level. Other gases and nutrients in the system also undergo a similar mass transfer mechanism. At the inter-particular level, substances are transferred from the air-filled pores in the solid matrix to the growing microorganism whilst at the intra-particular level, substance are transferred within the solid matrix [22].

The effectiveness of mass transfer in any SSF system is dependent on the nature of the substrates and the materials being transferred. This is because concentration gradients of oxygen, enzymes, nutrients and products are developed as microorganisms grow on the substrate. Any change in these concentration gradients is a potential source for limiting mass transfer. For instance, if the void spaces within the solid matrix are mostly filled with water, as opposed to air, growth of the microorganisms will be hindered and thus the rate of product formation will be greatly decreased. To evaluate intra-particular mass transfer limitations, the effectiveness factor, η, may be used [7]. This factor, which has a maximum value of 1 is defined as:

$$\eta = \frac{r_{obs}}{r} \tag{2.4}$$

Where r_{obs} is the observed reaction rate with diffusion limitations and r is the reaction rate in the absence of any diffusion limitations.

2.2.2 Heat Transfer Aspects

The removal of waste metabolic heat is perhaps the most critical factor in large scale SSF processes and is limited by the heat capacity of the substrate [2]. In addition, the porosity and size of the substrate bed decreases as fermentation progresses [34], further limiting heat transfer. Traditional methods of conduction and convection provide limited heat removal capability for packed beds over 15cm in diameter [3] due to the poor thermal conductivity of most substrates. In aerated packed beds, axial convection and evaporation are the main heat transfer mechanisms, but need to be highly regulated to avoid drying the substrate bed. Periodic mixing, which distributes the generated heat more evenly across the substrate bed, would also need to be highly regulated to minimize mechanical damage to the microorganisms present.

The major difficulty associated with heat transfer is that it occurs at two different levels within the bioreactor – intra- and inter-particularly. The result of this is non-uniform localized heating that increases the temperature gradients in the system. Thus the rate of heat transfer is hampered by both local and global heat transfer rates and the rate at which heat is transferred between phases, for example, transfer from the particle surface to the gas phase [2]. Many studies done to model heat transfer aspects of SSF systems have assumed thermal equilibrium between phases in order to simplify the models [18].

The coupling of heat transfer with other SSF processes such as water transport and aeration further complicates the task of modeling this phenomenon and thus predicting its behavior in other systems. As a result of these numerous intricate relationships, many models have been simplified by ignoring one or more processes that contribute to the overall behavior of a given reactor system [35]. One of the more comprehensive studies has modeled a two-phase system that incorporates both heat

and water transfer [36]. Although including all these relationships increases the complexity of the model, it eliminates a frequently used assumption in these models: that the phases are in equilibrium. As a result of the different processes occurring in each phase, the authors described the energetics of the gas phase with the following equation [36]:

$$\varepsilon \cdot \rho_g \left(C_{Pg} + \varphi_g \cdot C_{Pv} \right) \frac{\partial T_g}{\partial t} + \left(C_{Pg} + \varphi_g \cdot C_{Pv} \right) \cdot G \frac{\partial T_g}{\partial z} = -ha(T_g - T_s) \qquad (2.5)$$

Where:

ε = Void fraction (m^3-void-space m^{-3})

ρ_g = Gas phase density (kg-dry-air m^{-3})

C_{Pg} = Dry gas heat capacity (J kg-dry-air^{-1} K^{-1})

φ_g = Water content of the gas phase (kg-water kg-dry-air^{-1})

C_{Pv} = Water vapor heat capacity (J kg-water-vapor^{-1} K^{-1})

T_g = Temperature of the gas phase (°C)

t = time (s)

G = Inlet air flow rate (kg-dry-air s^{-1} m^{-2})

z = Axial position (m)

ha = Heat transfer coefficient (J s^{-1} m^{-3} K^{-1})

T_s = Temperature of the solid phase (°C)

The first term on the left hand side is the change in energy content of the gas phase as it relates to changes in the gas temperature (T_g). The second term on the left hand side represents the convective movement of dry gas and water vapor. The right hand side of the equation represents the transfer of energy by convection between the gas and solid phase.

Recognizing that the processes occurring in the solid phase include the production of metabolic heat, the evaporation of water to the gas phase and convection to the gas phase, the authors derived the following energy balance to describe the solid phase [36]:

$$S \cdot (C_{Ps} + \varphi_s \cdot C_{Pw}) \frac{\partial T_s}{\partial t} = ha(T_g - T_s) - \lambda \cdot K'a(\varphi_s - \varphi_s^*) + Y_Q(S \frac{\partial b}{\partial t} + b \frac{\partial S}{\partial t}) \quad (2.6)$$

Where:

S = Volumetric concentration of total dry solids (kg-total-dry-solids m^{-3})

C_{Ps} = Heat capacity of the dry solid (J kg-dry-solid^{-1} °C)

φ_s = Water content of the solid phase (kg-water kg-total-dry-solids^{-1})

C_{Pw} = Heat capacity of the liquid water (J kg-water^{-1} °C)

λ = Enthalpy of evaporation of water (J kg-water^{-1})

$K'a$ = Mass transfer coefficient (kg-water s^{-1} m^{-3})

φ_s^* = Water phase equilibrium (kg-water kg-total-dry-solids^{-1})

Y_Q = Heat yield from growth (J kg-biomass^{-1})

b = Concentration of biomass (kg biomass kg-total-dry-solids^{-1})

The term on the left hand side of this equation expresses the assumption that the heat capacities of the substrate and the biomass formed are equal. The first term on the right hand side represents convection to the gas phase; the second term is movement to the gas phase via evaporation and the third term represents the evolution of metabolic heat.

Although the model presented above followed relatively well with experimental trends observed for the water activity of the solid, the mass of biomass

produced, and the solid and gas temperatures, it only dealt with axial heat transfer despite being applied to a system that was intermittently mixed.

2.2.3 Heat Generation

Microbial growth and activity in SSF reactors produces significant amounts of heat that must be properly managed. In composting, this heat generation, which leads to rapid increases in temperature is favorable for material degradation, but in other SSF processes such as enzyme production, huge amounts of heat are lethal [23]. Heat generation is a direct result of microorganism metabolic activity, which cannot be directly measured, but may be indicated by a number of different factors [26]. Some authors; however, doubt the direct proportionality between heat production and microbial growth [37], but provide no alternatives to this relationship.

The factors which have been used in the development of numerous heat and mass transfer models as indicators of microbial activity include oxygen consumption rate [18], carbon dioxide depletion rate [26, 29], and biomass growth rate [24, 36]. There are assumptions incorporated in the use of any of these components in model development and so the selected indicator of metabolic activity depends on the desired outcome of the researcher [37]. Due to the complexity of balancing the intricate components in metabolic heat production, many studies have developed empirical sub-models to represent this phenomenon in various models [37, 38]. One such study [18], which developed an empirical component to describe heat generation, first developed the relationship between temperature and oxygen consumption rate (RO_2):

$$RO_2 = RO_{2,0} + a(1 - e^{-c\bar{T}}) \quad (2.7)$$

where $RO_{2,0}$ is oxygen depletion rate at time zero and position zero, a and c are constants obtained from temperature data regression and $\bar{T}$ is a given temperature

minus the temperature at time zero. This equation was then used to evaluate the heat generation term ($\dot{q}_{gen}$) by using the equation:

$$\dot{q}_{gen} = Q(RO_2) \quad (2.8)$$

where Q is a heat generation factor.

This study was able to present a model that very closely followed experimentally observed trends in temperature and humidity, but only through the use of an analytical solution that contained many simplifications; a numerical solution was not presented.

Other researchers performed a heat balance on the composting of greenhouse tomato plant residues in a bench scale batch reactor to determine the amount of heat produced [39]. The authors determined that a cumulative total of 2131.6 KJ of heat was produced from a reactor that was approximately 0.02 m^3 in volume over a period of about 40 hours at a heat production rate of 16.4 KJ/g DM, which falls within the median range of other literature values [39].

2.2.4 Reactor Design

Reactors designed for SSF systems are built to overcome as many of the challenges associated with heat and mass transfer as possible. For this reason, there exist a number of different designs, each of which focuses on minimizing the effect of a known process constraint or limitation. Since these complications have been well documented, there are certain criteria that must be met by a well-designed SSF reactor. These requirements [3, 9, 11, 22] are to:

(a) Contain the substrate bed – Of importance is the nature of the material used to build the reactors. It must be durable enough to withstand increases in temperature, pressure drops across static piles, and corrosion. The material must also be of low cost and harmless to the process.

(b) Prevent unwanted contamination from entering the reactor – This is of particular importance where sterile environments are required for product growth, such as in the food industry, but is difficult to achieve as a result of solids handling. In addition, the conditions for organism optimal growth, such as pH, flow rate and moisture content may be affected via this contamination.

(c) Have control systems for temperature, heat removal, air flow rate and humidity – The ability to regulate all of these factors that affect the growth of microorganisms is essential for proper product formation. This is perhaps the most difficult aspect of the bioreactor design because gradients (temperature, heat, concentration) can still develop even with control mechanisms in place.

(d) Prevent the uncontrolled release of process microorganisms – Many SSF processes produce pathogens from fungal spores [22] and thus need to be exhausted efficiently to prevent the release of these organisms into the atmosphere.

(e) Maintain uniformity in the substrate bed – This is most commonly achieved via mixing during the process and serves as a deterrent for the formation of local temperature zones and thus leads to fewer and narrower thermal gradients.

(f) Scale up easily – The design employed on the bench scale must also be readily applicable on the large scale for the industrial production of the materials whilst maintaining low cost and low labor intensity.

Once an SSF reactor is built it may be run continuously, in batch, or in fed-batch mode, with batch reactors being the most common. In batch reactors, the solid substrates are mixed and then fermented without the addition of any new material, whereas in continuous reactors, new material is added regularly to the

Table 2.4 Selection criteria for bioreactor design [3]

Organism Growth Type	Agitation Type	Bioreactor Type
Fast	None	Packed bed
Fast	Gentle	Rotating Drum
Fast	Intermittent – Fast	Stirred drum; stirred bed; gas-solid fluidized bed; rocking drum
Slow	None	Tray fermentation
Slow	Slow-Fast	Stirred bed; rotating or stirring drum

Fast growing indicates an organism that grows within 1-5 days, and slow growing refers to an organism that grows in 1-4 weeks

fermenters while fermented material is constantly removed. In fed-batch operations, new material is added periodically to the initial raw material, mixed and allowed to ferment for a given time period. In comparing fed-batch operations to batch operations for the composting of mixed organic substrates, Nakasaki et al. [19] determined that organic material degradation was more efficient in batch mode over a period of 8 days.

Bioreactors currently used for SSF may be static or moving and may or may not employ forced aeration through the substrate bed. The nature of the substrate, inoculum, and product determine the type of bioreactor selected for a given process. Table 2.4 [3] provides a guide to the choice of bioreactor, taking into consideration issues such as product type, substrate and organism properties.

2.3 Composting – An Exemplary SSF Process

2.3.1 Definition and Uses

Composting may be defined as the aerobic microbial biodegradation of organic matter. This degradation occurs as a result of the activity of a community of microorganisms present in the system. Composting is used to reduce the volume of many municipal solid wastes that are biodegradeable. The end result of a composting process is a stable, usable product that is free of pathogens and plant seeds [32].

In aerobic composting, air is employed in the degradation of organic substrates to produce carbon dioxide, water and heat. Anaerobic composting processes can be done and this is accomplished in the absence of oxygen yielding methane, carbon dioxide, organic acids and alcohols [32]. The nature of these products increases the

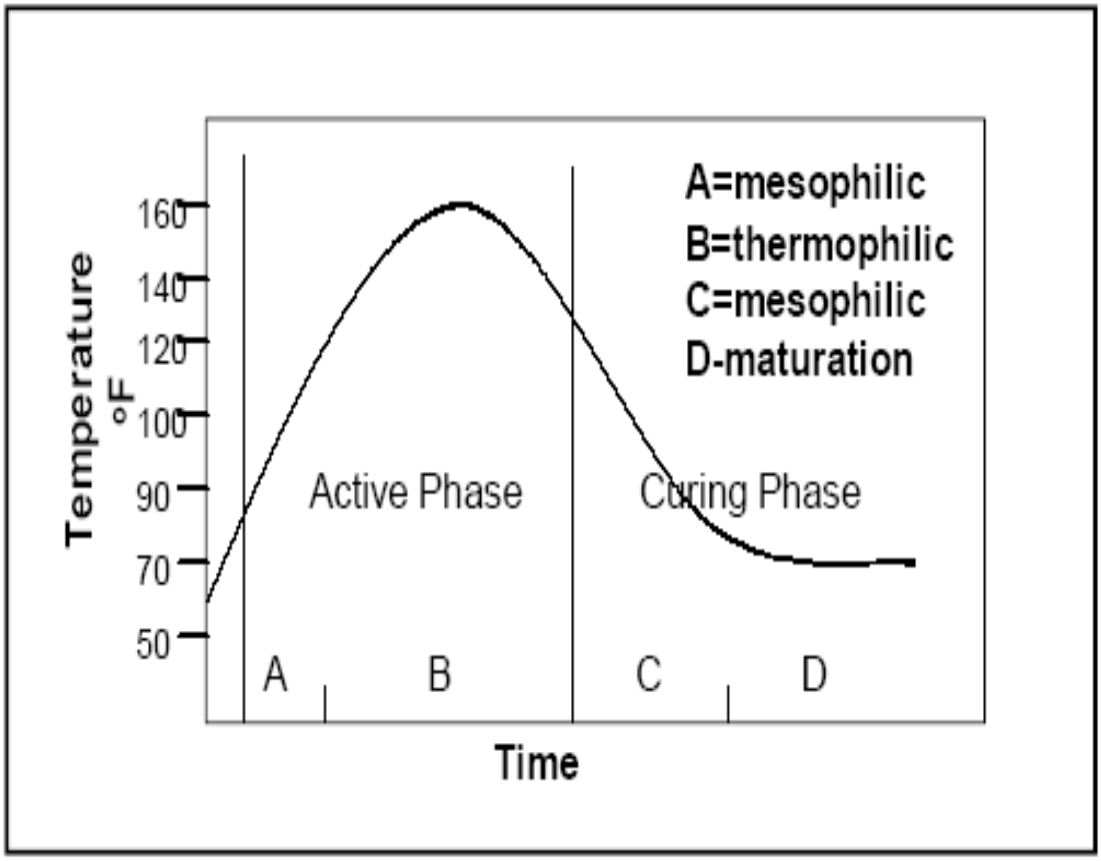

Figure 2.1 The phases of composting [1]

ejection of strong odors – a problem that is minimized in aerobic composting; for this reason, most composting processes are carried out under aerobic conditions.

The process of composting occurs in 3 different phases (Figure 2.1). The first stage is the mesophilic phase. At this stage, temperatures are moderate in the range of 10℃ to 45℃ (50℉ to 113℉) [1] and mesophilic organisms commence the composting process by breaking down readily soluble and degradable components. The next stage, termed the thermophilic phase or the active phase is characterized by elevated temperatures in the range of 54℃ to 66℃ (130℉ to 150℉) [1] and possibly higher temperatures. In this active phase, thermophiles break down more complex compounds such as proteins, fats, cellulose and hemicellulose and in the process, kill off pathogens. In the final stage of composting, the temperature cools back down to mesophilic conditions and the compost enters the maturation or curing phase where organic substrates continue to degrade and are converted into biologically stable humic substances [1]. The amount of time taken for each phase is dependent on the size and conditions of the compost pile. Some compost piles may go through all 3 phases within several hours while others may take up to several months to get to the maturation phase.

2.3.2 Compost Microbial Community

The process of composting is driven by the microbial activity of organisms that lead to the degradation of the substrate. The shift in environmental conditions in compost piles from mesophilic to thermophilic also represents a shift in the microbial community structure from mesophilic to thermophilic organisms. The main organisms

responsible for composting are bacteria and fungi, while all other microbes play minor roles in the decomposition process [32].

Bacteria dominate most compost piles throughout all the phases of composting. As the smallest known free-living organisms, they account for 80 - 90% of the microorganisms typically found in a gram of compost, and are responsible for the majority of the heat generation and degradation during the process [40]. Table 2.5 shows some quantitative data for an aerated composting process and indicates the increased presence of bacteria as a function of temperature. The domain, bacteria, encompasses a large number of prokaryotic microorganisms which come in different shapes and sizes and at different points during the composting process [41]. Mesophilic bacteria predominate during the first and last stages of composting, while thermophilic bacteria dominate during the active phase where the pathogenic prokaryotes are killed. A number of studies have been done to track the temporal population dynamics of bacteria in compost systems [42-50]. Notable bacteria within the compost pile include actinomycetes, which are filamentous bacteria responsible for degrading complex compounds like cellulose, lignin, chitin and proteins [40]. Also notable is the *Bacillus* genera which dominates during the thermophilic phase [51]. In this group of microorganisms, *Bacillus subtilis* has been studied [4] for use in numerous applications.

Table 2.5 Microbial Populations During Aerobic Composting [32]

Microbe	Mesophilic Initial Temp < 40°C	Thermophilic 40-70°C	Mesophilic 70°C to cooler	Number of species identified
Bacteria				
Mesophilic	10^8	10^6	10^{11}	6
Thermophilic	10^4	10^9	10^7	1
Actinomycetes				
Thermophilic	10^4	10^8	10^5	14
Fungi				
Mesophilic	10^6	10^3	10^5	18
Thermophilic	10^3	10^7	10^6	16

*Values are given in number per wet gram compost

One of the first studies completed to quantify bacterial population dynamics in compost did so via the use of 16S rRNA probes [42]. This study built upon previous work [43] that qualitatively profiled changes in the bacterial community using genetic fingerprinting [42]. For this investigation, the researchers designed small sub-unit rRNA probes to quantify the relative abundance of bacterial populations and found a trend in the bacterial population, which, for the most part, tracked the temperature profile. The researchers also found a shift in the dominant species, with Pseudomonas-type genes being most prevalent up to 72 hours and then domination by low G+C Gram-positive bacteria by 84 hours. This shift in population complemented changes observed for the temperature and carbon dioxide production profiles. The information gained from this study is extremely relevant to compost microbial community studies because it gives significant credence to the important role that bacteria play in composting processes and it also gives an indication of what groups have a major presence. Moreover, this assessment was accomplished through probe hybridization – one of the few methods that give quantitative data about the make-up of the community. Due to the highly sensitive nature of rRNA, working with it could prove difficult, and the researchers may have lost quite a bit of information while carrying out their protocol. The quantification of bacterial population dynamics could have been completed with rDNA probes that are less sensitive to work with. Although many of the genes in this study were unidentified, the study sets the stage for the need to explore the role of other microorganisms, such as fungi, in the composting process.

Fungi work with bacteria to degrade organic material during composting. This group of microorganisms is highly adaptable and can grow under a vast variety of conditions, thereby facilitating their presence during all phases of composting. Fungi are ubiquitous in nature and have low moisture and nitrogen requirements [32]; thus, making them ideal organisms for cellulosic compost piles, which tend to be low in

nitrogen. Fungi are broadly classified as molds (aerobic) and yeasts (both aerobic and anaerobic) and can withstand a broad range of pH conditions [32]. During composting, fungi facilitate substrate degradation by bacteria, by breaking down tough debris such as plant polymers [40].

The mode of growth of filamentous fungi (molds) puts them at a distinct advantage in competitive-nutrient environments [41]. The filaments (*hyphae*) grow by extending at the tip and by branching out [41]. In so doing, the tips are able to extend to areas that are more nutrient rich; thus, enabling filamentous fungi the ability to colonize solid substrates better than unicellular organisms [2]. In addition, the rate at which branching occurs produces an exponential kinetic growth pattern of biomass [2]. This is because the growth pattern allows the hyphal tips to excrete concentrated hydrolytic enzymes at the solid surface that break through the solid substrate, providing the fungi with access to nutrients available within the substrate particles [2]. Fungi are divided into five different groups, as shown in Table 2.6, but most of the groups are phylogenetically similar [41].

Due to the difficulty in obtaining data that tracks the succession of fungal communities in compost, only a limited number of studies [48, 52, 53] have been completed for this group of microorganisms. The study by Dees and Ghiorse [48] investigated the microbial diversity of thermophilic compost by using both culture-based and cultivation-independent techniques. This was accomplished by isolating 16S rRNA

Table 2.6 Fungi Classifications [41]

Group	Common Name	Hyphae	Typical Representatives	Habitats
Ascomycetes	Sac fungi	Septate	*Neurospora, Saccharomyces, Morchella*	Soil, decaying plant material
Basidiomycetes	Club fungi, mushrooms	septate	*Amanita, Qgaricus*	Soil, decaying plant material
Deuteromycetes	Fungi imperfecti	Septate	*Penicillium, Aspergillus, Candida*	Soil, decaying plant material, surfaces of animal bodies
Oomycetes	Water molds	Coenocytic	*Allomyces*	Aquatic
Zygomycetes	Bread molds	Coenocytic	*Mucor, Rhizopus*	Soil, decaying plant material

sequences from compost samples through extraction, PCR amplification, cloning, sequencing, and ARDRA (Amplified rDNA Restriction Analysis) screening. The researchers were able to PCR- amplify the bacteria and actinomycetes isolates from their samples at 50℃, 60℃ and 74℃; whereas the fungal isolates were only amplified at 50℃ and archaea resulted in no amplifications. These amplifications led to the result that gram positive bacteria governed the thermophilic phase of composting and that fungal growth disappeared at temperatures of 60℃ and higher [48]. Interestingly, the results from the culture-based techniques did not overlap with those from the cultivation-independent techniques, but rather the results complemented each other. Provided that their ARDRA analysis was done correctly, the lack of complete congruency between the two different methods used highlights some of the flaws of using culture based methods; namely, the preferential growth of certain bacterial groups coupled with the selected growth suppression of others as a result of the culture conditions and environment [53]. The use of a culture-based method could also account for the researchers' observations that their most abundant isolates were from groups not typically associated with compost piles. In addition, the findings of the study were limited by the availability of library clone sequences to which the isolates could be compared for identification. Although the results of this study agree with previous culture-based findings, the relevance of this work is minimal because advances in molecular biology tool and techniques have eliminated the need to work with biased culture-based methods.

Another study, done by Hansgate et al.[52] on fungal community dynamics, employed F-ARISA (Fungal-Automated rRNA Intergenic Spacer Analysis) and 18S rRNA gene cloning and sequencing to explore fungal populations in the initial stages of composting. The combination of these methods allowed the researchers to identify more fungal sequences than the previous study by Dees et al., and thus led to the

observance of a shift in the fungal community structure between 48 and 60 hours, despite the small size of the A-OTUs (ARISA Operation Taxonomic Units). The researchers also verified the effect of fungi on the system pH by identifying the presence of *Rhizopus* and *Amylomyces* which are lactic acid producing fungi. This study, perhaps the first of its kind, provides much needed insight into the role of fungi in composting systems. This is extremely useful because it documents the temporal changes in the fungal community during the initial stages of composting. This information; although qualitative, provides a foundation upon which further fungal dynamics may be established; such as the use of fungal probes to quantify major fungal species. The use of the ARISA method also allows for high throughput analysis – a critical asset in managing numerous samples for temporal studies.

2.3.2.1 Molecular Biology Tools for Characterizing Compost Microbial Communities

Several methods have been developed to satisfy the need for microbial community information in compost and soil samples. The choice of method depends on the level of detail desired, the sample size, and the time available for sample processing. Molecular biology techniques have surpassed the use of classical microbiological methods and biochemical methods in the identification of microbial groups because of the higher efficiencies and more accurate results. Microbiological methods employ culture-based protocols that may be laborious and biochemical methods, such as quinone profiling [54], DNA reassociation kinetics and Bisbenzimidazole-Cs-Cl-gradient fractionation, do not have broad applications because 99% of microorganisms have not been isolated [55], so databases for

comparative purposes are of limited availability. For this reason, molecular biology techniques are leading the way towards obtaining microbial data.

Most molecular biology techniques employ Polymerase Chain Reaction (PCR) as an essential step in the process of genetic fingerprinting. PCR is defined as the exponential amplification a DNA fragment [56]. This amplified product can then be separated based on nucleic acid content to produce a pattern of bands on a gel that will provide an indication of the diversity of the microbial community [56]. These fingerprinting methods which lead to characterization of microbial communities include Amplified ribosomal DNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal-restriction fragment length polymorphism (T-RFLP), length heterogeneity polymerase chain reaction (LH-PCR), ribosomal intergenic spacer analysis (RISA), single-strand conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD), functional PCR and direct cloning and sequencing. Of these methods, several have been used to evaluate compost microbial communities, and will be discussed in later paragraphs.

Despite the broad application of these molecular biology methods, the results are inherently biased because of the use of PCR during the process. PCR has several limitations that may hinder the accuracy of the results. Firstly, PCR is highly sensitive to environmental conditions and tiny amounts of contamination can lead to quantitatively different results [56]. In addition, polymerases, which are used to elongate the DNA strands, may incorrectly insert or delete nucleotides, producing incorrect sequences that go unnoticed because the polymerases are unable to proofread the strands [56]. Errors such as these become exponential because of the numerous repeated cycles that characterize PCR. Furthermore, PCRs of longer products are less efficient and less accurate [56] – a particularly troubling issue in intricate microbial

communities because of the differences in size and copy number of the various organisms. Additionally, PCR introduces many chimeric sequences, which occur when a foreign DNA strand is annealed to a prematurely terminated amplicon and is copied to completion in subsequent PCR cycles [56].

DGGE (Denaturing Gradient Gel Electrophoresis) is used to separate rDNA in a polyacrylamide gel containing a linear gradient of DNA denaturing compounds, based on differences in G-C content of the sequences [55]. As the DNA moves along the gel, the increased concentration gradient forces it to become single stranded, but it does not become completely denatured because of the presence of a GC clamp that is incorporated into one of the primers for the PCR amplification. Compost research completed using DGGE has produced microbial diversity data and identification when coupled with 16S rDNA sequencing [49, 50, 57]. The most recent of these studies [49] sought to characterize the microbial community of a composting process in a Hazaka system. Surprisingly, the study showed no significant changes in the microflora during the composting process based on the DGGE profile even though the material had composted for 30 days. This unusual result may be due to the use of the Hazaka system which regulated the temperature at $60-76℃$ during the thermophilic phase and at $45℃$ during the mesophilic phase. Such regulation of the temperature during composting would not allow for the natural selection of microorganisms which are necessary for the degradation process. Amazingly, the authors did not present any explanations for the lack of community shifts, but overshadowed this by presenting the bacterial genera that comprised their samples. Another indication that composting conditions may not have been ideal for microorganism differentiation was that the lack of any noticeable change in the pH for the entire 30 day period. Although, this study did a good job of identifying species in the bacterial community that were present in

their samples, the overall result provided no new insight into the changing roles of microorganisms during composting.

A major advantage of the DGGE method is that bands can be cut out from the gel and then sequenced to obtain phylogenetic information [55]. Also, it is relatively easy to determine the changes in the microbial community through the absence or presence of bands on the gel [49]. The downsides associated with DGGE include laborious calibrations to ensure optimal separation conditions and DNA fragment lengths are limited to 500bp [56]. In addition, large amounts of DNA – as much as 500ng - are needed for good resolution with DGGE. Furthermore, PCR biases can lead to incorrect conclusions about the components of a given sample by either under or over- representing certain groups [56].

Similar to DGGE is TGGE (Temperature Gradient Gel Electrophoresis) which separates rDNA in the exact manner as DGGE, but uses temperature at the denaturing gradient as opposed to chemical denaturants [56]. The advantages and limitations of TGGE are the same as that of DGGE [55]. To this author's knowledge, no microbial community analyses have been completed on compost samples using TGGE, but several have been done on soil samples [58].

In the SSCP (Single Strand Conformation Polymorphism) method, PCR-amplified DNA fragments are denatured and separated on non-denaturing gels based on the folded structures of the single strands of DNA [55]. Although SSCP is used primarily to identify polymorphisms and mutations in human genes, it has found application in microbial community studies [56]. SSCP has also been used to track microorganism community dynamics in compost samples [53, 59]. The more recent of these works [59] used SSCP in combination with 16S rRNA sequencing to determine the microbial complexity of a compost system consisting of domestic organic wastes, wood chips and yard trimmings. Seven samples were collected over a

period of 23 days and after analysis revealed that a significant shift occurred in the community profile in the initial curing period. Considering that composting goes through three different stages, it is somewhat surprising that this study only found one change in the community profile. Also strange was the initial temperature of the compost reactor - 41°C. The proximity of this value to thermophilic temperatures could explain why the authors did not observe a shift from the mesophilic phase to the active phase. Furthermore, the authors did not present the dynamics of physical state variables such as temperature and pH and so it was not possible to correlate the observed shift in the microbial community to those variables. Despite the inability of the authors to sequence their most abundant clones, they have presented SSCP as a viable alternative to DGGE to monitor microbial community dynamics during composting.

A major disadvantage of using SSCP is the re-annealing of DNA strands during electrophoresis, after being initially denatured [56]. This can lead to complex structures that can produce more than one band on a gel in addition to other possible bands added through PCR biases [56]. Furthermore, the sensitivity of the SSCP method is affected by electrophoretic conditions such as temperature and the gel matrix and SSCP only works optimally for short DNA fragments in the range of 150-400bp [55]. Also, SSCP requires very high specificity for rRNA genes because the folded conformations of these genes are very similar [56]. SSCP is advantageous because it is simpler to carry out than DGGE [56]and more samples can be processed at once through the creation of larger gels [59].

The RISA (Ribosomal Intergenic Spacer Analysis) method creates a community profile of the PCR-amplified genetic region between 16S rRNA and 23S rRNA on the basis of species-specific length polymorphisms in the region[56]. RISA has been automated (Automated Ribosomal Intergenic Spacer Analysis, ARISA) to

increase resolution and analytic power [60]. This technique was employed in a study [43] to develop bacterial community fingerprints during the initial stages of composting. Nine samples were taken over a period of 96 hours for each reactor and through subsequent analysis, the authors observed 2 major shifts in the community population – one between 12 and 24 hours and the other between 60 and 72 hours. The first shift corresponded with an increase in temperature and a drop in pH while the second shift corresponded with increases in both temperature and pH. Through the phylogenetic identification of the most common groups, the authors were able to correlate the species of bacteria to the observed physical phenomenon. This study, although qualitative, provides a glimpse of the intricate relationship between biotic and abiotic phenomena. The information obtained from this study is critical towards further understanding the dynamics responsible for changes observed in the initial stages of composting. Similar to that study, another investigation [52] (discussed in the previous section) used a variation of ARISA to characterize fungal communities. This variation, called fungal ARISA (F-ARISA) focuses on the rDNA region that contains the two internal transcribed spacers (ITS) and the 5.8S rRNA gene [60].

One of the major limitations of using RSIA and its variations is that it only provides a general assessment of the microbial community through the community fingerprint [60]. RISA must be used in conjunction with other methods to identify major species of interest which may not be identifiable due to the limited information available in intergenic spacer databases [56]. Furthermore, PCR biases particularly affect RISA because shorter fragments may be preferentially amplified even though intergenic spacer regions are of varying lengths [56].

The flexibility of RISA in terms of the number of variations of the method [60] that can be used, make it an attractive option. In addition, this method, once automated leads to rapid, high-throughput analysis [56] which makes it ideal for

obtaining high resolution data in complex systems that require numerous sampling points. ARISA also produces highly specific profiles that are readily comparable at different time points [56]. Moreover, ARISA may be used with other high-throughput technologies, such as capillary electrophoresis systems that increase the rapidity and reproducibility of the method [60].

ARDRA (Amplified rDNA Restriction Analysis), also known as restriction fragment length polymorphism (RFLP) is a method in which PCR-amplified DNA is subjected to restriction digestion analysis based on the theory that differences in gene sequences will create unique binding sites for various restriction enzymes [56]. A community profile is created after the digested fragments are run on a gel [55]. This method was employed in one composting study [48] (discussed in previous section) to determine the microbial diversity during the thermophilic phase. One of the major limitations of using this method is that the members of the microbial community may be overestimated through the presence of more bands on the gel than the number of amplified DNA sequences [55]. Also, similar to ARISA, ARDRA must be used in conjunction with another technique to detect and identify the specific groups present in the profile [56]. Furthermore, ARDRA may become very time consuming because each experiment needs to be optimized to find the best combination of restriction enzymes that work for the system of interest [56]. Despite these limitations, ARDRA is advantageous because it is rapid, once optimized, cost-effective and it uses two parameters to separate rDNA genes: size and sequence [56].

The aforementioned methods provide excellent qualitative results about the genetic fingerprints of microbial groups in composting systems, but must usually be used in conjunction with another technique for species identification. Table 2.7 summarizes the significance of the above mentioned techniques and other PCR based methods.

Probe hybridization is a molecular biological method that does not require the use of PCR, and is available for providing information about the microbial community in a compost sample. In probe hybridizations, a labeled fragment of a known DNA sequence is hybridized to its complimentary strand in an environmental sample. The labeling may be done via radioactivity or chemiluminescence to detect and visualize the presence of specific species in the microbial community. According to Spiegelman et al. [56], there are 3 main reasons for using oligonucleotide probes: (i) to investigate the presence of various taxonomic groups in the community, (ii) to measure the relative abundance of specific taxa, and (iii) to determine the spatial distribution of species or groups present. Probe hybridizations may be used (i) with clone libraries in the identification of groups of interest [61], (ii) along with other fingerprinting techniques [61] and (iii) directly in order to investigate the presence of taxonomic groups.

To obtain quantitative information from probe hybridization, the environmental sample must first be denatured and immobilized onto a positively charged membrane. In addition to the sample, a known amount of a standard must also be blotted onto the membrane. The probe is then hybridized to this membrane and the intensities of the signals obtained from the samples, either through radioactivity or chemiluminescence, are compared to those of the standards to provide relative abundance data.

Probe hybridizations provide flexibility in obtaining taxonomic information because of the breadth of information that may be gleaned from the data. In addition, the high specificity of the probes greatly minimizes the possibility of mismatch errors and the probes are relatively easy and cheap to produce. The greatest limitation of probe hybridization is the possibility of errors during the probe synthesis. The probing will only be as efficient as the probe design for which there may be limited amounts of

sequence data available in databases. In addition, probing is limited by the difficulty in detecting lesser known organisms [56]. Furthermore, in compost samples, probe hybridizations are complicated by the presence of humic acids, which are often co-extracted with nucleic acids through the extraction procedure [50]. The presence of humic acids affects the quantification of DNA, the performance of DNA in PCR amplification and DNA-DNA hybridizations [62]. A study [62] done to determine the effects of humic substances on DNA quantification and hybridization found that the Hoechst dye gave more reliable results when using a fluorescence detection procedure. The study; however, concluded that treating the samples with PVPP (polyvinylpolypyrrodidone), reduced the amount of humic substances present, thereby allowing for the use of the samples in hybridizations with little interference from humic contaminants [62].

2.3 Summary

Composting may be regarded as the most complex SSF process because of the numerous microbial communities that work together in very intricate relationships to degrade organic matter. Through this microbial activity, waste metabolic heat is produced; thereby, elevating the temperature within the compost pile. The proper management of this generated heat, along with the management of mass transfer aspects is essential for controlling the behavior of SSF processes. In composting, one must first understand how the microbial communities interact to degrade organic material in order to be able to control and eventually predict compost reactor behavior. Molecular biology has evolved to produce numerous high-throughput tools that facilitate the characterization of microbial communities both qualitatively and quantitatively. These tools allow for the tracking of microbial communities over time, the identification of groups present, and the amounts of each group present.

Table 2.7 Summary of PCR-based Molecular Biological Methods [56]

Method	Description	Community Profile	Taxonomic Identification	Quantitative	Limitations	Advantages
ARDRA	Separates amplified 16S molecules by restriction patterns	yes	possible	no	Optimization required	Rapid; minimal requirements
DGGE	Separates amplified 16S molecules by % G-C content	yes	possible	no	Labor intensive; complex profiles possible	Single base pair resolution
TGGE	Separates amplified 16S molecules by % G-C content	yes	possible	no	Labor intensive; complex profiles possible	Single base pair resolution
T-RFLP	Separates amplified 16S molecules by restriction patterns	yes	yes	yes	Optimization required	High throughput; immediate, digital analysis
LH-	Separates amplified 16S	yes	possible	no	Some ambiguity	Great simplicity and

Table 2.7 (Continued)

PCR	molecules by length				in results	speed
RISA	Separates amplified 16S-23S intergenic region by length	yes	possible	possible	Small database; especially sensitive to PCR artefacts	Great simplicity and speed; automation possible
SSCP	Separates amplified 16S ssDNA by sequence-dependent higher order structure	yes	no	possible	Can be a delicate procedure	Great simplicity and speed; automation possible
RAPD	Sequence-independent profiling based on random PCR priming	yes	no	no	Non-generalizable profiles	Fast, easy and inexpensive; high statistical power possible
Functional PCR	Several PCR-based analyses using amplified catabolic genes; indirect functional assay	n/a	n/a	no	Limited taxonomic information; limited databases	Better-suited to specialized investigations

Furthermore, these methods can determine which microbial groups are active at a given time and how these groups evolve during the course of the process. In characterizing compost microbial communities, most of the research has focused on the use of qualitative methods to describe process dynamics. In order to turn molecular biology into a predictive science, quantitative data must also be extracted; hence the need for the research presented in this thesis. By quantifying the microbial groups present, one can determine the amounts and types of microbes responsible for a given amount of heat generated. From the quantification of the heat generated, one can then develop a model that incorporates microbial activity in order to predict reactor behavior.

Chapter 3

Materials and Methods

In this study, both the engineering and biological aspects of aerated compost reactors run at different moisture contents were investigated. The following sections discuss the reactor construction, operation and instrumentation; as well as the sampling procedure and sample processing via DNA extraction, purification, blotting, hybridization and quantification.

3.1 Reactor Design and Construction

Three bench scale reactors with a working volume of 50L each were constructed from a high density polyethylene (HDPE) pipe with an inner diameter of 0.3m. HDPE is durable, non-toxic, non-corrosive, of high tensile strength, and tough enough to withstand temperatures up to 120°C. In each reactor, 7 sampling ports of diameter 3.2cm were placed 10cm apart, along the length of the reactor to facilitate spatial material sampling, as shown in the two dimensional image of Figure 3.1. These ports were threaded and sealed with a threaded cap during the composting process. The sampling ports were placed at 4 different positions along the circumference of the reactor to reduce the number of times the reactor bed would be disturbed in the same location during sampling. A perforated plate (Figure 3.2) was placed at 0.2m from the bottom of the reactor on top of a PVC ring to support the substrate bed. The sampling ports were placed at 0.1m, 0.2m, 0.3m, 0.4m, 0.5m, 0.6m and 0.7m above the perforated plate and were numbered accordingly.

Stainless steel racks (Figure 3.5) were custom made to fit inside of the reactors with the support of small brackets to provide an immobile support for thermocouples. The racks were further supported by the perforated plate, into which the ends of the stainless steel racks were placed.

The reactors were mounted onto a 0.63m tall plywood structure constructed to support the reactors and to allow the air inlet pipe to be positioned mounted at the base of the reactors to allow for the inlet of air and for the collection of leachate (see figure 3.3). The plywood frame contained openings that allowed the reactors to sit upright on the frame.

To minimize radial temperature gradients and heat losses to the environment, the reactors were insulated with a foil and fiberglass duct insulation (Thermwell Products Co. Inc., Mahwa, NJ). The insulation was 0.05m (2 in.) in thickness, 0.3m (12 in.) wide and 4.6m (15 ft) long. The insulation was cut into sections to fit the circumference of the reactors and then the sections were glued to flexible window mesh (Lowes, Ithaca, NY) for support. This netting was then wrapped around the reactors for the duration of the process.

A ventilation system was designed and implemented to minimize the ejection of odors into the immediate environment. The system consisted of flexible tubing being placed at the exhaust of each reactor, which consisted of a pipe fitting that was inserted into the reactor cap that covered the reactor once the composting process had started. The flexible tubing was then connected to a sturdier PVC pipe that was fed into an underground building exhaust system. Figure 3.3 shows a schematic of the overall reactor set up with the ventilation system, while figure 3.4 shows the set up with the 3 reactors used per run.

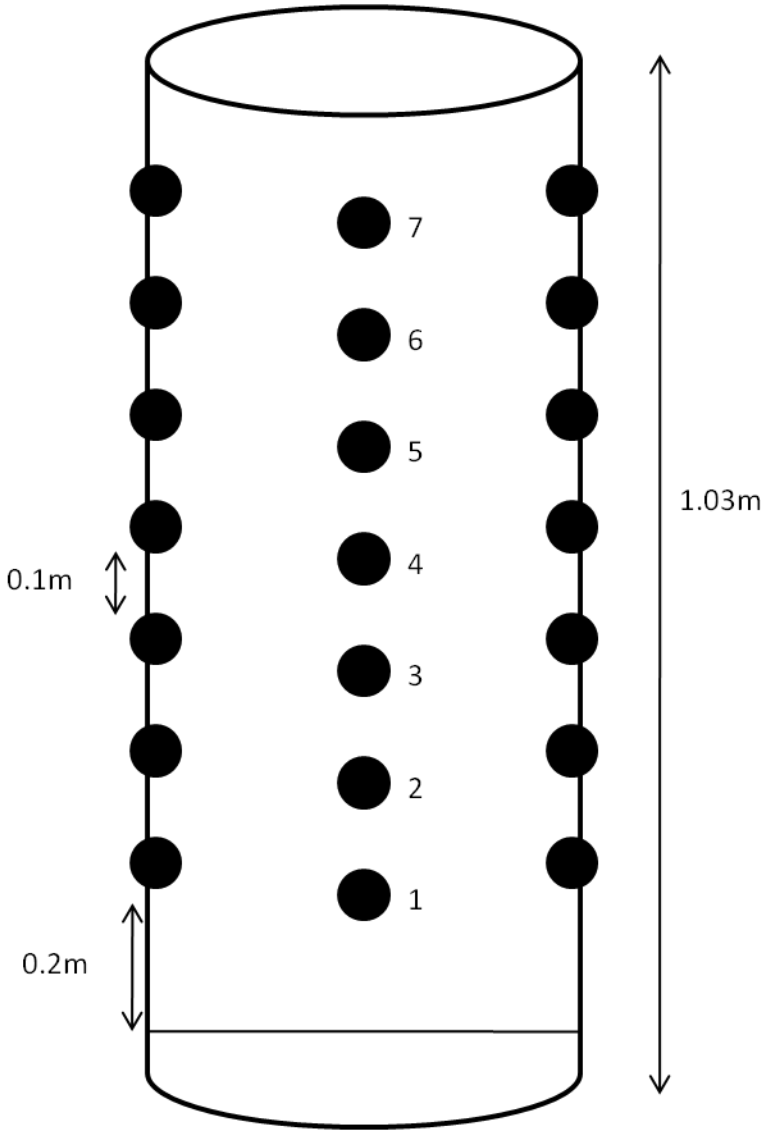

Figure 3.1 Schematic of reactor design

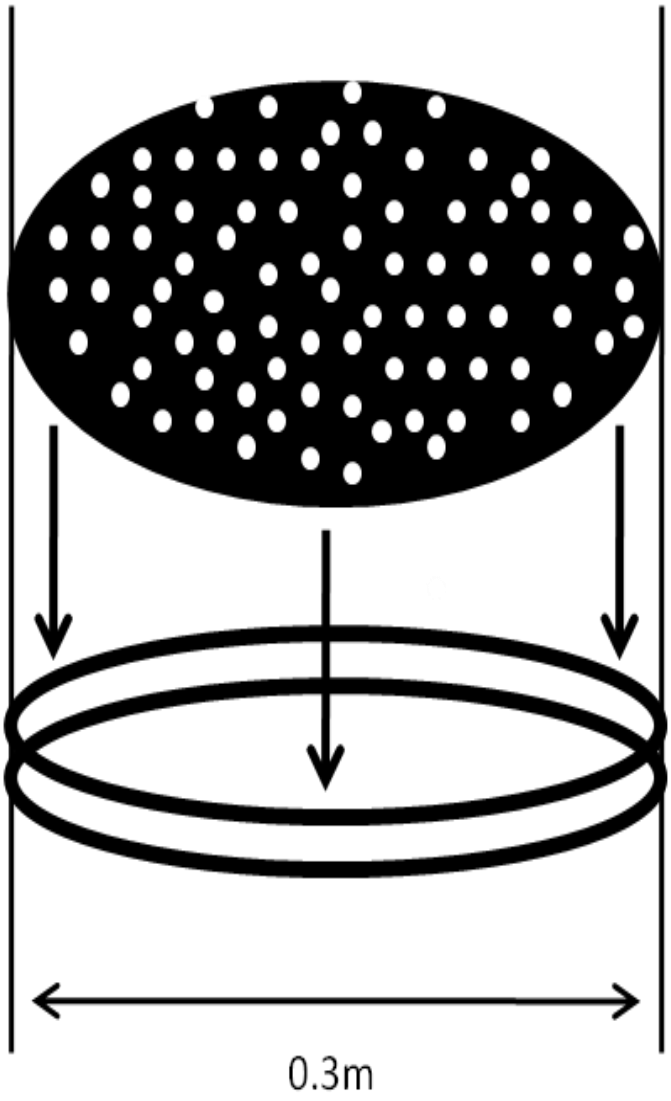

Figure 3.2 Perforated plate set up

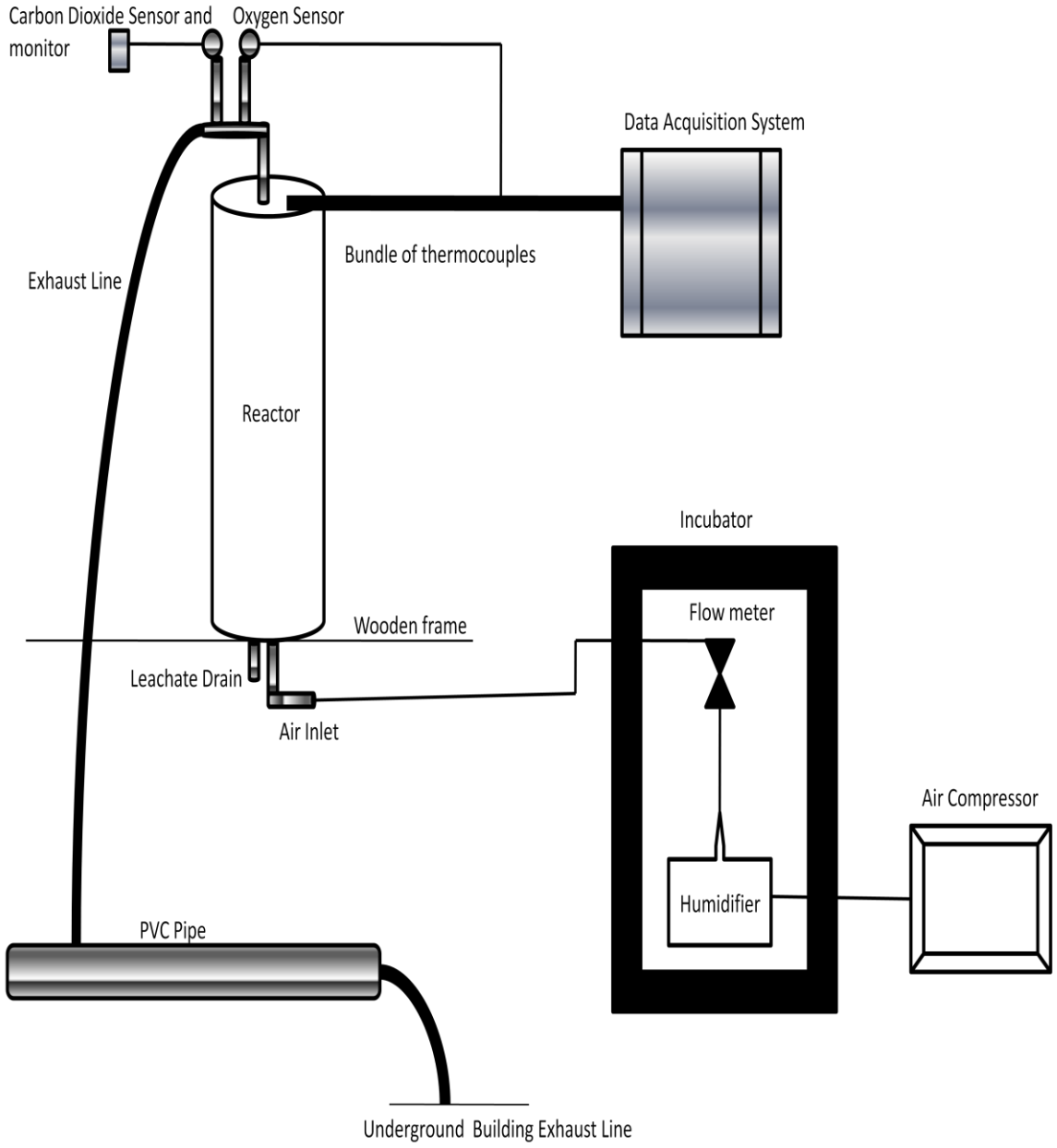

Figure 3.3 Overall reactor set up

Figure 3.4 Overall System Set Up

3.2 Substrate Preparation

A synthetic food waste, consisting of dog food and switchgrass was prepared. The dog food (Big Red Puppy Food, Pro-Pet Inc., Syracuse, NY) was chosen because its chemical composition is similar to that of cafeteria waste. The switchgrass (Stickle Farm, Ligonier, PA) was of the shelter variety and was selected as a bulking agent and carbon source for the composting process. Table 3.1 gives some of the physical properties of the two substrates.

Prior to mixing the two substrates together, the switch grass was size-reduced using a Buffalo Hammer Mill (Schutte-Buffalo Hammer Mill, Buffalo, NY) through a 2cm (3/4 inch) screen. The substrates were then mixed together in a C:N ratio of 15:1, resulting in a mass ratio of 1:1. The amount of water needed to produce initial moisture contents of 60%, 65% and 70% wet basis was then calculated. Twenty four hours prior to the start of the experiment, the substrates were weighed and placed in different storage containers. Seventy percent of the total required water volume was added to each of the switchgrass bins and 20% of the total required volume was added to each of the dog food bins. Table 3.2 shows the raw material requirements. The individual substrates were hand-mixed with the water and allowed to sit overnight. Tap water was used to reach the desired moisture contents because the existence of microbes in tap water would provide a source of inoculum for the reaction; however variable. Three hours prior to the start of the experiment, the switchgrass was again individually hand-mixed and the dog food was broken by hand into smaller pieces. The two substrates for a given moisture content were then hand-mixed together to produce a uniform mixture. A 250g sample of old compost from

Table 3.1 Physical properties of substrates [63, 64]

Substrate	Carbon (% of dry solids)	Nitrogen (% of dry solids)	Moisture (% wet basis)	Volatile solids (% of dry solids)
Dog Food	44.6	5.3	7.0	89.5
Switchgrass	44.0	0.5	15	80.1

Table 3.2 Raw Material

Moisture Content % wet basis	Mass of switchgrass (Kg)	Mass of dog food (Kg)	Volume of water (L)
60	3.5	3.5	10.5
65	3.5	3.5	13
70	3.5	3.5	16.3

previous experiments was also manually mixed in with the uniform substrates as an inoculums source. After hand-mixing, the mixture was then mixed mechanically for 15 minutes using a cement mixer (General Electric, Trenton, Canada), after which the homogenized mixture was loaded into the reactors. Figure 3.5 shows the mixed switchgrass-dog food substrate. A sample was collected as the initial time point sample.

3.3 Air Flow Regulation and Humidification

Air into the reactors was first compressed using a silent air oil free compressor (Werther International Inc., Houston, TX) with a compressor outlet pressure in the range of 100-300 kPa. The air was then humidified by bubbling it through a 20L carboy full of water (Figure 3.4). The flow rate of the humidified air was then regulated using precision valve flowmeters (Gilmont Instruments, Barrington, IL) at a flow rate of 20 L air/min before it entered the reactors. This air rate was chosen as a mid-range value for a 50L reactor, based on previous composting work done in our research group. The air humidifier and flow meters were enclosed in an incubator that was kept at 25℃ to maintain constant air inlet conditions.

3.4 Temperature Measurement

Copper-constantan thermocouple wire (PP-T-24, Omega Engineering, Stamford, CT) was used to measure temperature. The tips of the thermocouple were spot-welded together to maintain a robust junction during the composting process. At the other end of the wire, the thermocouples were attached to male thermocouple connectors that were used to connect the thermocouple wire to the data acquisition board. The spot-welded

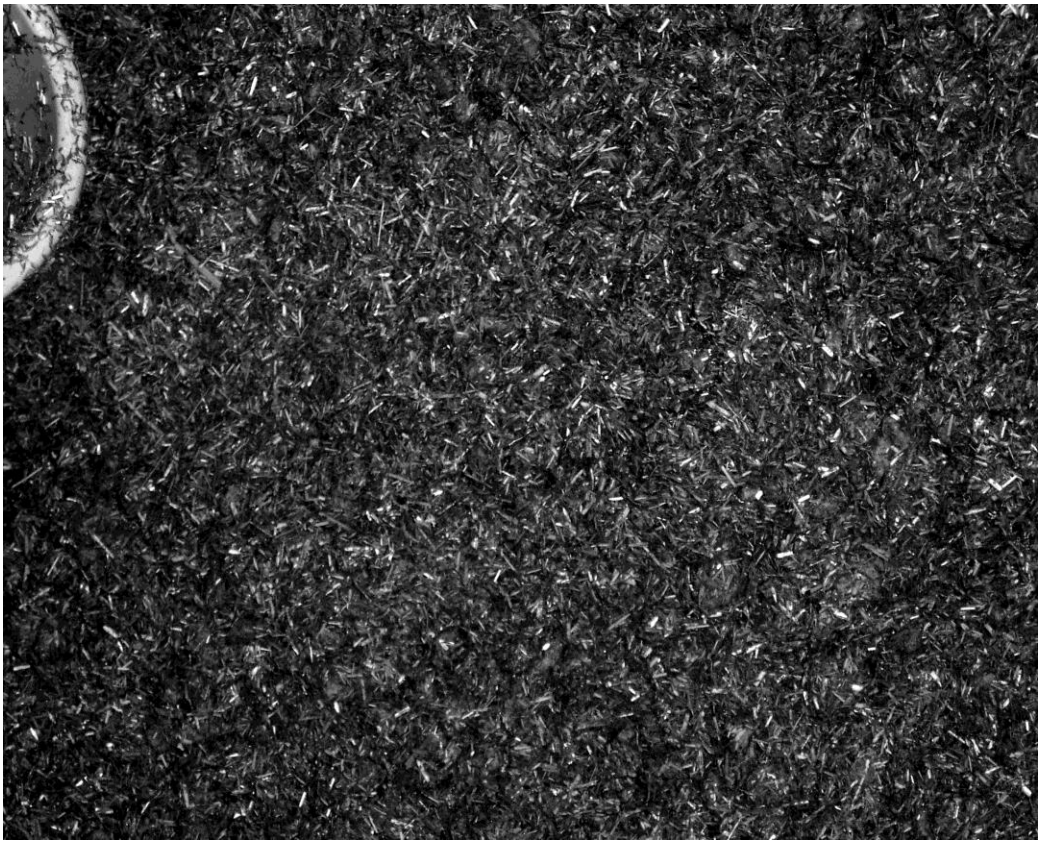

Figure 3.5 Mixed switchgrass – dog food substrate

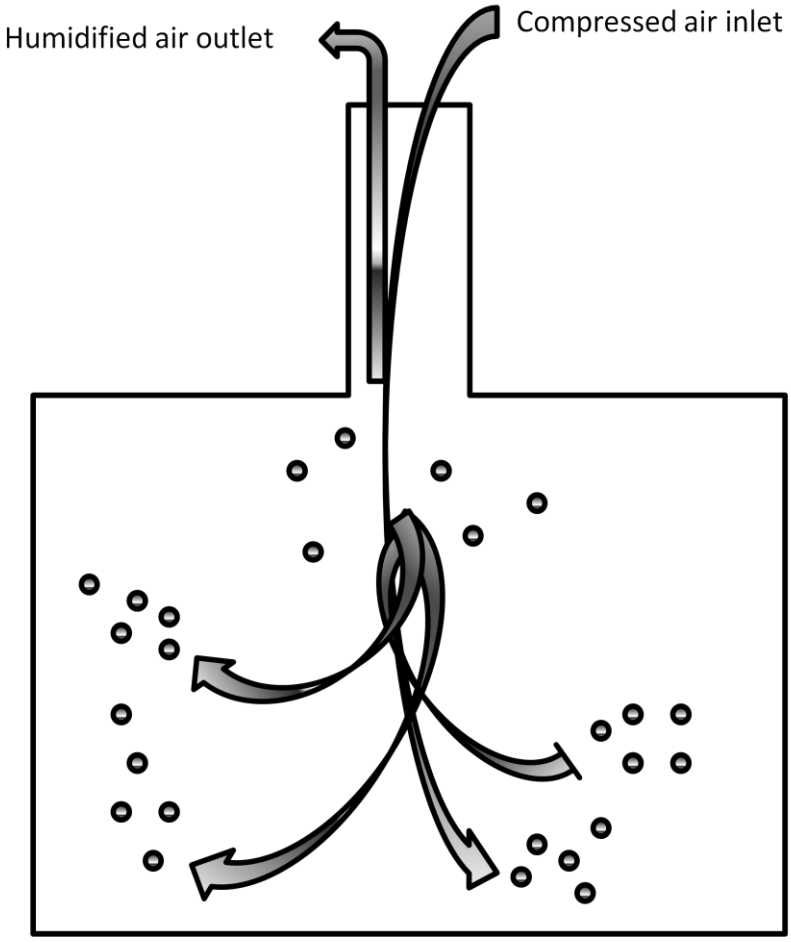

Figure 3.6 Humidified air set up

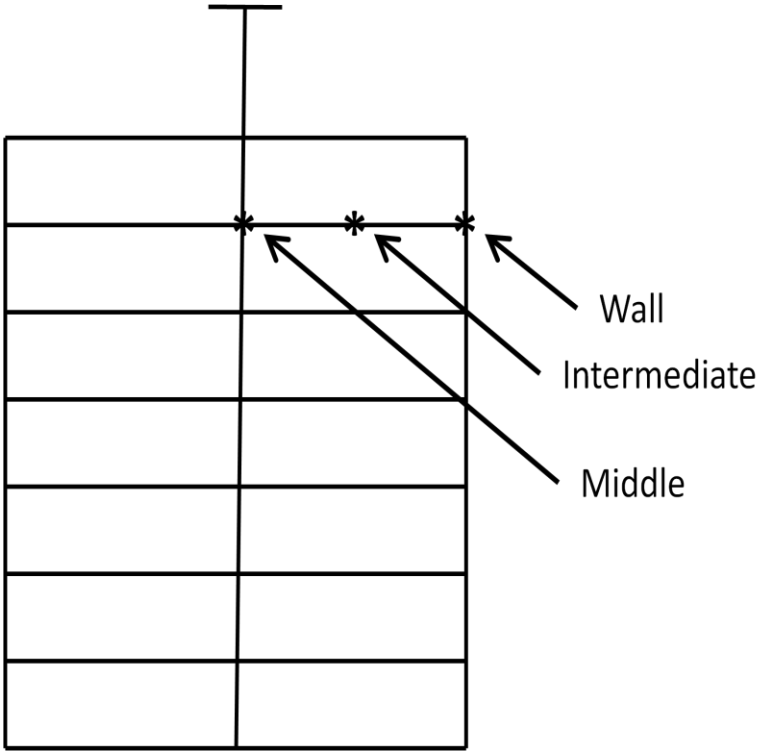

Figure 3.7 Stainless steel thermocouple rack

ends of the thermocouples were attached to a stainless steel rack using 10cm (4 inch) nylon cable ties (General Electric, Oklahoma City, OK). As demonstrated in Figure 3.7, each of the 7 rows of the stainless steel rack had a thermocouple placed at three different positions: 1 in the middle, 1 at the wall and 1 at the halfway point between these two, referred to as the intermediate position. The sides on which the thermocouples were attached alternated along the length of the rack in order to avoid creating an artificial preferential flow of air in the reactors. The thermocouples were bundled together at the center of the rack to minimize random distributions of air flow. A hole was drilled into the cover cap of the reactor to allow the outlet of the bundle of thermocouples into the data acquisition system.

3.5 Effluent Gas Measurement

Both oxygen (O_2) and carbon dioxide (CO_2) concentrations were monitored continuously at the exit of the reactor. The O_2 concentration was measured using O_2 sensors designed for high humidity environments by NeuwGhent Technology (NeuwGhent Technology, LaGrangeville, NY). The O_2 sensors had been calibrated to give output in the range of 000 to 500 to represent its 0 to 5 volts dc. To obtain the O_2 concentration, the metered readings were converted using the following equation:

$$\% O_2 = \left[1 - e^{-\left(\frac{V_O}{17{,}387}\right)}\right] \times 100 \qquad (3.1)$$

where V_o is the O_2 signal voltage output. The O_2 sensors were placed at one of the outlets from the reactor and connected to the data acquisition system.

The carbon dioxide concentration was measured using a hand-held Vaisala CO_2 meter (Vaisala, Woburn, MA) with a probe attachment that was secured to

another reactor outlet. The CO_2 sensors were obtained with data logging capability and CO_2 concentrations were recorded every 5 minutes for the duration of the composting process.

3.6 Data Acquisition System

Temperature and oxygen concentration measurements were monitored and recorded using a computer based data acquisition system. The system was comprised of 1 CIO-DAS08 analog and digital I/O board (Measurement Computing, Middleboro, MA) and 3 CIO-EXP32 data acquisition boards (Measurement Computing, Middleboro, MA). Two of the data acquisition boards were dedicated to thermocouples, while the remaining board housed the oxygen sensors.

A LabVIEW (National Instruments, Austin, TX) program was written to display and record the data being collected. The program was designed to display on-screen the temperature and oxygen concentrations of each individual sensor in 2 minute intervals. In addition to the displayed values of given sensors, the program also displayed the sensor values graphically in real time as the process progressed. The values of each sensor were then recorded to an Excel spreadsheet every 5 minutes, with the value recorded being an average of the values of the previous 5 minutes.

3.7 Sample Collection for Moisture Content, pH and DNA Extraction

Samples were collected from 6 ports of each reactor at pre-defined times for each reactor run for the duration of the run (at least 96 hours). Samples from port # 7 were not collected because it had been noticed from past experiments that as the composting process proceeded, the decrease in bed height left port # 7 bare mid-way through the experiment so data from that port would have been incomplete. Between 3 – 5 grams of solids were removed from each port at a given time point.

Once the samples were collected, 1 gram was weighed out into a vial for pH measurements. Ten milliliters of distilled water was added to each of the samples, and the mixture was mixed vigorously with a stirring rod for approximately 1 minute to form a slurry, and then allowed to equilibrate for another minute. pH measurements were done using a standard electrode pH meter (Thermo Orion, Beverly, MA) which was calibrated every 24 hours.

Thirty milligrams of each sample was also weighed out and placed into pre-labeled 2ml screw-top tubes. Prior to labeling, the bottom of the tubes had been filled with approximately 0.5g of 0.5mm diameter glass beads (Biospec Products Inc., Bartlesville, OK) and one 5mm glass bead (Biospec). The tubes were then autoclaved and labeled for compost sample collection. The tubes containing the samples were consequently stored at -20℃ until they were used in the DNA extraction procedure.

The remaining solids for each reactor sample, termed the wet mass (m_w) were then weighed and the masses were recorded for moisture content (MC) calculations. The samples were allowed to dry for 24hours in an oven at a temperature of 100℃ and then weighed again to obtain the dry mass (m_d) of the samples, which was also recorded. The following equation was then used to calculate the moisture content of each sample:

$$\%MC = \frac{m_w - m_d}{m_w} \times 100\% \qquad (3.2)$$

3.8 DNA Extraction

The frozen compost samples were thawed at 4℃ to begin the DNA extraction procedure. Eight hundred micro-liters of extraction buffer was added to each tube and the tubes were agitated used a bead beater Disruptor genie (Scientific Industries, Bohemia, NY) for 4 minutes. The extraction buffer consisted of : (i) 100mM EDTA

(ethylenediamine tetraacetic acid) – used to inactivate metal-dependent enzymes that could damage DNA, (ii) 100mM NaCl (sodium chloride) – needed to draw water out of cells by osmosis, (iii) 100mM Tris (trishydroxymethylaminomethane) – necessary to keep DNA protonated and soluble in water, (iv) 5% PVPP w/v (polyvinylpolypyrrodidone) – used to remove impurities, namely humic acids, from solution, (v) 2% SDS w/v (sodium dodecyl sulfate) – anionic surfactant needed to denature protein and (vi) 2% CTAB w/v (Centrimonium bromide / hexadecyltrimethylammonium bromide). The bead beater was used to lyse the cells and the tubes were centrifuged for 2 minutes at 14,000 ×g using a bench top centrifuge. Eight hundred micro-liters of phenol:chloroform:isoamyl alcohol, 25:24:1, v:v:v solution was added to the sample to separate out the DNA from the impurities. The tubes were agitated for 1 minute using the bead beater to evenly disperse the phenol/chloroform/isoamyl solution and centrifuged for 2 minutes at 14,000 ×g to separate out the DNA based on solubility. The top layer (aqueous phase) of the centrifuged tube was then transferred to a 2ml phase lock gel tube (5 Prime, Gaithersburg, MD) that contained 800µl of chloroform:isoamyl alcohol, 24:1, v:v solution. The solution was homogenized by inverting the tube several times and the tubes were centrifuged for 5 minutes at 14, 000 ×g. The chloroform solution was used to purify the DNA by extracting out the phenol based on density differences – the heavier phenol solution would be the bottom layer, while the lighter, aqueous DNA layer would stay on top. The phase lock tubes were used to separate the two phases, thereby making it easier to distinguish the two. The phase lock tubes contained a gel which lay at the bottom of the tube before centrifugation, and then came to rest at the interface of the two phases by the end of the centrifugation time. The chloroform purification was subsequently repeated to further extract any remaining phenol. Next, the aqueous layer was transferred to a 1.5ml Eppy tube containing 800µl of

isopropanol. The solution was gently homogenized by inversion and the tubes were placed on ice for 15 minutes. The tubes were centrifuged for 20 minutes at 4℃ to precipitate out the DNA. After centrifugation, the supernatant was discarded and 100µl of 70% ethanol was added to each tube to wash the pellet. The tubes were then centrifuged for 30 seconds at 10,000 ×g and the ethanol wash was repeated. All remaining ethanol was completely discarded and the tubes were allowed to air dry for a few minutes. At that point, 100µl of nuclease free water was added to each tube and the tubes were vortexed to re-suspend the DNA pellets. A 5µl aliquot of each sample was then withdrawn and diluted in 95µl of distilled water to perform absorbance readings using a BioTek PowerWave HT Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT) at: (i) 230nm – to detect any residual phenolic compounds, (ii) 260nm – to detect DNA and (iii) 280nm – to detect the presence of proteins. The spectrophotometer was calibrated and corrected for a 1cm pathlength before each new use. The remainder of the sample was stored at -20℃.

In addition to extracting DNA from the compost samples, DNA was also extracted from pure cultures of yeast, *E.coli* and fungi to serve as standards during the hybridization. These cultures were extracted using a MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Absorbance readings were also performed using the BioTek Power Wave HT Microplate Spectrophotometer and the samples were stored at -20℃.

3.9 Dot Blots

Positively charged Bright Star Plus nylon membranes (Ambion, Austin, TX) were first cut o size – 22.5cm in length and 6cm in width. The frozen compost, yeast, *E.coli* and fungi DNA samples were thawed at 4℃ and then the compost DNA samples were diluted by adding 10µl of the sample to 90µl of 2X SSC Buffer in a

1.5ml Eppy tube. The yeast, *E.coli* and fungi were diluted in 2X SSC Buffer to obtain samples containing (i) 500ng, (ii) 1µg, (iii) 5µg, (iv) 10µg, and (v) 20µg of DNA. The extracted fungi DNA was not very concentrated and so the samples for fungi ended at 10µg. The diluted samples were then denatured by boiling for 5 minutes at 100℃. The tubes were then immediately placed on ice to avoid recombining the DNA strands. The tubes were centrifuged briefly and returned to the ice. Next, the orientation of the pre-cut membrane was marked and labeled. Following that, the dot blot vacuum apparatus, Minifold II Slot-Blotter (Schleicher and Schuell, Keene, NH), was set up. Two pre-cut pieces of blotting paper (Schleicher and Schuell, Keene, NH) were wetted in 2X SSC Buffer for about 30 seconds and then placed on the vacuum apparatus assembly. The nylon membrane was also wet in 2X SSC Buffer and placed on top of the blotting paper. The final assembly of the dot blot apparatus (Figure 3.8) was completed according to the manufacturer's instructions and a vacuum was pulled through the membrane. The 100µl volumes of denatured, diluted samples were then applied to pre-designated wells. After all the samples had been loaded on the membrane, a 100µl aliquot of 2X SSC Buffer was applied to each of the wells to remove any DNA that got stuck to the wells. The vacuum was then stopped and the apparatus disassembled. Each membrane was blotted with compost DNA samples and the respective standard that would be probed for. The membrane was removed and baked at 80℃ for 15 minutes to allow for the cross linking of the DNA. The dried membrane was kept in Saran wrap at room temperature until the start of the hybridization process.

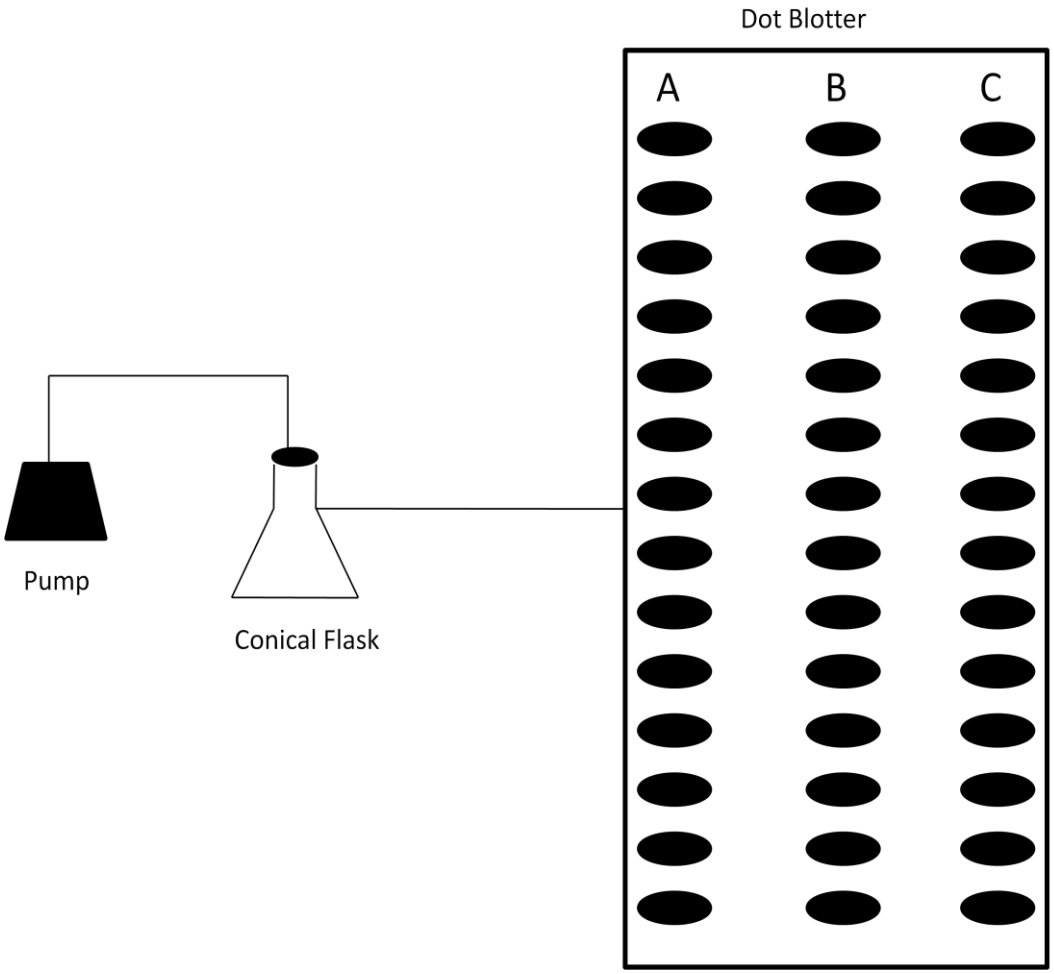

Figure 3.8 Dot Blot apparatus set up

3.10 Probe Design and Synthesis

Three probes were selected for the hybridizations – yeast, fungi and bacteria, which represent the groups playing a dominant role in the composting process. The relevant information for each of the probes used is summarized in Table 3.3. Once the sequences of the probes were determined, the probes were synthesized and purified by IDT DNA (Integrated DNA Technologies Inc., Coralville, IA).

3.11 Hybridization

The pre-hybridization process began by warming up 14ml (10ml buffer/ 100cm^2 membrane) of Northern Max hybridization buffer (Ambion, Austin, TX) for 15 minutes at 37°C, using glass hybridization bottles (VWR International, West Chester, PA) placed in a hybridization oven (VWR International, West Chester, PA). Once the hybridization buffer was warmed up, the membrane was sealed in the hybridization bottle and incubated at the probe hybridization temperature for at least 30 minutes. The hybridization temperatures used for the 3 different probes were: (i) bacteria - 45.5°C, (ii) fungi - 43°C and (iii) yeast - 35.8°C. These hybridization temperatures, T_m, were calculated using the following formula provided by the hybridization buffer manufacturer:

Table 3.3 Hybridization probe data

Probe Name	**Sequence 5′ - 3′**	**Target Group**	**Melting Temperature(℃)**	**Reference**
S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	Universal Bacteria	55	Schloss et al., 2004
NS8	TCCGCAGGTTCACCTACGGA	Universal fungal	56	Peters et al., 2000
PF2	CTCTGGCTTCACCCTATTC	Yeast	51	http:www.microbial-ecology.net

$$T_m = 35℃ + 0.8(\%G + C) - \frac{500}{duplex\ length} - 15℃\ (for\ mismatch) \quad (3.3)$$

Once the pre-hybridization procedure was complete, the probe was diluted in 500µl of hybridization buffer to bring the probe to a final concentration of 0.1nM (10ng/ml) (14µl of probe per 140cm^2 membrane). The diluted probe was consequently added to the pre-hybridization buffer in the hybridization bottle. The bottle was re-sealed and it was ensured that the membrane was surrounded by the hybridization buffer. The membrane was then incubated overnight (14 – 24hr) at the probe hybridization temperature.

3.12 Detection of Probe Hybridization

The non-isotopic chemiluminescent detection of the probes was accomplished using a BrightStar BioDetect kit (Ambion, Austin TX) using the following outlined protocol. At the end of the hybridization period, the membrane was washed twice for 5 minutes each in 1X Wash Buffer using 1ml of Wash Buffer per cm^2 membrane per wash. The membrane was incubated twice for 5 minutes each in 1X Blocking Buffer at room temperature, using 0.5ml Blocking Buffer per cm^2 membrane per incubation. Next, the membrane was incubated for 30 minutes in 1X Blocking Buffer, using 1ml Blocking Buffer per cm^2 membrane. Diluted Streptavidin-Alkaline Phosphatase (Strep-AP) was prepared by mixing together 14ml of 1X Blocking Buffer and 1.4µl of Strep-AP per 140cm^2 membrane. The membrane was incubated for 30 minutes at

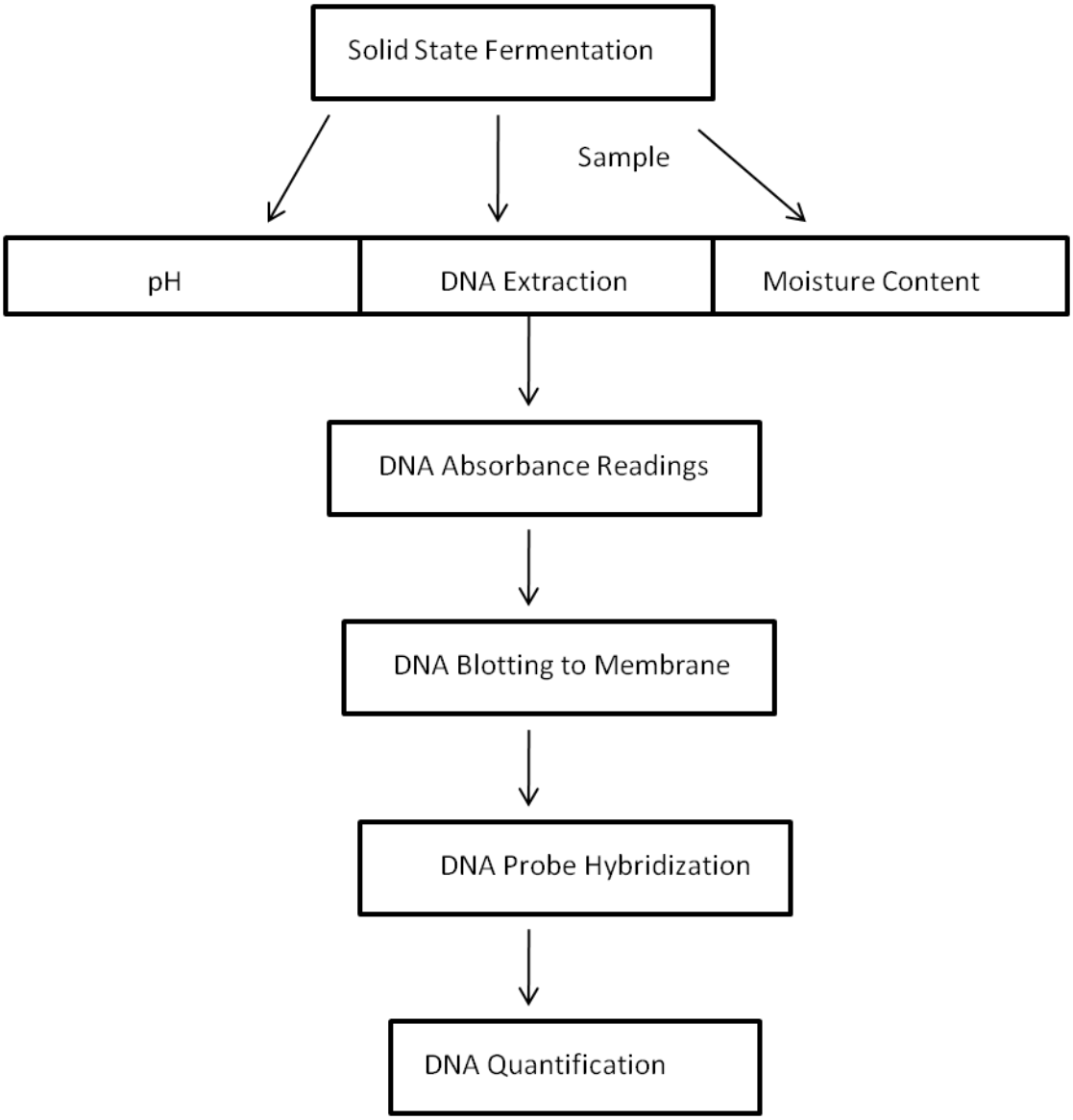

Figure 3.9 Major Process Steps

room temperature in the diluted Strep-AP solution. Then, the membrane was incubated for 10 minutes in 1X Blocking Buffer at room temperature, using 0.5ml Blocking Buffer per cm^2 membrane. Afterwards, the membrane was washed 3 times for 5 minutes each in 1X Wash Buffer, using 1ml 1X Wash Buffer per cm^2 membrane per wash. Following this, the membrane was incubated twice for 2 minutes each in 1X Assay Buffer, using 0.5ml Assay Buffer per cm^2 membrane per incubation. Consequently the membrane was incubated for 5 minutes in CDP-Star using 5ml CDP-Star per 100 cm^2 membrane. The excess CDP-Star was shaken off by quickly blotting the membrane on filter paper without letting the membrane dry. The membrane was covered in a single layer of Saran Wrap, covered with foil, and allowed to sit for 1 – 2 hours. The light emission from the membrane was captured using a G:Box HR imager (Syngene, Frederick, MD) for 1 hour. The observed bands were then quantified using the imager software, GeneSnap Tools (Syngene, Frederick, MD).

The overall process scheme is shown in Figure 3.9.

Chapter 4

Results & Discussion

The physical and biological data presented in this chapter are used to gain a better understanding of the intricate relationships that contribute to reactor performance and observed trends. The most readily observed trend was that most of the reactors went through all the phases of composting: (i) mesophilic phase, (ii) active/thermophilic phase, and (iii) mesophilic phase as depicted in Chapter 2 within 96 hr, with the exception of a couple of reactors which only made it to the thermophilic phase during different reactor runs. Not only did the reactors go through all the phases, but they produced similar behaviors and trends when replicated; thus performance reproducibility was established and documented. From previous work it was also observed that by the end of 96 hr, the sampling port at the top of the reactor located at 0.7m above the perforated plate (Figure 3.1) did not contain any samples due to shrinkage of the substrate bed during composting. This led to the port at 0.6m above the perforated plate being taken as the top of the reactor. The results and analyses of observed phenomena are presented in the next few sections according to the averaged value of the measured variable.

4.1 Temperature

One of the first objectives in measuring temperature was the determination of the temporal radial profiles of the reactors. Figure 4.1 shows the temperature profile at 0.6m above the perforated plate of the 3 different reactors: 70%, 65% and 60% moisture content wet basis (MC w.b.). This figure is just one example of a similar

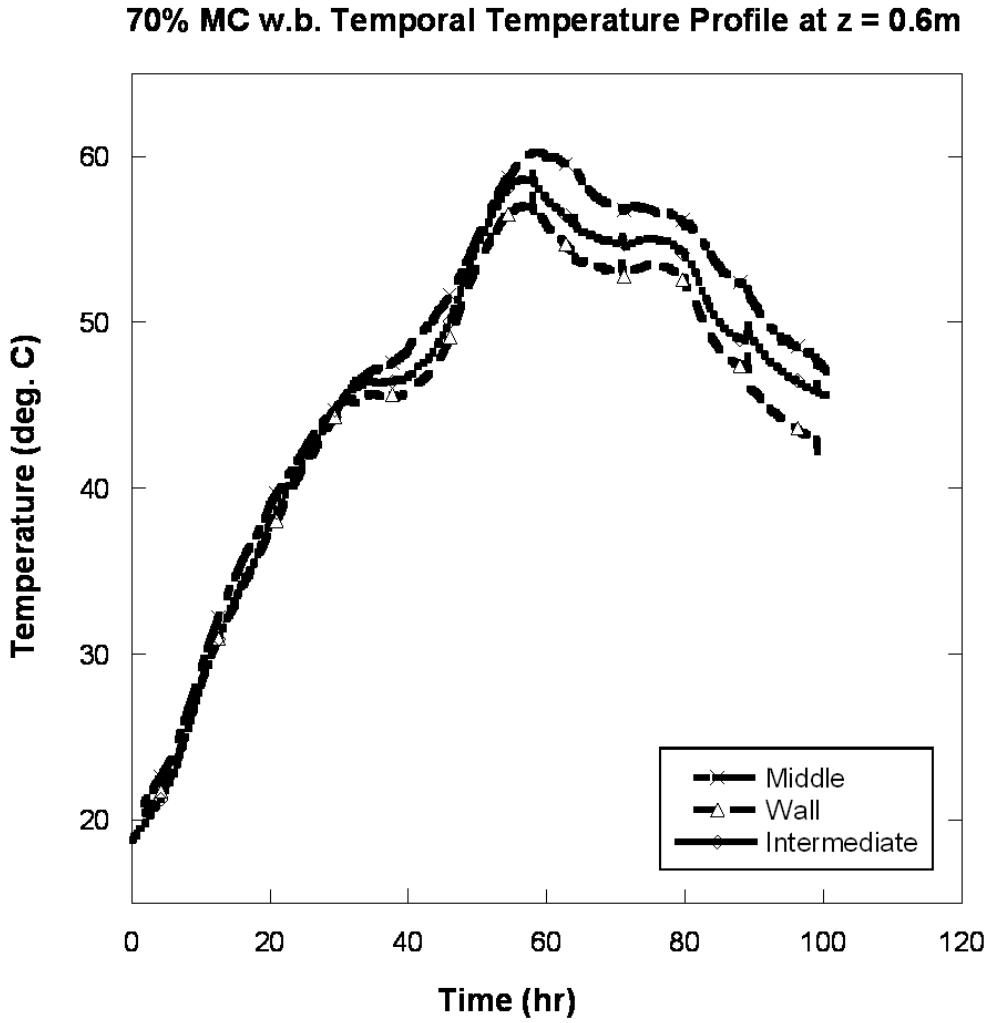

Figure 4.1 (a) Radial temperature profiles at 0.6m above perforated plate in 70% MC w.b. reactor

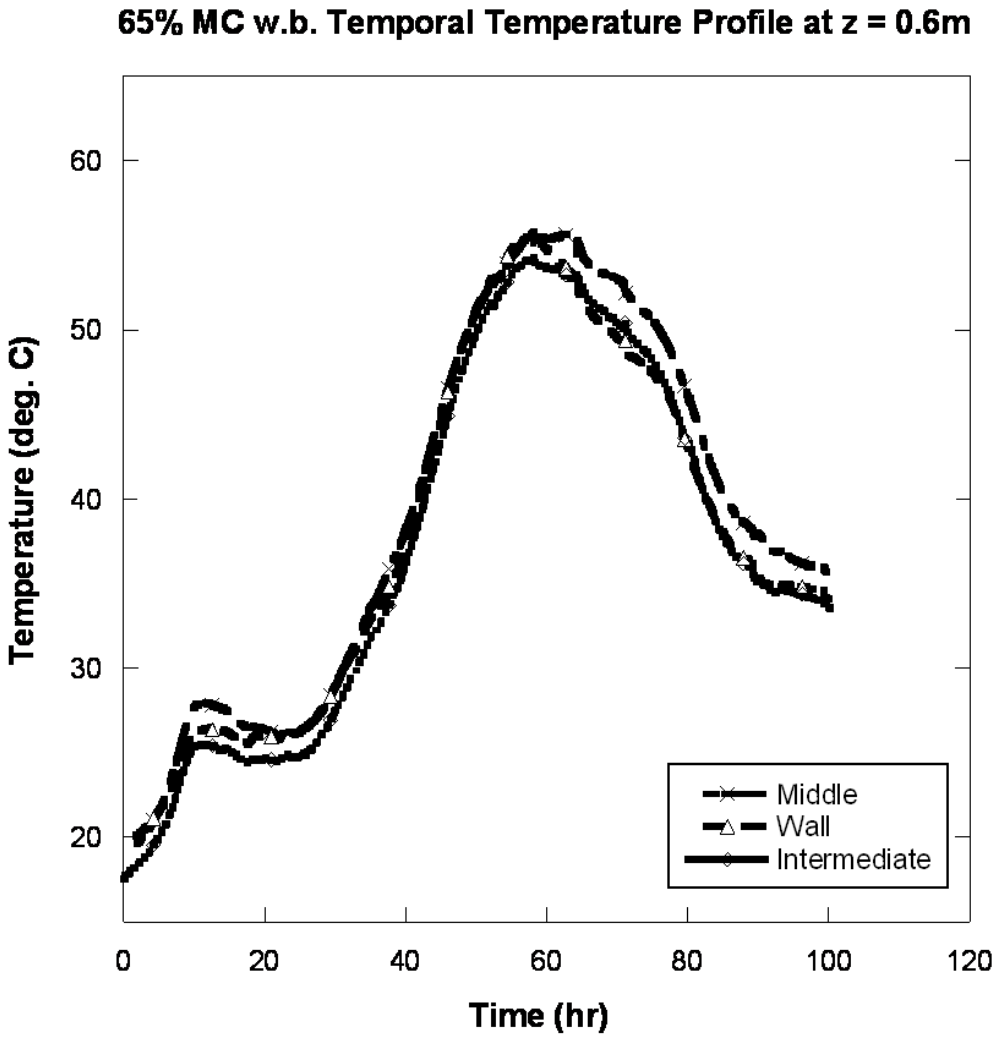

Figure 4.1 (b) Radial temperature profiles at 0.6m above perforated plate in 65% MC w.b. reactor

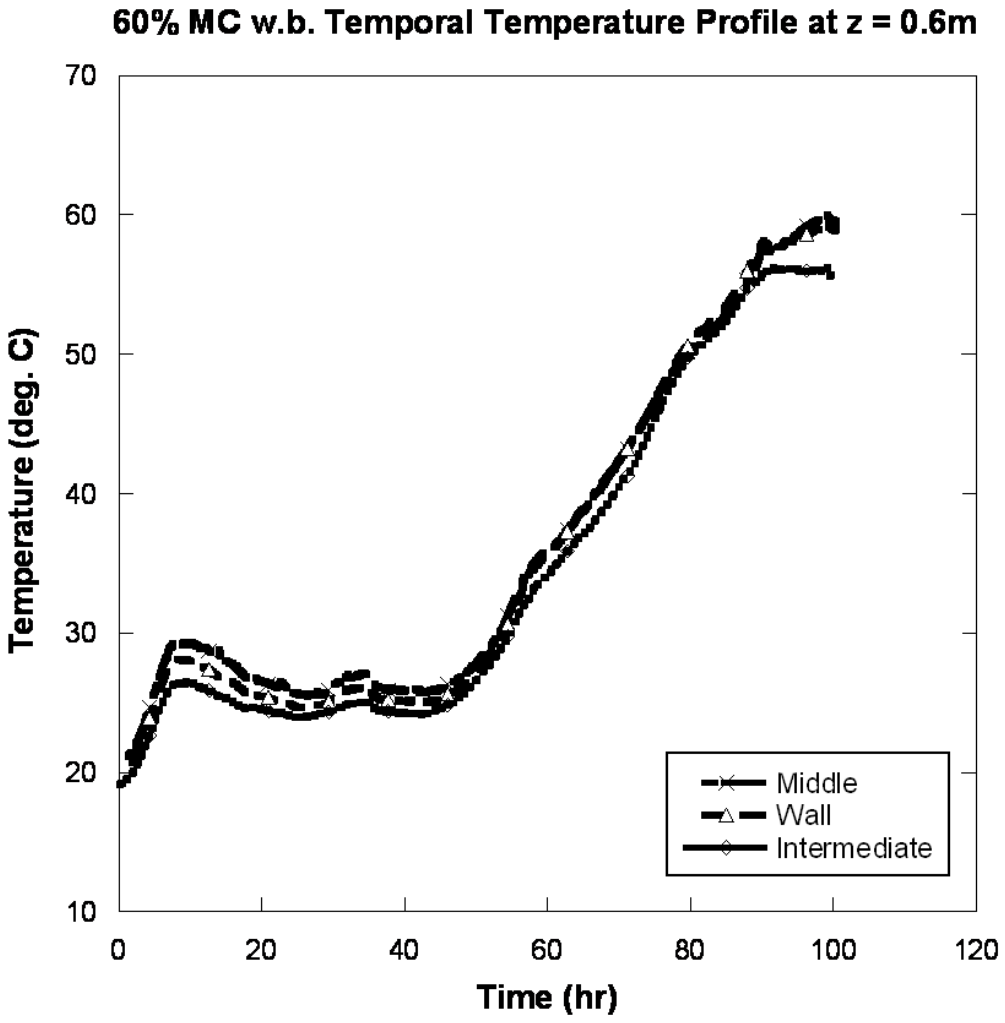

Figure 4.1 (c) Radial temperature profiles at 0.6m above perforated plate in 60% MC w.b. reactor

trend observed for many of the other points in the reactor over the different runs. The calculated radial gradients (not shown) between the center and the wall of the reactors was less than $1℃/cm$ for most of the points along the reactor. This trend confirms that radial gradients were minimized for the duration of the process, indicating that the bulk flow of energy may be modeled as a one dimensional heat transfer problem and that the temperature profile could be accurately described by the temperatures at the middle of the reactor.

Comparing the temperature profiles on the basis of different moisture contents led to the observation that the system with the highest initial MC reached the highest peak temperature during the process. The highest temperature observed throughout all of the reactors and the different runs was $62℃$ in the 70% MC w.b. reactor, for which the starting temperature was recorded as $20℃$. Figure 4.2 shows the temperature profiles for the 3 different moisture contents at 3 different heights within the reactor, providing both temporal and spatial variation in the temperature profiles as a function of time. The spatial variation shows that over time, the positions towards the top of the reactor consistently had higher temperatures when compared to points below them. This may be due to the cooling effect of the inlet air; however, as the air travels from the bottom of the reactor to the top, its heat capacity, as well as its evaporative cooling capacity drops, thereby limiting its ability to remove heat from the top part of the reactor and resulting in axial temperature gradients. The cooling influence of the inlet air on temperature profiles is somewhat noticeable up to 0.3m above the perforated plate as shown in Figure 4.2(b). Temporally, the reactors show a relatively rapid self-heating process from an ambient temperature of $22℃$ to a temperature of $50℃$ within 30 hours. Temperatures of greater than $50℃$ were maintained for as many as 59 hours in some cases before dropping. For the most part, the 60% MC w.b. reactor had the lowest temperatures, while the 70% MC w.b. reactor

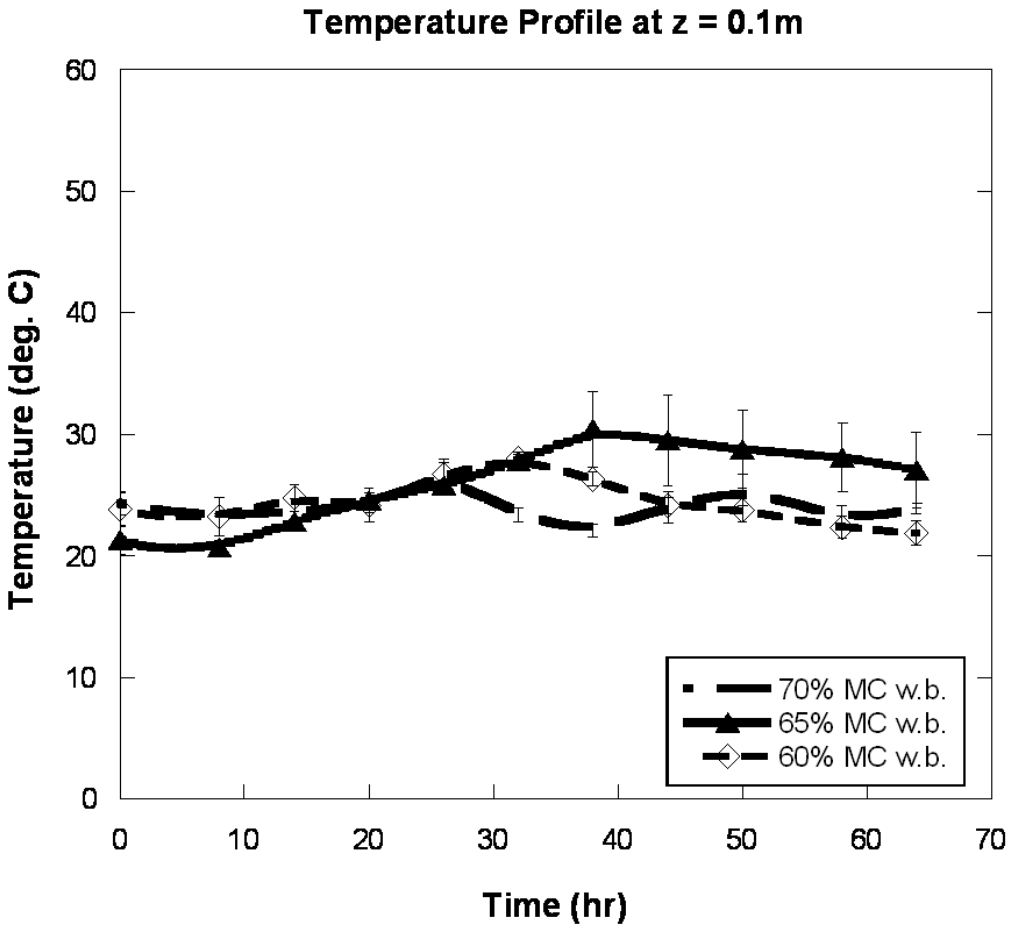

Figure 4.2 (a) Temporal temperature profile as a function of initial MC w.b. at 0.1m above the perforated plate

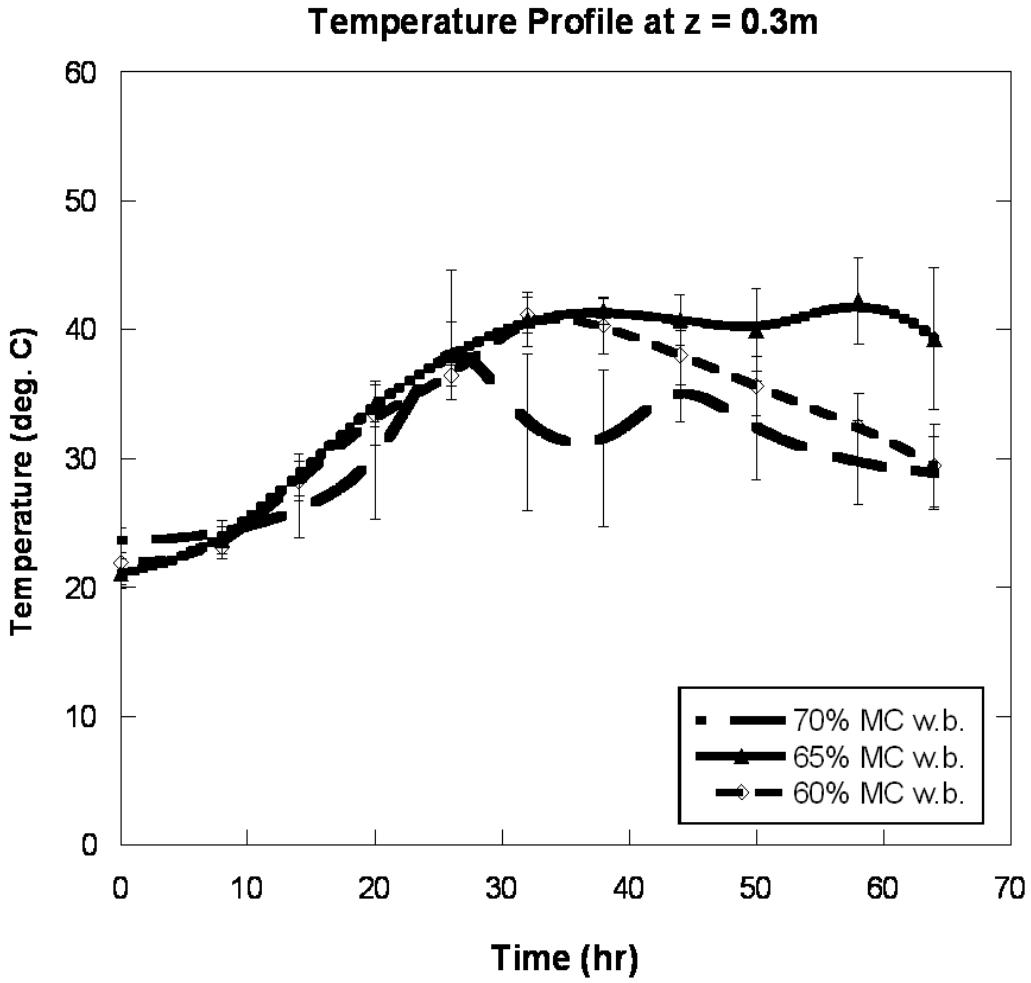

Figure 4.2 (b) Temporal temperature profile as a function of initial MC w.b. at 0.3m above the perforated plate

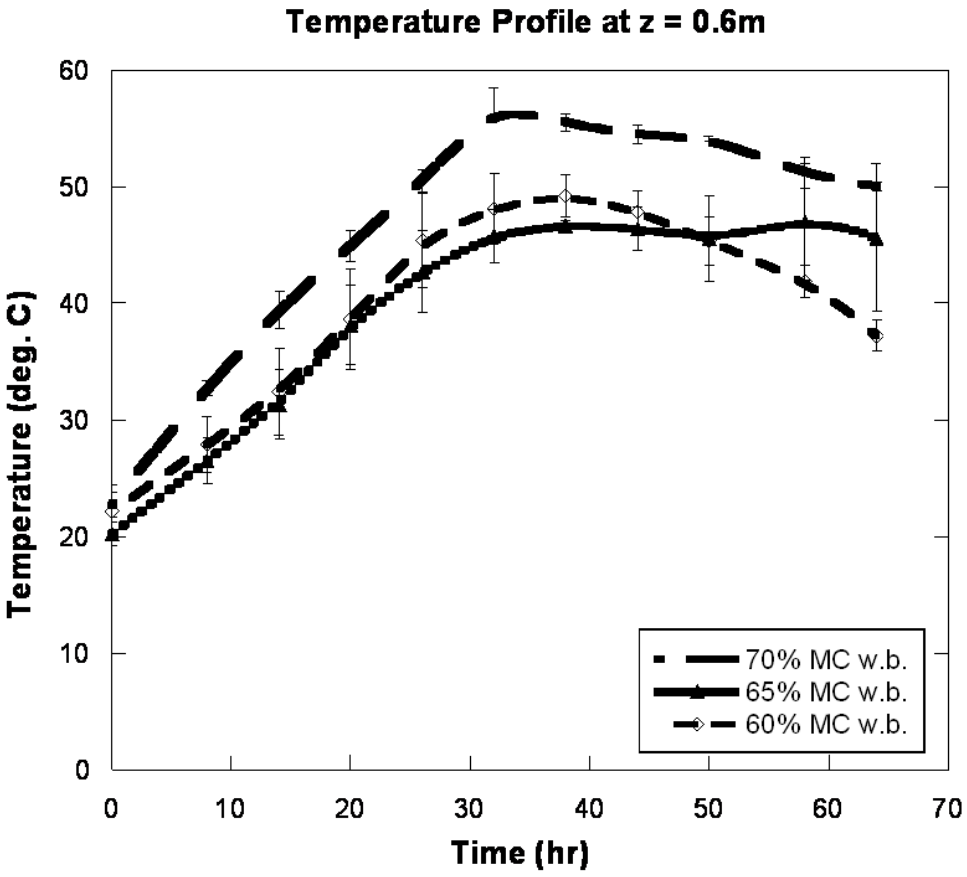

Figure 4.2 (c) Temporal temperature profile as a function of initial MC w.b. at 0.6m above the perforated plate

had the highest temperatures, with the 65% MC w.b. reactor falling in between those two. The observed increases in temperature reflect an increase in microbial activity within the reactors and is suggestive of the evolution and succession of the microbiological communities.

Figure 4.2 also shows some fairly noticeable error bars for the replicates – these errors provide an indication of the system variations between reactor runs, given that the physical initial conditions were the same. These system variations could be due to a number of factors including variable ambient conditions ($\pm$ 5°C in temperature) of the room in which the reactors were contained and the presence of different initial microbial communities. The most variability was observed for the 65% MC w.b. reactor which took the longest time to ramp up to thermophilic conditions during all 3 runs.

4.2 O_2 and CO_2 Evolution

The O_2 consumption and CO_2 evolution began almost immediately after the aeration was turned on. The effluent O_2 concentration started at a value of 19% and continuously dropped in accordance with the rise in temperature suggesting an increase in microbial activity. The lowest observed value for all of the runs completed was 16%. This small drop in O_2 concentration suggests that aerobic conditions were maintained for surfaces in contact with the gas and suggests that within the substrate bed there was enough oxygen to support microbial growth and activity. Figure 4.3 shows the average oxygen consumption and the average CO_2 evolution profiles obtained for the 3 different moisture contents. The 65% and 70% MC w.b. reactors maintained lower O_2 levels for extended periods of time whereas the 60% MC w.b. reactor exhibits an upturned parabolic behavior in order to return to atmospheric conditions, with the converse being true for the CO_2 profiles. In all three cases, the

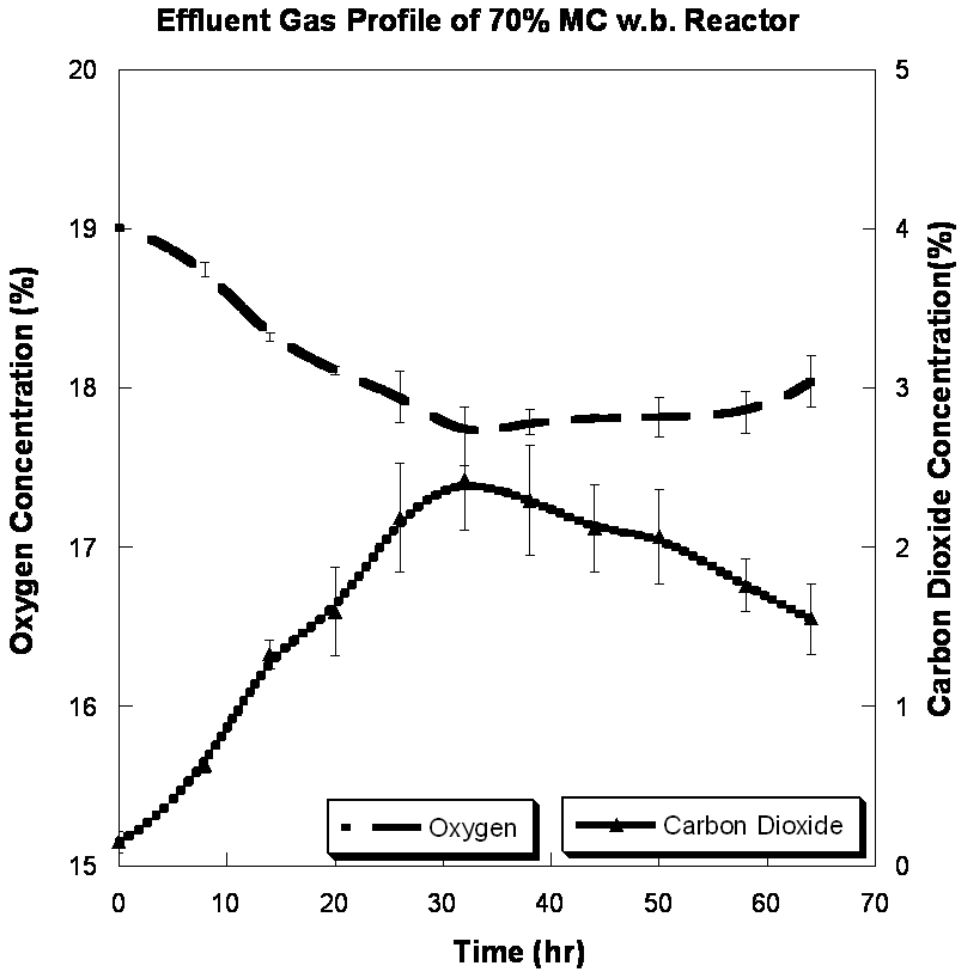

Figure 4.3 (a) Effluent gas profiles for 70% MC w.b. reactor

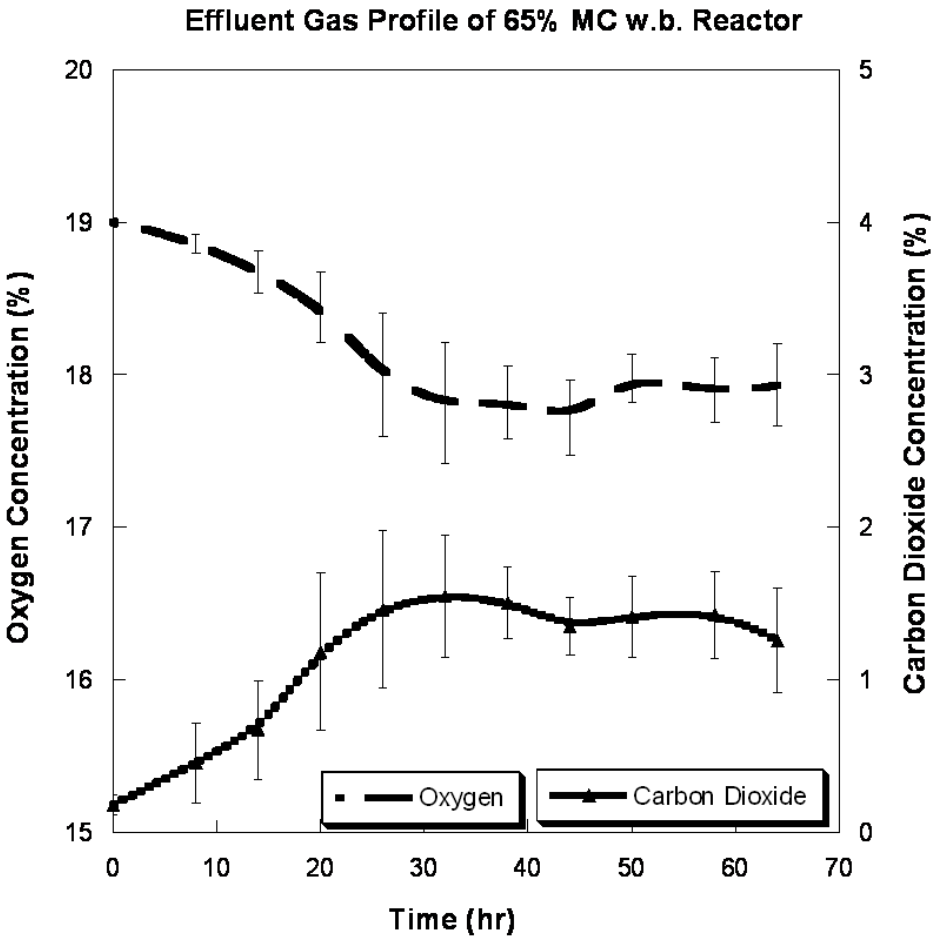

Figure 4.3 (b) Effluent gas profiles for 65% MC w.b. reactor

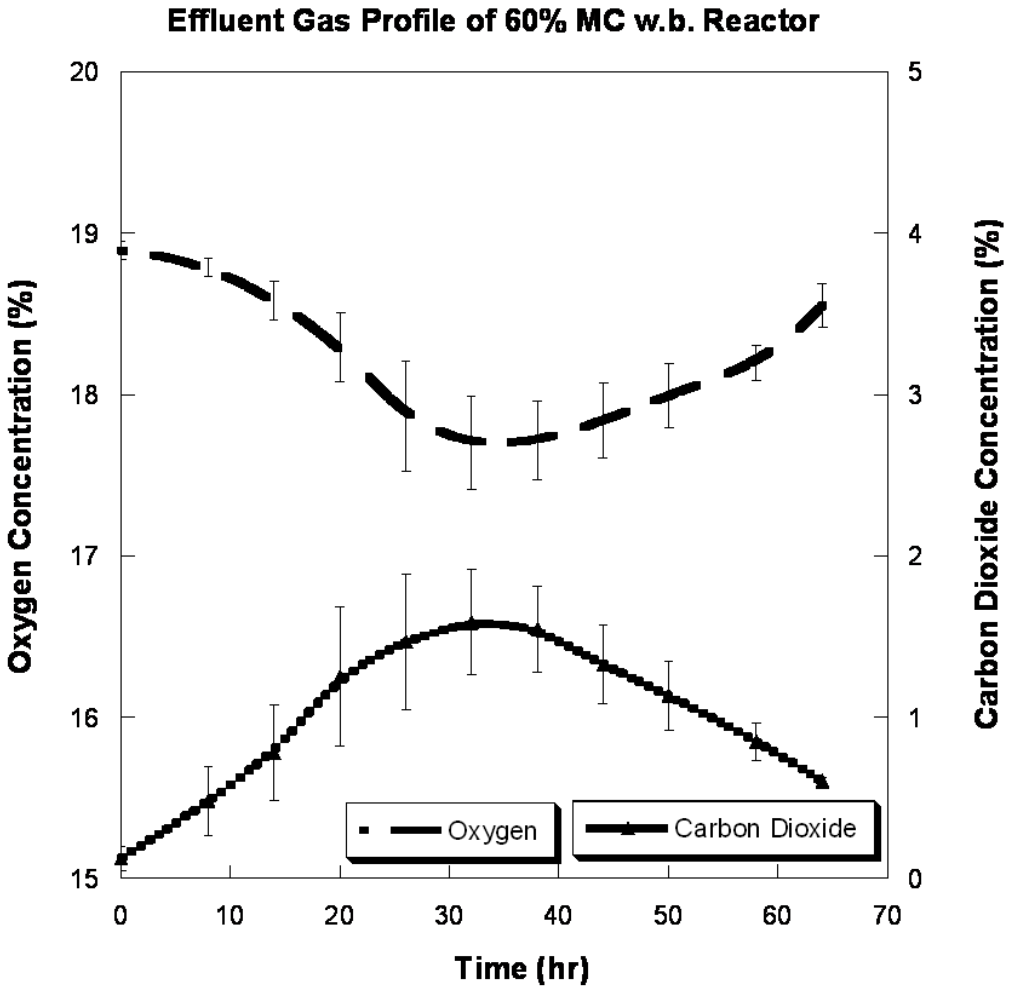

Figure 4.3 (c) Effluent gas profiles for 60% MC w.b. reactor

CO_2 production profile mirrored the O_2 consumption profile suggesting a balanced relationship between the input and output of the microorganism. A better parameter for evaluating microbial growth would be the O_2 consumption rate whose calculated values indicate how much oxygen is transferred from the gas phase to the solid substrate as a function of time. The oxygen consumption rate (OCR) was calculated using the following equation:

$$OCR = O_i - O_e \quad (4.1)$$

where O_i is the oxygen influent rate (moles/min) and O_e is the O_2 effluent rate (moles/min). The O_2 concentration in the effluent gas was calculated using the following equation:

$$O_e = \frac{P \times Q \times C}{R \times T} \quad (4.2)$$

where:

P = atmospheric pressure (atm)

Q = aeration flow rate (L/min)

C = concentration of oxygen in effluent (mole fraction)

R = universal gas constant (atm*L/mol*K)

T = temperature at given time point (K)

The O_2 concentration in the inlet gas was calculated using the same equation as for O_e with the exception that the concentration of O_2 in the influent, C, remained constant at a mole fraction value of 0.209 (mole/mole). The resulting O_2 consumption rate profiles are shown in Figure 4.4 and it again shows the highest activity being found in the reactor with the highest initial moisture content. The OCR peaked at about 30 hours into the process, in keeping with the peak observed from the temperature profiles. The small values obtained for the oxygen consumption rate provide an indication of the recalcitrant nature of switchgrass as a substrate – a common issue with cellulosic biomass.

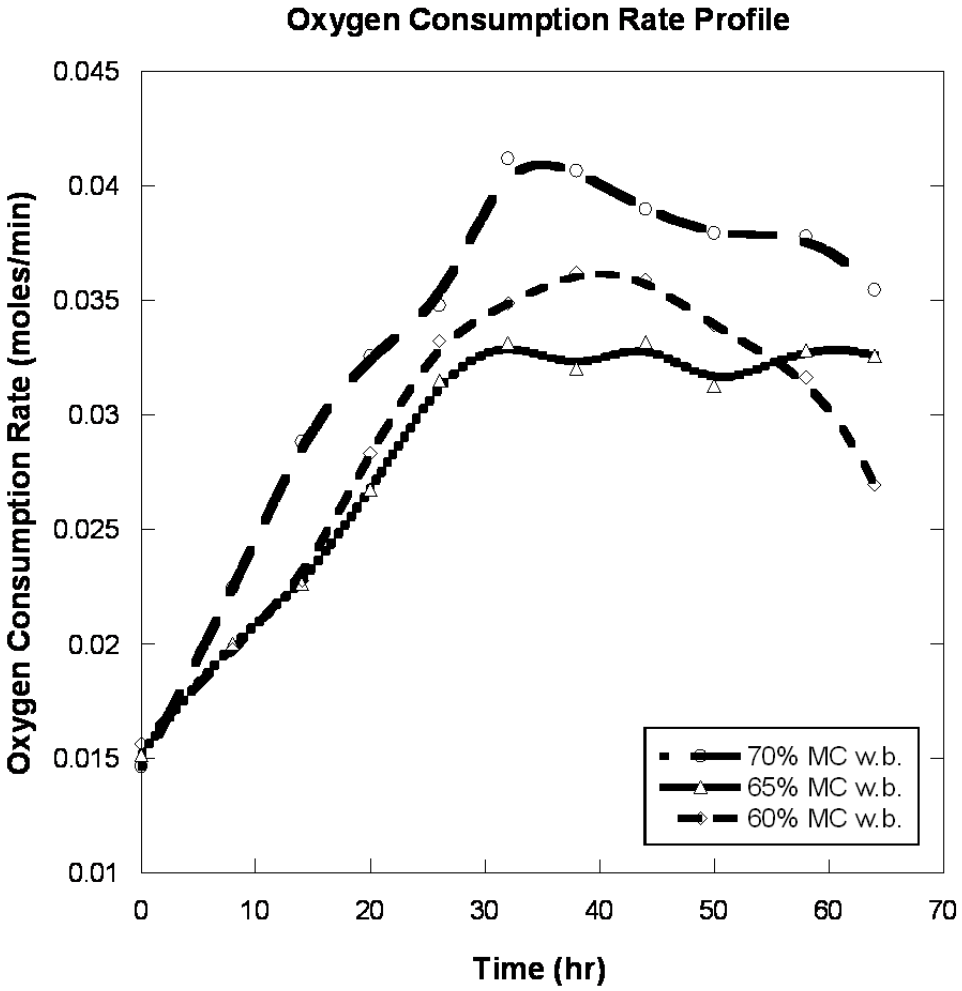

Figure 4.4 Oxygen Consumption Rate

Both oxygen and carbon dioxide concentrations were measured only at the exhaust of the reactors and so their trends provide only a broad view of the overall system behavior. It would be interesting to obtain gaseous concentration data at the same ports that samples were taken from in order to better correlate observed patterns. This would; however, be difficult to do with the current system because of the difficulty associated with obtaining oxygen sensors that would be able to withstand high moisture and acidic pH environments over an extended period of time. In addition, further instrumentation of the reactors could impede the bulk flow of air within the system.

4.3 pH

Due to the acidic nature of dog food and switchgrass, the initial pH of the mixed substrate was acidic, and became increasingly acidic as the process started, but then rapidly climbed to basic conditions after 12 hours as shown in Figure 4.5. Despite some fluctuations in the pH profiles, all the reactors followed the same trend of a dip in pH after start up, followed by a rise to alkaline environments with the highest moisture content reactor possessing the highest pH values. It also appears that there are 2 peaks in the pH profiles for all the reactors – one at 12 - 18 hr and the other at 36 hr, indicating a shift in the microbial population at those times. Spatially there were no significant changes in pH values at a given time point as one moved from the bottom of the reactor to the top. This is noticeably different from the temperature profile where there were significant changes in the temperature values as one moved from the bottom of the reactor to the top.

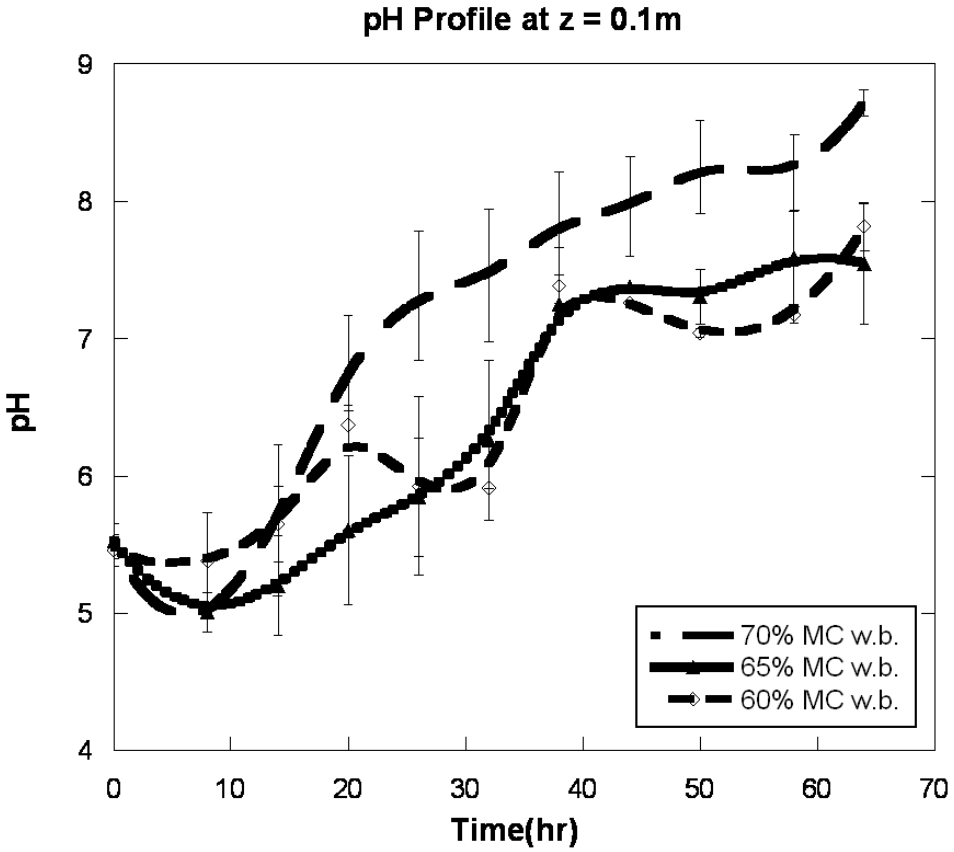

Figure 4.5 (a) pH profiles for varying moisture content at 0.1m above the perforated plate

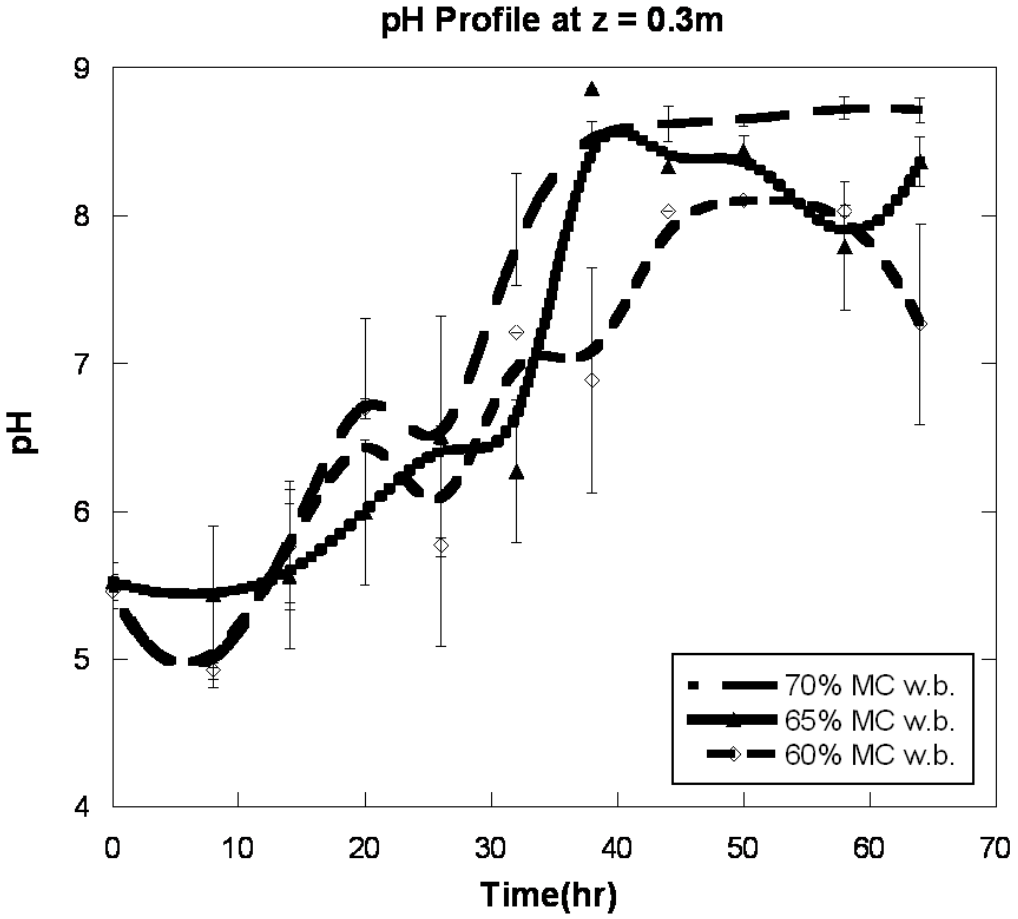

Figure 4.5 (b) pH profiles for varying moisture content at 0.3m above the perforated plate

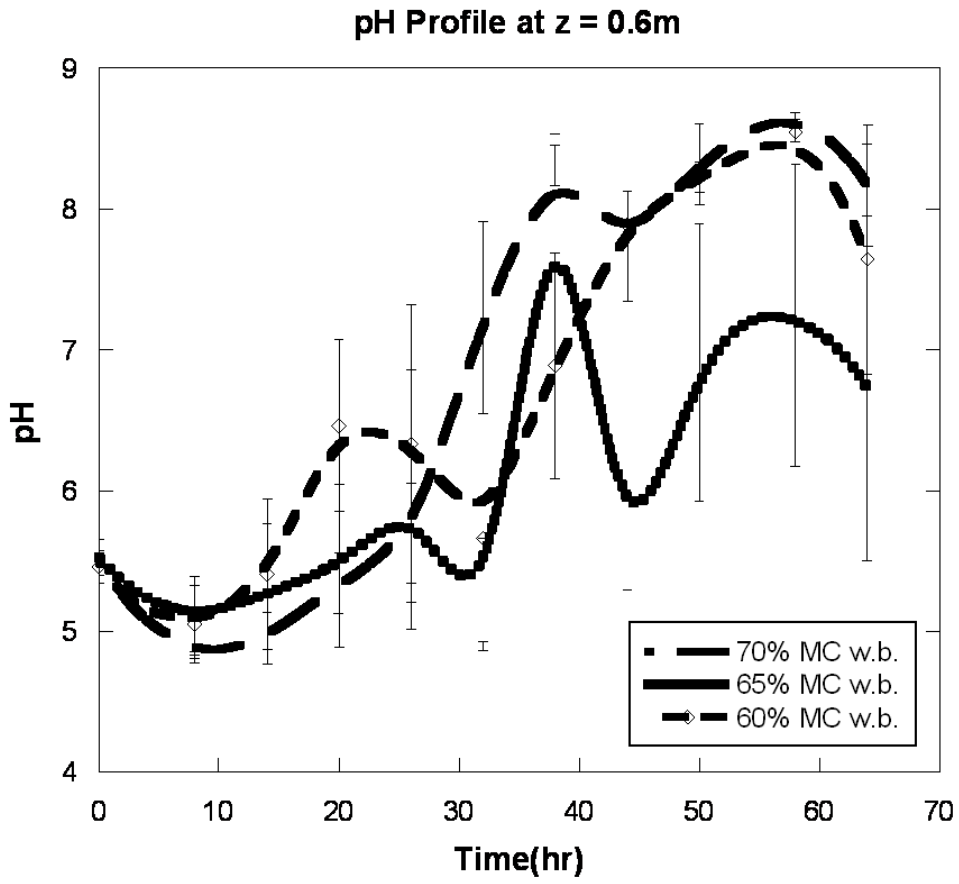

Figure 4.5 (c) pH profiles for varying moisture content at 0.6m above the perforated plate

The rapid increase in pH suggests that ammonia was being produced from the degradation of proteins, further indicating that the rate of protein degradation increased during the thermophilic stage. The production of ammonia was also evident from the odor of the

exhaust gas as thermophilic conditions were reached. There is also the added possibility that the ammonia reacted with carbon dioxide and water to produce ammonium ions and bicarbonate ions which could be further decomposed to produce hydroxide ions that increased the system alkalinity.

4.4 Moisture Content

The moisture content of the reactors remained fairly constant during the entire process as shown in figure 4.6. At 0.3m above the perforated plate, there is a drop in the moisture content at the end of the process where evaporation within the substrate bed led to some drying of the bed; however at a height of 0.6m within the bed, there is a slight increase in moisture content at the end of the process indicating greater microorganism activity. Beyond that, there were no significant spatial differences in the reactors. The average initial moisture content values were found to be slightly higher than the set point values; but this did not appear to affect the overall profile trends.

4.5 Population Dynamics of Compost Microorganisms

The presence of bacteria, fungi and yeast in the compost samples as investigated by probe hybridization revealed that these groups existed in large enough quantities to be detected by DNA probes in most of the samples. The bacteria and yeast hybridized samples produced bands

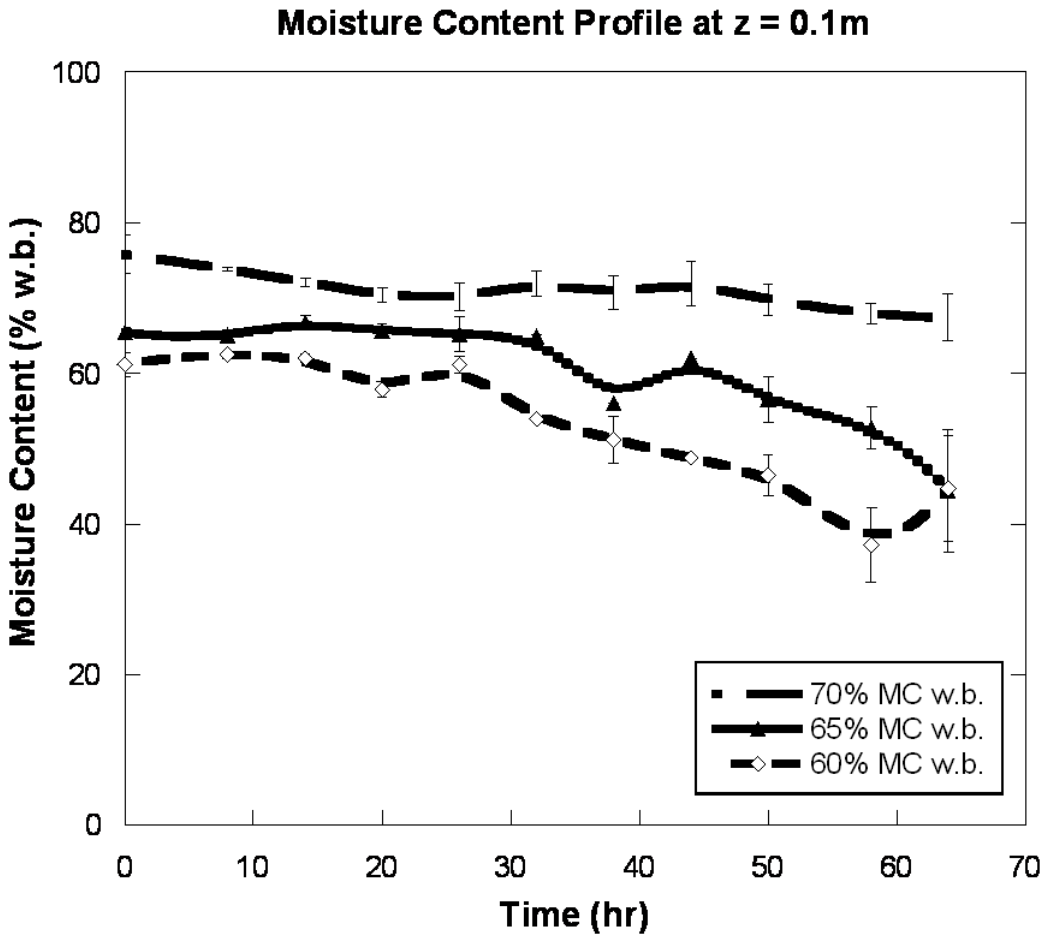

Figure 4.6 (a) Moisture content profiles for varying MC w.b. at 0.1m above the perforated plate

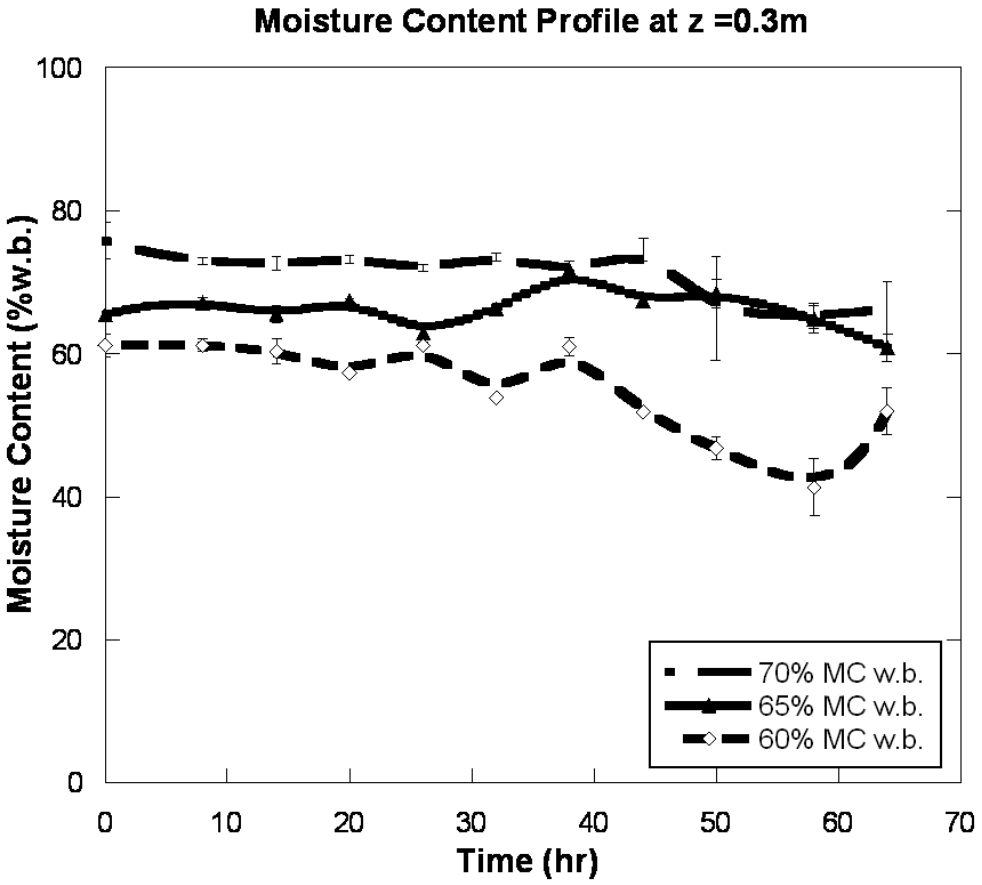

Figure 4.6 (b) Moisture content profiles for varying MC w.b. at 0.3m above the perforated plate

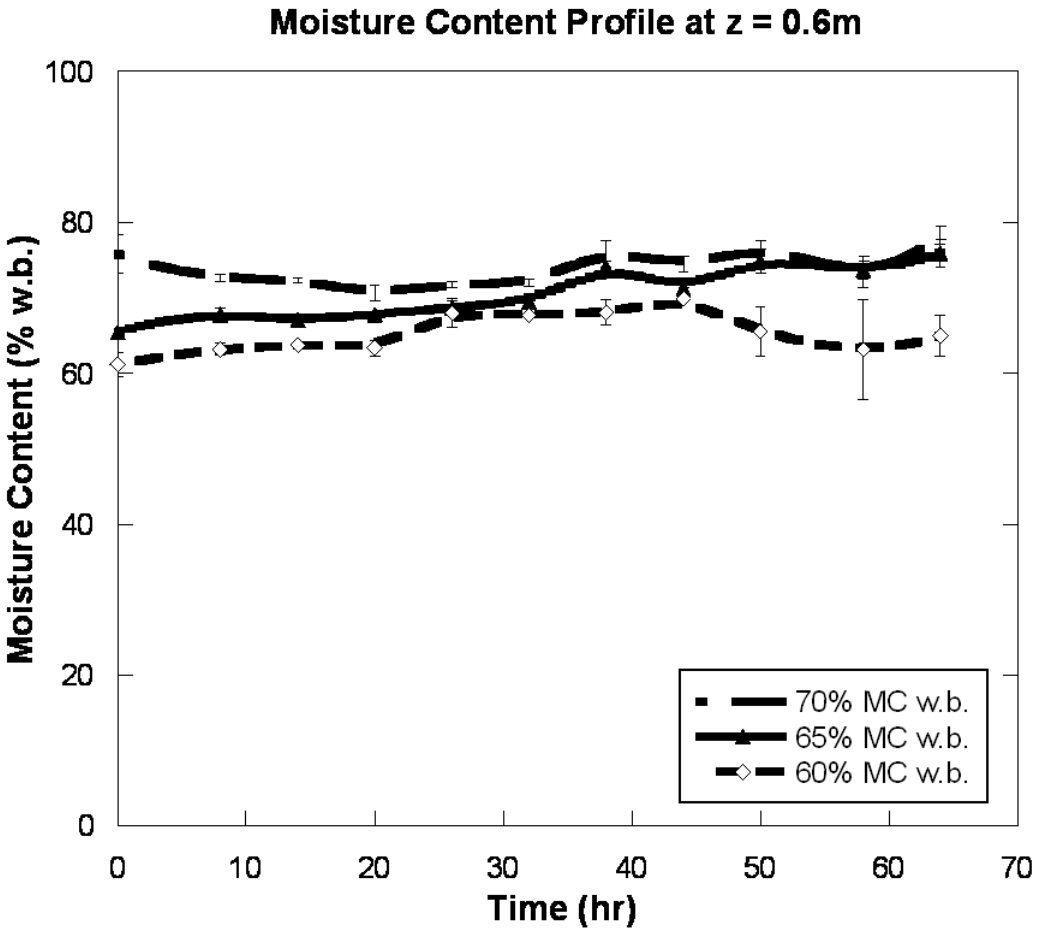

Figure 4.6 (c) Moisture content profiles for varying MC w.b. at 0.6m above the perforated plate

that were readily visible; however, the fungi hybridized membranes did not produce many readily visible bands. This could be due to the fungal probe not being specific enough for the fungal communities present in the compost pile, or it could be that the fungi were present in smaller quantities than those needed to produce bands by the detection procedure.

The use of chemiluminescence to detect the probe hybridization not only avoided the dangers of using radioactive isotopes, but also produced images that were easy to capture using a Charge-Coupled Device (CCD) camera instead of film. Figure 4.7 shows one such image captured for a membrane that was hybridized with the bacterial probe. The top left hand corner of the membrane contains bacterial standards that were used to quantify the bacteria present in the compost samples. To account for variability in the hybridization procedure, each membrane contained a set of standards, but it was found that the standards degraded slightly over time and this may have contributed some error to the quantification procedure.

The transition from mesophilic conditions to thermophilic conditions within the compost pile was evident from the increase in microbial population over time as shown in Figure 4.8. The average total DNA, comprising of bacteria, yeast and fungi produced one peak in the 70% MC w.b. reactor at 30 hours, in keeping with the peak observed in the temperature profile. The profile of the 65% MC w.b. reactor produced more peaks, although small, and the overall profiles were less distinct than that of the 70% MC w.b. reactor, suggesting greater microbial community succession in those reactors.

Figure 4.9 shows the profiles of the 3 quantified microbial groups: yeast fungi and bacteria at a height of 0.1m above the perforated plate for the different moisture contents. This graph shows a general increase in the combined amount of the quantified groups over time with peaks observed at 30-44hr, in keeping with peaks

observed in the temperature profile. At this height in the reactors, it is seen from Figure 4.9 that the highest detected amount of DNA was 8.8μg of yeast in the 60% MC w.b. reactor. This is indicative of the ability of fungi to grow extensively in low moisture environments. The 70% MC w.b. reactor had the highest combined amount of the three groups at most time points.

Shown in Figure 4.10 is the DNA profile of the three reactors at a height of 0.3m above the perforated plate. Similar to the trend observed at 0.1m, the combined amounts of DNA for all three reactors increased with time, with peaks being observed at 30-44hr for all the reactors. The largest amount of DNA was detected for bacteria at a value of 17.7μg at 32hr, which mirrors a peak observed in the oxygen consumption rate at that time point. For the different moisture contents, the 60% MC w.b. reactor had the lowest amount of quantified groups, with the maximum value of 6.5μg for yeast being lower than the maximum value obtained at a height of 0.1m.

At the top of the reactor (0.6m above the perforated plate), a maximum DNA value of 20.8μg for bacteria was observed for the 60% MC w.b. reactor. At this height, the peak amounts of combined DNA groups increased with increasing moisture content values. The 70% and 65% MC w.b reactors decreased in their total values when compared to values at 0.3m; however, the 60% MC w.b. reactor showed an increase in the total amount of quantified DNA.

The initial microbial population of the reactors appears to have been dominated by fungi, with the exception of the 65% MC w.b. reactor in which there was a predominance of bacteria at the beginning (see Figure 4.9). This difference in the initial community compositions most likely explains the reason for the extended lag phase observed in the 65% MC w.b. reactor for 2

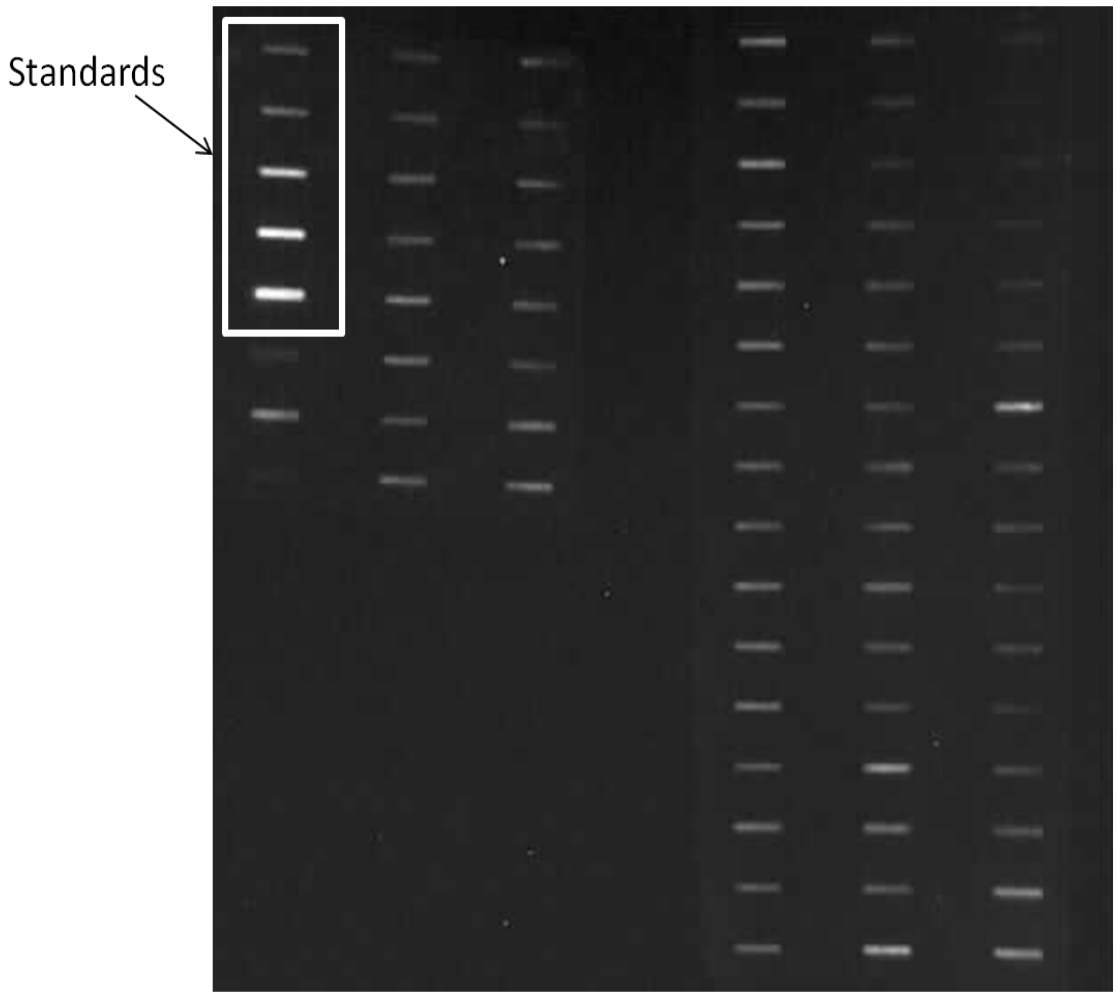

Figure 4.7 Image of bacteria Hybridized Membrane

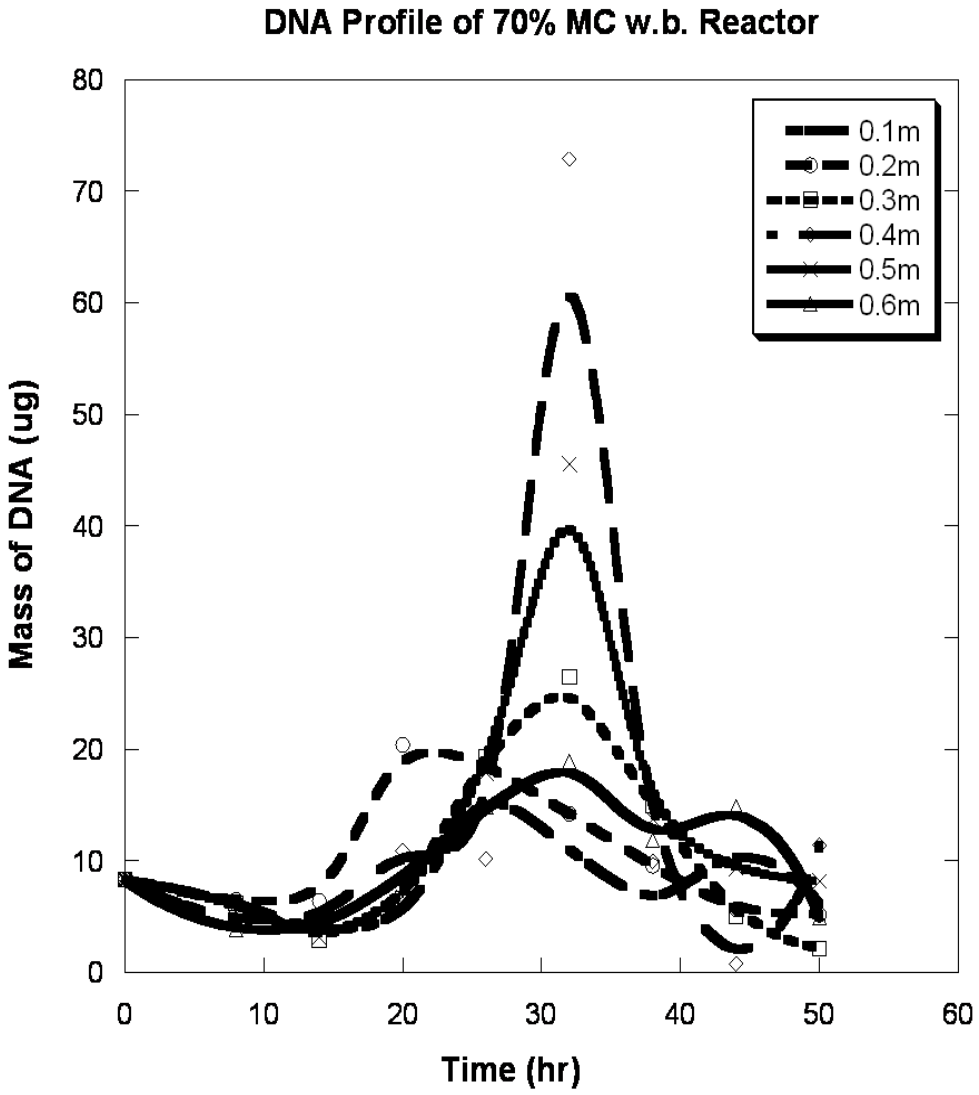

Figure 4.8 (a) Total DNA (bacteria, fungi and yeast) for 70% MC w.b. reactor

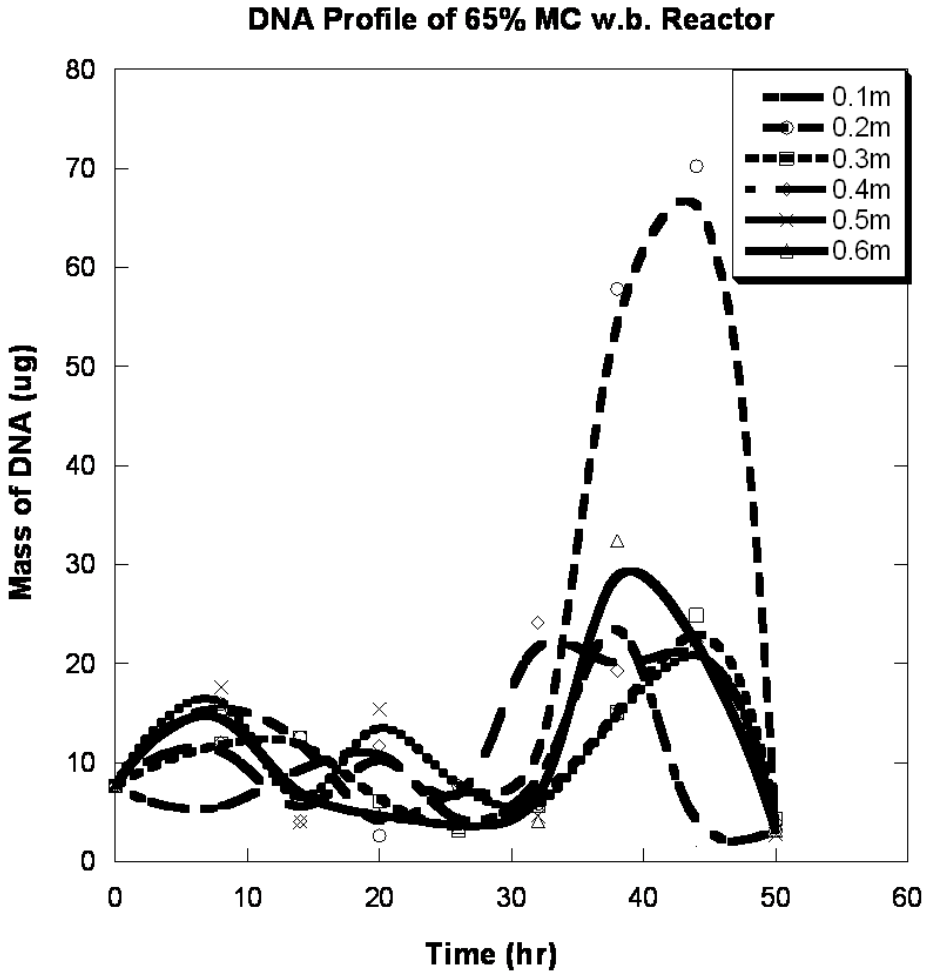

Figure 4.8 (b) Total DNA (bacteria, fungi and yeast) for 65% MC w.b. reactor

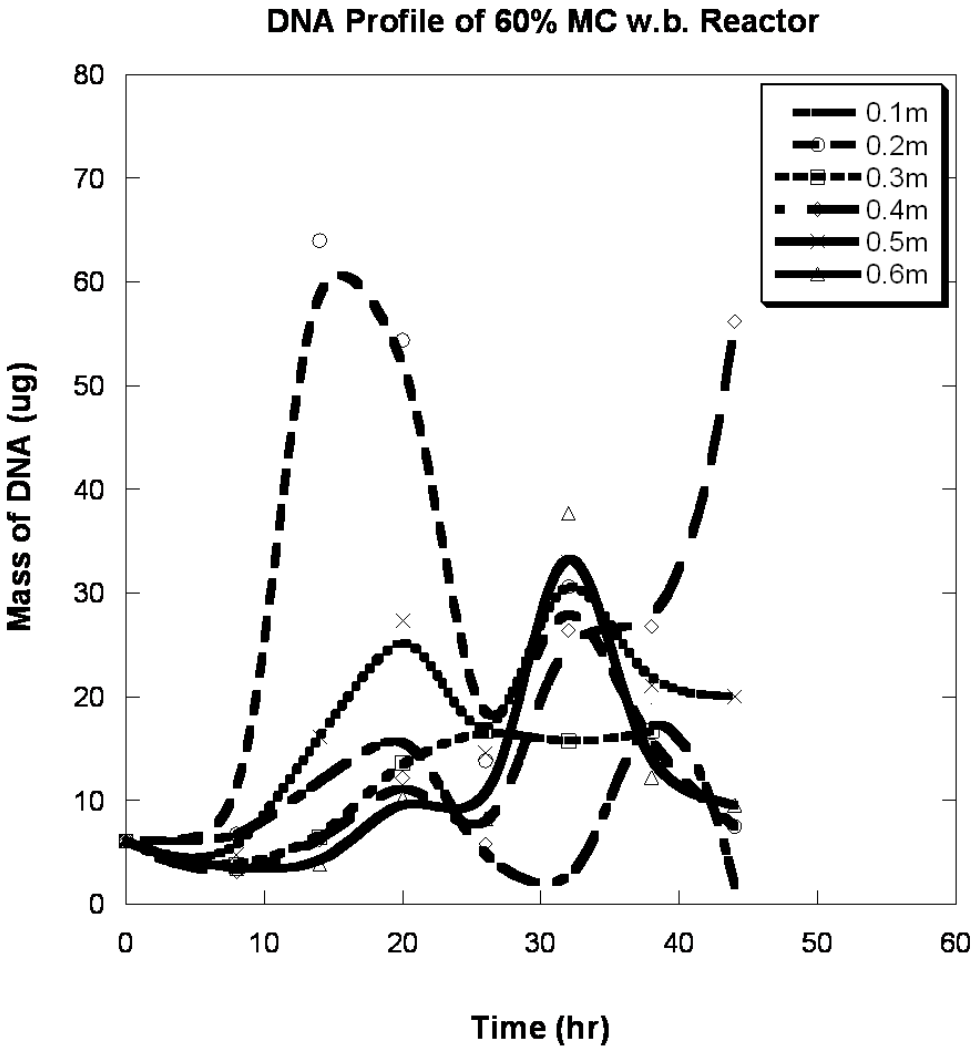

Figure 4.8 (c) Total DNA (bacteria, fungi and yeast) for 60% MC w.b. reactor

of the 3 runs carried out. The initial community composition for the 3 different reactors is shown in figure 4.9 at time 0. The data shows that the starting moisture content of the systems is a key factor is determining the quantity of the microbial population present at the beginning because there is an increase in the average quantity of microbial DNA as a function of moisture content, with the 70% moisture content reactor possessing 8.3μg of total DNA, the 65% moisture content reactor possessing 7.7μg and the 60% moisture content reactor containing 6μg of initial DNA.

Bacteria have often been considered dominant during composting, but this data shows that bacteria only really dominated at peaks corresponding to the highest temperatures during the initial stages of composting. Figures 4.9, 4.10 and 4.11 show the variation of the 3 quantified groups with time according to their heights in the reactor. For the 70% and 60% MC w.b. reactors, there is a steady increase in the amount of bacteria present with a maximum being reached in keeping with peak temperatures; however the 65% MC w.b. reactor starts off with a bacterial content which is twice that of the fungi that decreases over time and then spikes up at the peak temperature. This interesting pattern again most likely accounts for the long lag phase observed in that reactor before it ramped up to its peak temperature.

Interestingly, fungi and yeasts were detected at all points in space and time for all of the reactors whereas bacteria were not detected at all points in the process for the data shown in figures 4.9, 4.10 and 4.11. This may be due to an artefact of the probe hybridization method, as opposed to being an intrinsic result from the data. At one of the extreme points, yeasts were present in 40 times as much mass as bacteria. For the most part, fungi only surpassed bacteria at the beginning of the process, but they generally followed the same trend as the bacteria – that is, there was a steady increase in their quantities over time with peaks being observed in keeping with peak temperatures, even though their overall amounts were lower than that of bacteria. This

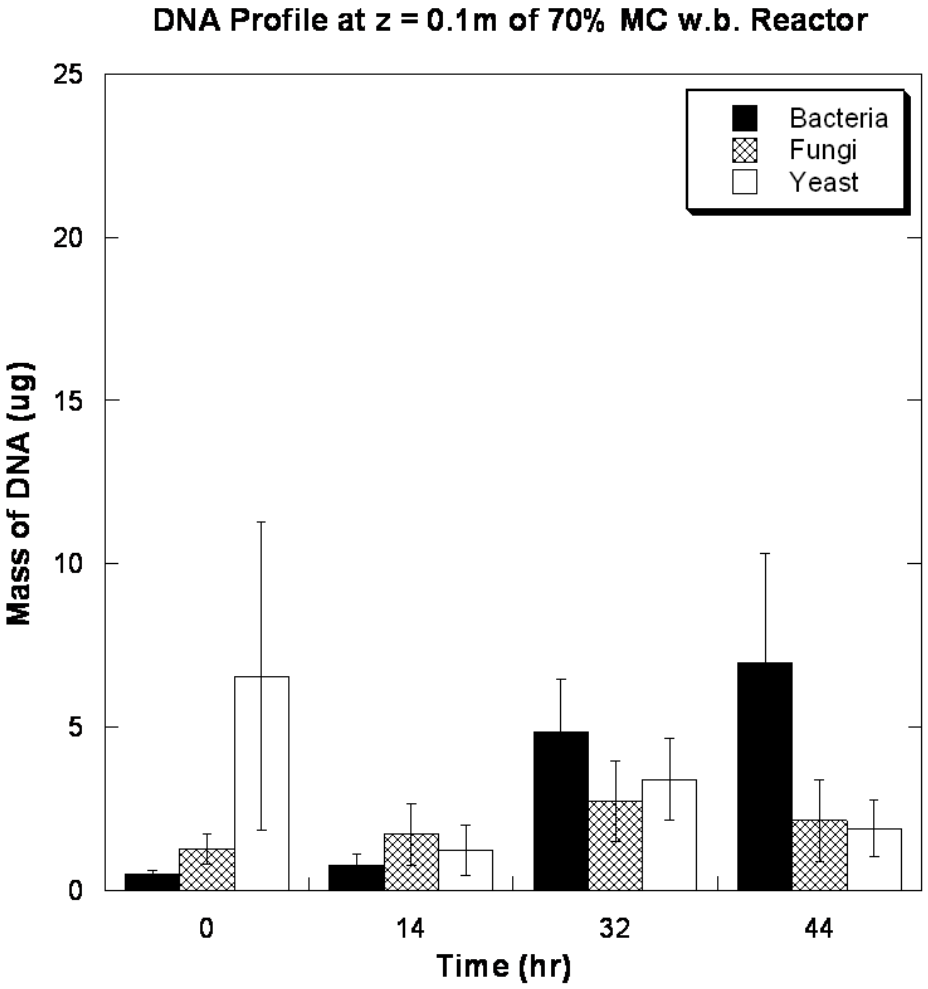

Figure 4.9 (a) DNA Population distribution of 70% MC w.b. reactor at a height of 0.1m above the perforated plate

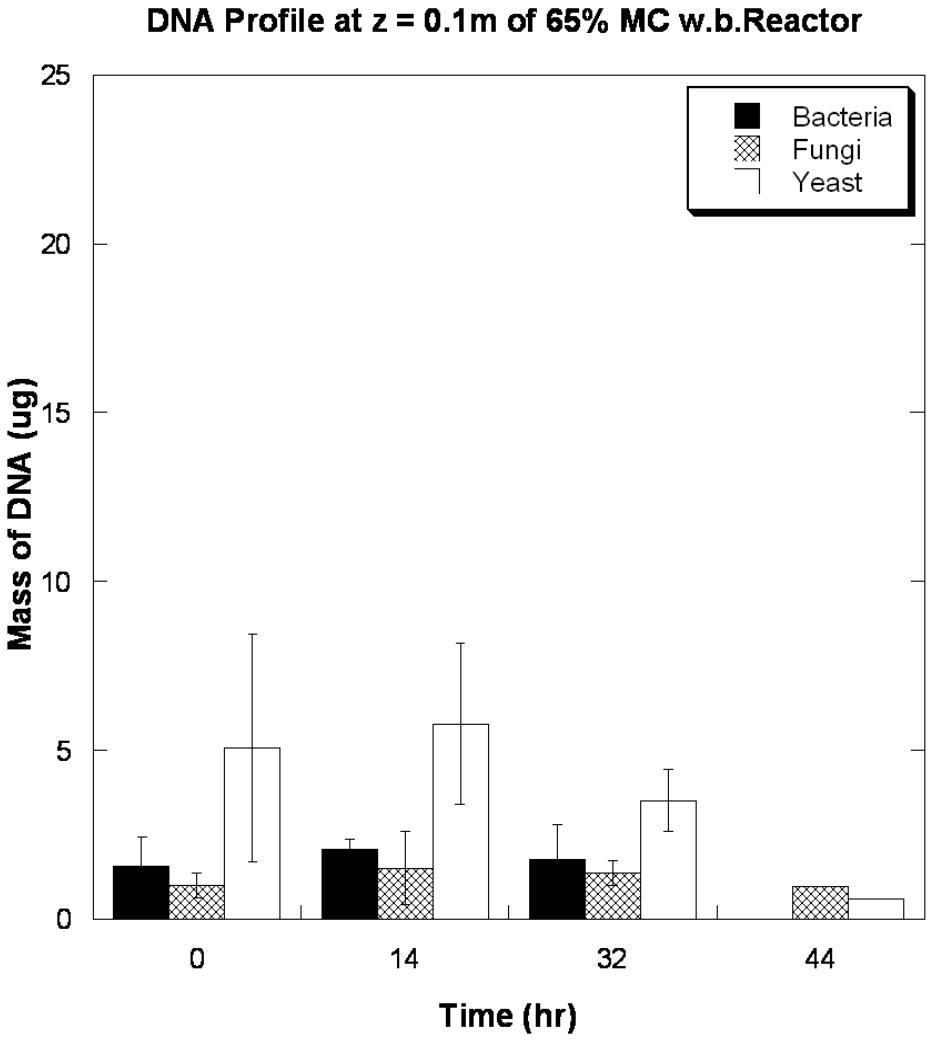

Figure 4.9 (b) DNA Population distribution of 65% MC w.b. reactor at a height of 0.1m above the perforated plate

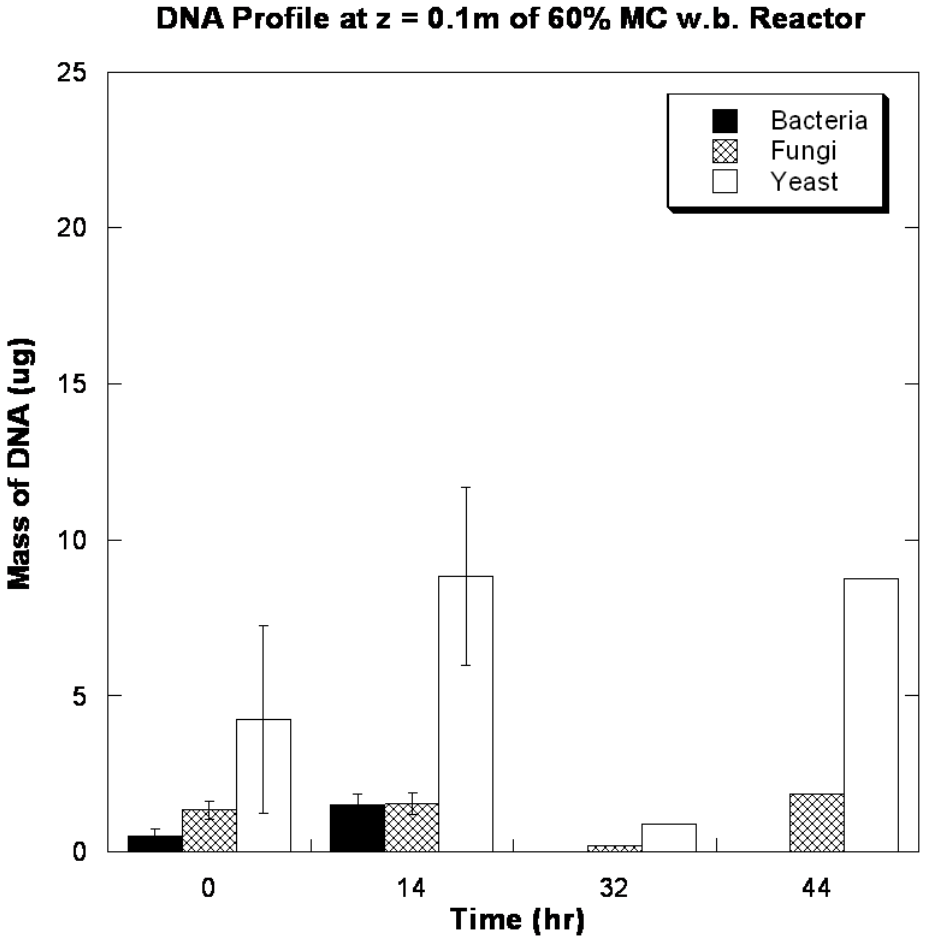

Figure 4.9 (c) DNA Population distribution of 60% MC w.b. reactor at a height of 0.1m above the perforated plate

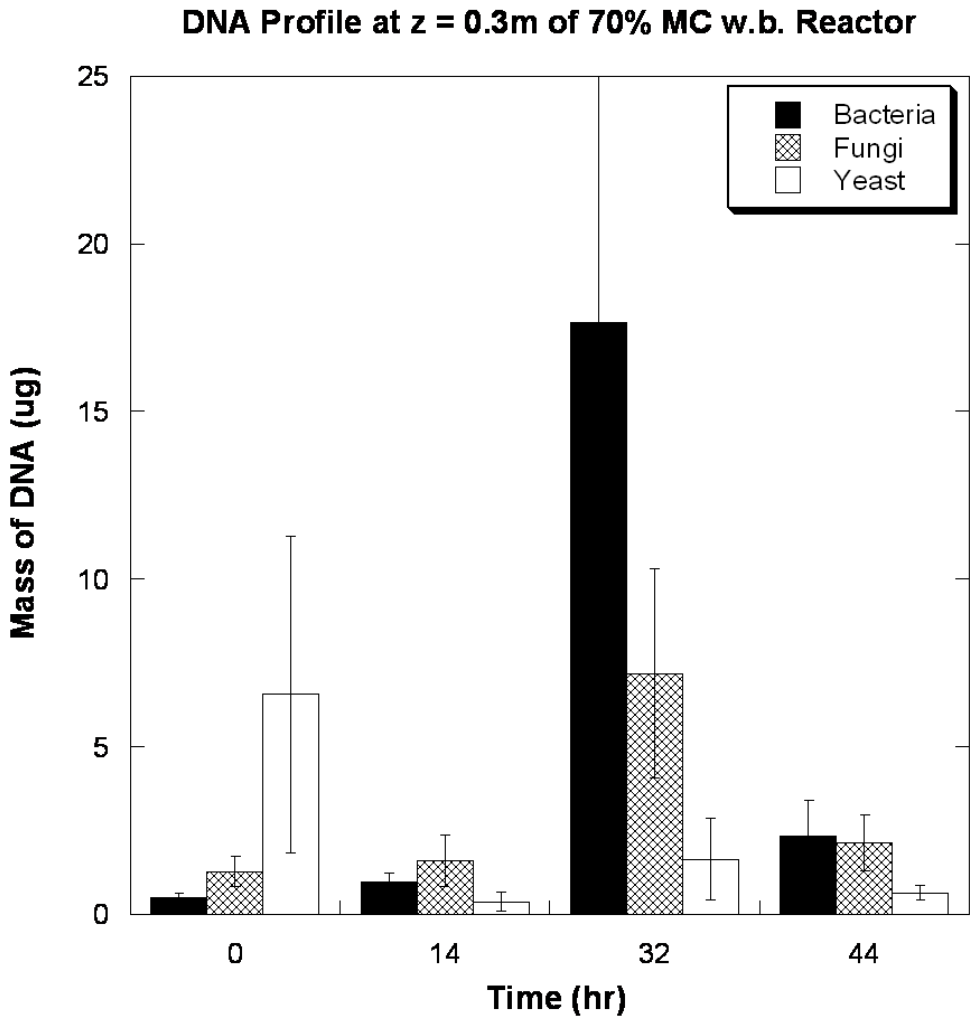

Figure 4.10 (a) DNA population distribution of 70% MC w.b. reactor at a height of 0.3m above the perforated plate

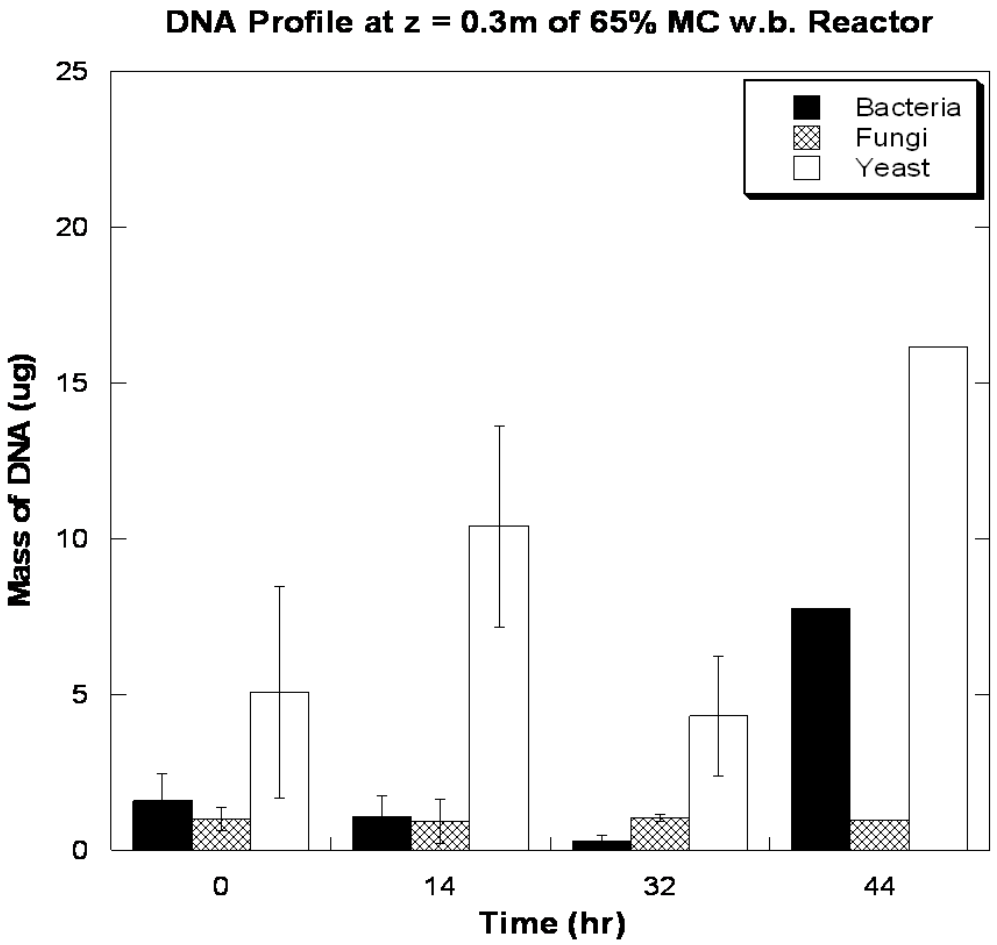

Figure 4.10 (b) DNA population distribution of 65% MC w.b. reactor at a height of 0.3m above the perforated plate

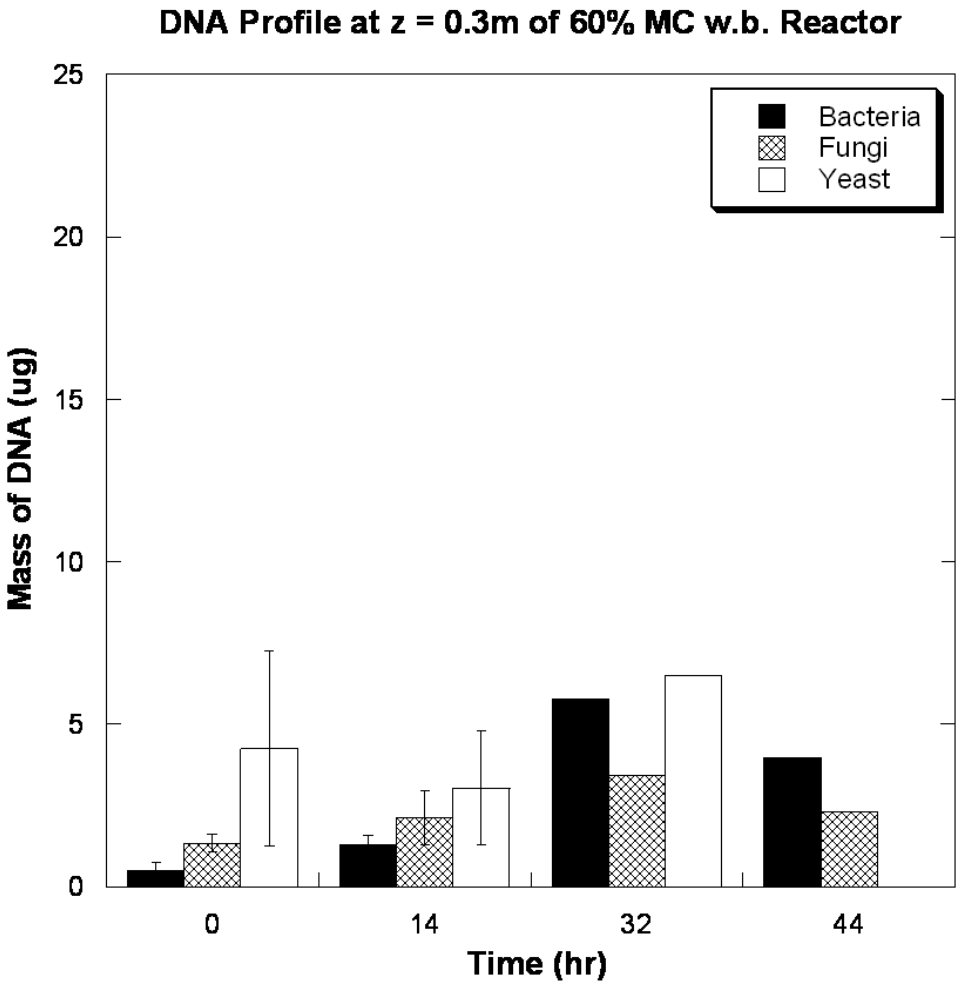

Figure 4.10 (c) DNA population distribution of 60% MC w.b. reactor at a height of 0.3m above the perforated plate

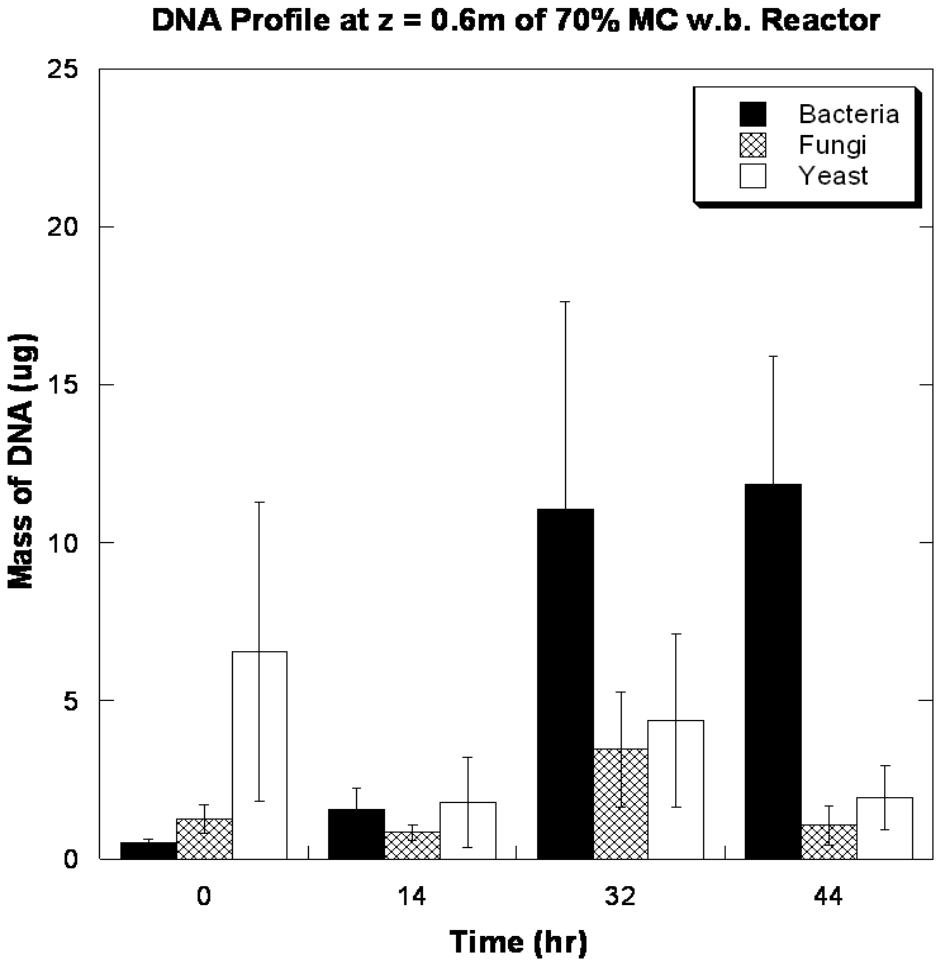

Figure 4.11 (a) DNA population distribution of 70% MC w.b. reactor at a height of 0.6m above the perforated plate

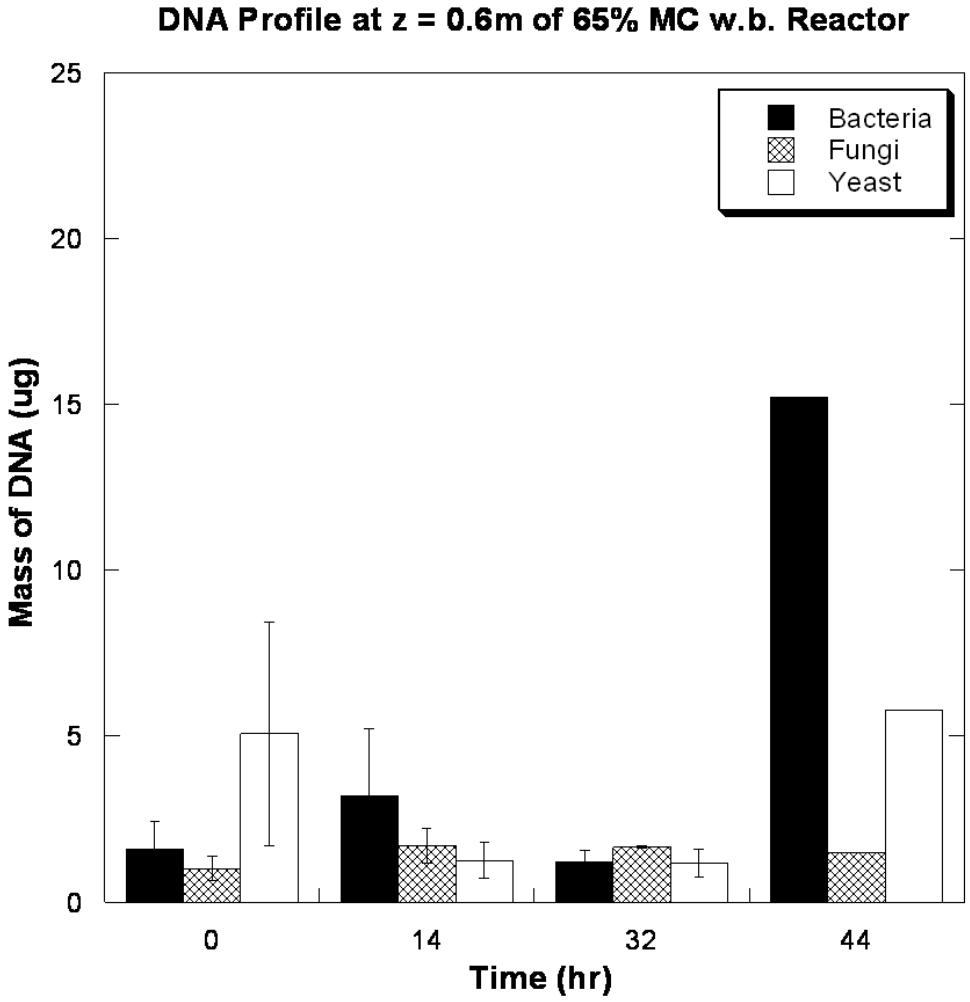

Figure 4.11 (b) DNA population distribution of 65% MC w.b. reactor at a height of 0.6m above the perforated plate

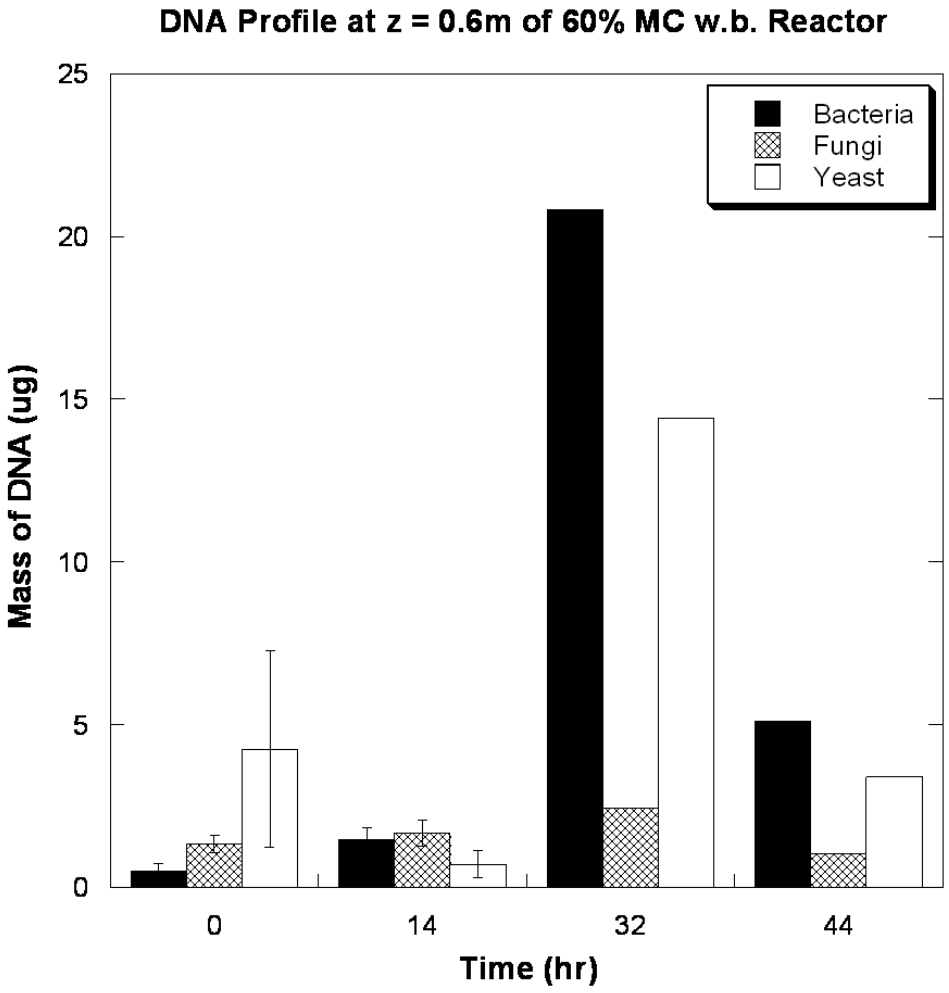

Figure 4.11 (c) DNA population distribution of 60% MC w.b. reactor at a height of 0.6m above the perforated plate

lends credence to the thought that fungi play a significant role in this composting process, at least for the temperatures recorded in the compost reactors. While yeasts were ubiquitous at all points and times sampled, there is no distinct pattern of behavior. The quantities of yeasts appear to vary randomly with space and time and the different moisture content reactors.

The highest average quantity of DNA recorded for any of the quantified groups was 20μg of bacterial DNA in the 60% MC w.b. reactor. This maximum occurred at the peak temperature of that reactor and at the highest sampling point in that reactor as well. This is in keeping with the observance of one major shift in the microbial community structure for the time points plotted in figures 4.9, 4.10 and 4.11 – that of the transition from mesophilic to thermophilic conditions as signified by the increase in microbial population. A closer examination of the populations at more time points (data not shown) indicates that the higher temporal resolution reveals smaller peaks in the microbial population that are consistent with those observed in the pH profile, implying the occurrence of other minor shifts in the microbial community.

4.6 Summary

Investigating both the physical and biological aspects of compost reactors sets the future stage for correlating these phenomena in an efficient manner that will lead to the characterization and prediction of reactor behavior as a function of the initial moisture content. From the data, it is seen that the highest moisture content reactor produced the most distinct profiles for the measured variables, suggesting that this reactor behavior would be the easiest to model and predict. The two lower moisture content reactors; however, exhibited more varied behavior for the measured variables, but trends were still observed. The physical variables measured for the different moisture content reactors were very consistent in their behaviors; thereby facilitating

their rapid correlation, such as paralleling rises in pH to increases in temperature. Even more significant is that the biological phenomena are consistent with the observed physical activity, showing a shift in the microbial population in keeping with the switch from mesophilic to thermophilic temperatures. Moreover, the differences in the measured variables observed for the small changes in the moisture content, signify the important role that initial moisture plays in the growth and activity of microorganisms.

To fully exploit the significance of the observed trends, a statistical analysis must be completed on the data to highlight the important parameters. Such a multivariate analysis is essential for determining the causes of shifts in the microbial populations and the significance and extent of those shifts. The observed microbial population trends are further limited by the potential presence of residual plant DNA, which would not represent viable microorganisms. This could present a false picture of evolving species over time, and to eliminate this possibility, the initial microbial composition of the substrates should be investigated before the start of the process.

Chapter 5

Conclusions & Future Work

This study is one of the first of its kind to provide quantitative data to describe the microbial community dynamics of a composting system using high resolution temporal and spatial data. This was done via the use of DNA probe hybridizations to quantify the amount of bacteria, fungi and yeast present in the system, based on different initial moisture contents. The simultaneous study of biotic and abiotic phenomena provides some insight into the relationships that drive observed behaviors and despite the complexity of those relationships, some conclusions could still be drawn to characterize the initial stages of composting.

Firstly, the quantitative microbial data obtained mimic the patterns and trends observed in the temperature, effluent gas concentration and pH in the different reactors. This result is consistent with previous quantitative work completed in our research group [42, 52], and it implies that the microbial activity may be used together with temperature, pH, effluent gas concentration and moisture content data to effectively build a model that could predict reactor behavior. This conclusion points to a direction I could potentially pursue for my research – that is, in the development of a model that uses the microbial population information in an empirical heat generation term that would then be used in the energy equation to predict temperature profiles. This could further be extrapolated to produce pH and effluent gas concentration profiles as well. In the longer term, the microbial population information could potentially be used on its own accord to predict the behavior of physical variables.

Another conclusion which may be drawn is that the detection of fungi and yeasts but not bacteria at all sampled spatial and temporal points indicate that the

contribution of fungi to microbial community development cannot be ignored. This quantitative data is consistent with qualitative patterns observed by Hansgate et al. [52] in characterizing fungal communities during the initial stages of composting. This further suggests that fungi may actually play an important role in the composting process – another area that I would like to pursue. To effectively investigate the role of fungi during composting, I would need to develop a more specific universal probe to capture the full range of fungal activity. This will involve searching through databases of already prepared probes and the testing of those probes to select the one which gives the best results with compost samples. If no such probe exists in databases; however, I would need to develop a molecular biology toolset that would allow me to construct my own probe.

It should be noted that there are a few factors that impact the validity of the results and conclusions presented in this work. The first of these is the high variability of the composting process. The use of the same brand of dog food minimized this variability but it was still evident, particularly in the temperature profiles in which the reactors took very different amounts of time to ramp up to thermophilic conditions even though the starting materials were the same. Secondly, biases were introduced into the method by using DNA-based techniques. This influenced the extraction, purification and hybridization of the compost samples in several ways including the preferential extraction of gram positive bacteria and the loss of microbial populations due to the procedures followed. The observance of fungi in the samples; however, shows that these inherent biases did not overpower the techniques used. Lastly, the results are limited by the specificity of the designed probes. Although the probes were designed from previous studies, their specificity for microbial DNA from the switchgrass-dog food mixture may not have been optimal because plant DNA and dog food DNA may also have been captured by the probes. Despite these limitations, the

results show that correlations can still be made between the physical variables and the microbial community data.

Another area that I would like to potentially investigate is the role of actinomycetes during the initial stages of composting. Actinomycetes are essential for breaking down cellulose and lignin during composting and their presence indicate that such degradation has occurred. By quantifying the amount of actinomycetes present during the composting process, I could conceivably determine the degradation pattern of the cellulosic switchgrass substrate. The greater implication for this would be the possible use of composting as a pretreatment method for switchgrass in the biofuel production process.

Also, I would like to be able to monitor the chemical composition of the compost pile during the initial stages of composting to investigate the degradation pattern of the substrate as a function of time and space. The high complexity of the mixed substrates that I use would be most conveniently studied using Fourier Transform Infrared Spectroscopy (FTIR) with online measuring capabilities. The information gained from this method will be critical in evaluating the effectiveness of the composting process.

The sheer complexity of the composting process presents numerous areas that could be pursued to make this process more reproducible and predictable. Through the application of state of the art technology, the full characterization of compost piles may be completed in the very near future.

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